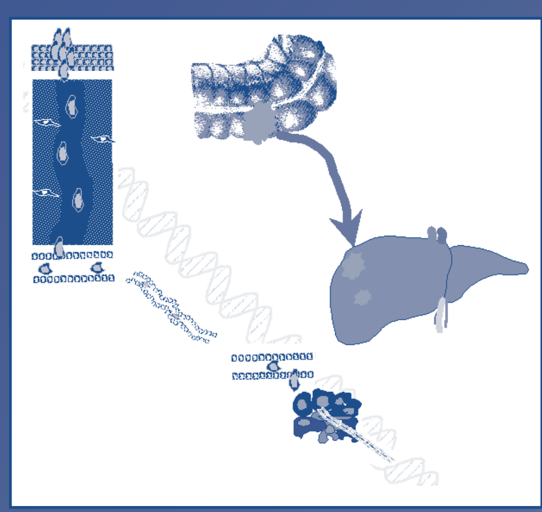
CANCER METASTASIS – BIOLOGY AND TREATMENT

DNA Methylation, Epigenetics and Metastasis

Edited by

Manel Esteller



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DNA Methylation, Epigenetics and Metastasis

Cancer Metastasis – Biology and Treatment

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PREFACE

Most of the cancer patients die because the tumoral cells do not “stick” in the original site, but instead detach, invade and disseminate throughout the bloodstream to distal sites, where these transformed cells start to proliferate and destroy again. In the last ten years, researchers have identified a number of important genes involved in these processes, including cadherins, laminins, heparan sulfates, inhibitors of proteases and angiogenesis and many others. The puzzling problem was that few genetic alterations in these genes had been described in human tumors, despite the common finding of down-regulation. CpG island hypermethylation-associated silencing has come to the rescue of several of these genes and has situated them in the forefront of the current cancer research. However epigenetic silencing is also much more than aberrant DNA methylation, a whole set of histone modifiers and chromatin remodelling factors “conspire” to maintain the repression of these tumor/metastasis suppressor genes. DNA demethylating agents and inhibitors of histone deacetylases are the first generation of epigenetic drugs to beat them. In this book, the current directions in the epigenetics of cancer progression and metastasis are comprehensively described. It is now the turn of the reader to take care of the future.

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Chapter 1

HOW CPG ISLAND HYPERMETHYLATION LEADS TO CANCER DISSEMINATION: THE SOUNDS OF SILENCE FOR TUMOR AND METASTASIS SUPPRESSOR GENES

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Abstract: CpG island hypermethylation is a common mechanism for gene silencing of tumor suppressor genes. A specific profile of gene hypermethylation occurs according to the tumor type and CpG island methylation of particular genes have been used for translational purposes. Genes inhibiting cell invasion and dissemination also undergo CpG island hypermethylation-associated inactivation. These metastasis suppressor genes can also become transcriptionally inactive by other epigenetic mechanism such as an aberrant histone code and a compact conformation of chromatin in a highly dynamic manner. The best characterized example is the E-cadherin gene, but the tumor/metastasis suppressor genes with CpG island hypermethylation in cancer include other members of the cadherin family (H-cadherin, R-cadherin, FAT), heparan sulfate genes (EXT1, GPC3, 3-OST-2), tissue inhibitors of proteinases (TIMP2, TIMP3, TFPI-2), axon guidance molecules (SEMA3B, SLIT-1, SLIT-2, SLIT-3), thrombospondins (THBS1, THBS2) and laminin genes (LAMA3, LAMB3, LAMC2). Preliminary data suggest that DNA demethylating drugs, reactivating these dormant methylated metastasis genes, have an effect against the development of metastasis.

Key words: CpG island hypermethylation, tumor suppressor genes, DNA demethylating drugs

CpG islands located in the promoter region of certain tumor suppressor genes, normally unmethylated at these regions in healthy cells, undergo a dense hypermethylation in cancer cells leading to gene silencing. However, not every gene is methylated in every tumor type, but strong specificity is apparent with respect to the tissue of origin (1-3). We have recently

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described the exquisite profile of hypermethylation that occurs in primary human tumors (2). Furthermore, the number of hypermethylated genes increases with the malignant potential (4). In recent years, we and other groups have extensively mapped an increasing numbers of gene CpG islands aberrantly hypermethylated in cancer (2,3). Such DNA methylation mapping has brought to light the existence of a unique profile of hypermethylated CpG islands that define each neoplasia (1-3,5). Following this lead, many groups are currently providing us with the "methylotype" or "DNA methylation signature" of each form of human cancer.

Methylation-associated silencing affects many genes in all existing cellular pathways (2, 3). As examples of DNA methylation markers of poor prognosis, we can mention that the Death Associated Protein Kinase (DAPK) and $p16^{INK4a}$ hypermethylation has been linked to tumor virulence in lung and colorectal cancer patients, respectively (6, 7). Not all hypermethylation events are "bad news": in neuroblastoma the CpG island hypermethylation of HOXA9 is associated with poor survival, but the hypermethylation of RARB2 is an excellent marker of good outcome (8).

However, one of the most attractive possibilities is the establishment of clusterings of CpG island hypermethylation in human tumors with prognostic value (8). Studying more than one-hundred fifty neuroblastomas and using an unsupervised hierarchical cluster analysis of all tumors based on methylation of 10 genes we separated the three clinically relevant groups of tumors (8). This type of analysis could be a good alternative to, or complement expression microarray analysis of human tumors, which requires fresh tissue and is expensive. A CpG island methylation clustering analysis cam accomplish the same goals in a more time and money efficient manner.

There are many genes with "classical" tumor suppressor function, that have also a predominant role in inhibiting cell invasion and dissemination. These genes, which we can regard as metastasis suppressor genes are not "safe" from undergoing CpG island hypermethylation-associated inactivation. An illustrative example is the E-cadherin gene (CDH1). E-cadherin germline mutations are responsible for the inherited form of diffuse gastric cancer and somatic mutations are characteristic of lobular breast cancer, but the major mechanism of E-cadherin loss in human cancer is epigenetic silencing through hypermethylation (9, 10). Epigenetics is the most adequate "Darwinian" way to inactivate a metastasis tumor suppressor gene, because it can be a dynamic process. Following this line of reasoning, it has been shown that for some primary tumors displaying E-cadherin hypermethylation, their corresponding metastasis were unmethylated at the E-cadherin gene (11). These data are in line with previous reports showing lack of E-cadherin expression in the original neoplasm, but re-expression of

E-cadherin in the distant metastatic sites. Thus, an idea is that the de-methylation and re-expression of E-cadherin is necessary for the right “fitting” of the metastatic cell in its new normal cellular neighbourhood.

E-cadherin was the first key that opened that door. We have now found methylation-inactivated genes in this double category of tumor/metastasis suppressors across a wide spectrum of cellular pathways: from cell adherence to proteinase inhibitors, and from glycoprotein production to tumor-endotelial interactions. However, the proliferation of new discoveries in this area cannot make us believe that all these genes are going to be hypermethylated. Many interesting and candidate genes in this field remain unmethylated in the cancer cells, such as CD44 and NM23. Other epigenetic mechanisms, such a shift in the histone modification pattern and a recruitment of chromatin remodelling factors with repressive activity may account for a loss of gene expression. This is, for example, again the case for E-cadherin in some instances, where gene inactivation in transformed cells can be mediated by the action of the transcriptional repressors Snail and Slug, which recruit histone deacetylases to the E-cadherin promoter (12,13).

My final goal in this chapter, and in the Edition of this book, is to provide a comprehensive view of those tumor/metastasis genes with hypermethylation-associated silencing in human cancer. The scenario that we have unmasked in the recent years shows that many genes in many cellular pathways are shut-down by this epigenetic aberration. These are the main culprits:

a. Cadherin genes

We have already described the E-cadherin history, but following its lead several members of this familiy have been found to undergo hypermethylation in neoplasms. The most studied members are H-cadherin (CDH13), CDH4 (R-cadherin) and Protocadherin (FAT). Inactivation of CDH13 by promoter hypermethylation is a common finding in most tumor types (14,15), whilst the aberrant methylation of CDH4 and FAT have been so far described in gastric and colorectal carcinoma (16,17).

b. Heparan sulfate synthesis

Germline mutations in the Exostoses-1 gene (EXT1) are found in hereditary multiple exostoses (HME) syndrome, which is characterized by the formation of osteochondromas and an increased risk of chondrosarcomas and osteosarcomas. EXT1 is a glycosyltransferase and its abrogation in human cancer cells occurs by CpG island-promoter hypermethylation, leading to the loss of heparan sulfate synthesis (18). The reintroduction of EXT1 into cancer-cell lines displaying methylation-dependent silencing of EXT1 induces tumor-suppressor-like features, e.g., reduced colony

formation density and tumor growth in nude mouse xenograft models (18). EXT1 CpG island hypermethylation is common in leukaemia and non-melanoma skin cancer (18). Two other genes in this network also manifest methylation-related silencing in human neoplasms: the Glypican-3 (GPC3) (19), a membrane-bound heparan sulfate proteoglycan, and the heparan sulfate D-glucosaminyl 3-O-sulfotransferase-2 (3-OST-2) (20).

c. Tissue inhibitors of proteinases

Tissue inhibitor of metalloproteinases (TIMPs) antagonize matrix metalloproteinase activity and can suppress tumor growth, angiogenesis, invasion, and metastasis. The most characterized members of this family are TIMP1, TIMP2, TIMP3 and TIMP4, whilst cases of TIMP2 and TIMP3 CpG island promoter hypermethylation have been described among different tumor types (2,21,22). A similar scenario can be drawn for another broad range proteinase inhibitor, TFPI-2, which it also displays epigenetic inactivation in glioma and primary pancreatic ductal neoplasms (23,24), demonstrating an association with the progression of the disease.

d. Axon guidance molecules

The semaphorin family of proteins plays a critical role in axonal guidance and contains a highly conserved 500 amino acid semaphorin domain at N-terminal. Semaphorins have been classified into eight subclasses, and five subclasses (3–7) are found in vertebrates. Two class 3 semaphorin genes, SEMA3B and SEMA3F, reside at 3p21.3, a hot-spot of loss of heterozygosity in human neoplasms, and have been shown to play a potential tumor suppressive role in tumorigenesis. It is thought that SEMA3B mediates its tumor-suppressing effects, at least in part, by blocking VEGF autocrine activity. Promoter methylation have been observed in the SEMA3B in non small cell lung cancer (25-26). A second family of axon guidance molecules is constituted by the SLIT genes. In Drosophila, the Slit gene product, a secreted glycoprotein, acts as a midline repellent to guide axonal development during embryogenesis. Slit plays a vital role in axon guidance by signaling through Robo receptors, but recent evidence suggests that Slit proteins may function in other settings because human and Xenopus Slit2 has been shown to inhibit leukocyte chemotaxis. Three human Slit gene orthologues that show methylation-associated silencing in different tumor types have been characterised thus far: SLIT-1, SLIT-2 and SLIT-3 (27).

e. Thrombospondins

The thrombospondins (THBS) are a family of proteins that regulate tissue genesis and remodeling. In many tumors, down-regulation of THBS-1 and

THBS-2 appears to be a prerequisite for the aquisition of a pro-angiogenic phenotype. The normal suppression of angiogenesis by both proteins involve multiple mechanisms including direct interaction with VEGF, inhibition of matrix metalloproteinases activation, inhibition of endothelial cell migration and induction of endothelial cell apoptosis. THBS1 and THBS2 undergo hypermethylation associated silencing across different tumor types (28), being particularly prevalent in gliomas, a neoplasm with a well-known higher level of neoangiogenesis.

f. Laminin genes

Different laminins profoundly affect tissue morphogenesis, starting around the time of embryonic implantation and extending through organogenesis and into the postnatal period. Collectively, they have revealed common functions that include the induction and maintenance of cell polarity, the establishment of barriers between tissue compartments, the organization of cells into tissues, and the protection of adherent cells from detachment-induced cell death. More than 12 laminin isoforms are presently known, which have a cell- and tissue-specific expression and are differentially recognized by integrins. However, during tumor invasion, loss of the basal membrane barrier occurs and a discontinuous pattern of laminin staining is observed. CpG island hypermethylation of Laminin-5 (LN5)-encoding genes (LAMA3, LAMB3, and LAMC2) has been reported in various human cancers, including breast, lung, prostate and bladder, occurring mainly in high stage and large size tumors (29).

Finally, I would like to conclude with a positive note. Most of the hypermethylation events I have described constitute molecular markers of progression and dissemination, in summary, poor prognosis. However, these are great targets for the new epigenetic drugs for the treatment of cancer that are emerging. We are at the dawn of an era when DNA demethylating drugs (30) could be an important weapon in our arsenal in the war against cancer. Hematological malignancies have provided a promising starting point supported by the USA Food and Drug Administration, but studies will surely extend to all solid tumors. However, we need to continue our research to develop more specific DNA-demethylating agents, in order to understand their biological effects and to determine whether they may be successfully combined with other epigenetic drugs, such as the inhibitors of histone deacetylases (31), and classical chemotherapy compounds. An effective treatment against the generation of metastasis by reactivating all the hypermethylated genes described is not a utopia.

REFERENCES

1. Costello J. F., Fruhwald M. C., Smiraglia D. J., Rush L. J., Robertson G. P., Gao X., Wright F. A., Feramisco J. D., Peltomaki P., Lang J. C., Schuller D. E., Yu L., Bloomfield C. D., Caligiuri M. A., Yates A., Nishikawa R., Su Huang H., Petrelli N. J., Zhang X., O'Dorisio M. S., Held W. A., Cavenee W. K., Plass C. Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. Nat Genet, 2000; 24:132-138.
2. Esteller M. A gene hypermethylation profile of human cancer. Cancer Res, 2001; 61:3225-9.
3. Esteller M. CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. Oncogene, 2002; 21:5427-5440.
4. Fraga M. F., Herranz M., Espada J., Ballestar E., Paz M. F., Ropero S., Erkek E., Bozdogan O., Peinado H., Niveleau A., Mao J. H., Balmain A., Cano A., Esteller M. A mouse skin multistage carcinogenesis model reflects the aberrant DNA methylation patterns of human tumors. Cancer Res, 2004; 64:5527-5534.
5. Paz M..F., Fraga M. F., Avila S., Guo M., Pollan M., Herman J. G., Esteller M. A systematic profile of DNA methylation in human cancer cell lines. Cancer Res, 2003; 63:1114-1121.
6. Tang X., Khuri F. R., Lee J. J., Kemp B. L., Liu D., Hong W. K., Mao L. Hypermethylation of the death-associated protein (DAP) kinase promoter and aggressiveness in stage I non-small-cell lung cancer. J Natl Cancer Inst, 2000; 92:1511-1516.
7. Esteller M., Gonzalez S., Risques R. A., Marcuello E., Mangues R., Germa J. R., Herman J. G., Capella G., Peinado M. A. K-ras and p16 aberrations confer poor prognosis in human colorectal cancer. J Clin Oncol, 2001; 19:299-304.
8. Alaminos M., Davalos V., Cheung N. K., Gerald W. L., Esteller M. Clustering of gene hypermethylation associated with clinical risk groups in neuroblastoma. J Natl Cancer Inst, 2004; 96:1208-1219.
9. Graff J. R. , Herman J. G., Lapidus R. G., Chopra H., Xu R., Jarrard D. F., Isaacs W. B., Pitha P. M., Davidson N. E., Baylin S. B. E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. Cancer Res, 1995; 55:5195-5199.
10. Yoshiura K., Kanai Y., Ochiai A., Shimoyama Y., Sugimura T., Hirohashi S. Silencing of the E-cadherin invasion-suppressor gene by CpG methylation in human carcinomas. Proc Natl Acad Sci U S A, 1995; 92:7416-7419.
11. Graff J. R., Gabrielson E., Fujii H., Baylin S. B., Herman J. G. Methylation patterns of the E-cadherin 5' CpG island are unstable and reflect the dynamic, heterogeneous loss of E-cadherin expression during metastatic progression. J Biol Chem, 2000; 275:2727-2732.
12. Bolos V., Peinado H., Perez-Moreno M. A., Fraga M. F., Esteller M., Cano A. The transcription factor Slug represses E-cadherin expression and induces epithelial to mesenchymal transitions: a comparison with Snail and E47 repressors. J Cell Sci, 2003; 116:499-511.
13. Peinado H., Ballestar E., Esteller M., Cano A. Snail mediates E-cadherin repression by the recruitment of the Sin3A/histone deacetylase 1 (HDAC1)/HDAC2 complex. Mol Cell Biol, 2004; 24:306-319.
14. Sato M., Mori Y., Sakurada A., Fujimura S., Horii A. The H-cadherin (CDH13) gene is inactivated in human lung cancer. Hum Genet, 1998; 103:96-101.

15. Toyooka K. O., Toyooka S., Virmani A. K., Sathyanarayana U. G., Euhus D. M., Gilcrease M., Minna J. D., Gazdar A. F. Loss of expression and aberrant methylation of the CDH13 (H-cadherin) gene in breast and lung carcinomas. Cancer Res, 2001; 61:4556-4560.
16. Miotto E., Sabbioni S., Veronese A., Calin G. A., Gullini S., Liboni A., Gramantieri L., Bolondi L., Ferrazzi E., Gafa R., Lanza G., Negrini M. Frequent aberrant methylation of the CDH4 gene promoter in human colorectal and gastric cancer. Cancer Res, 2004; 64:8156-8159.
17. Paz M. F., Wei S., Cigudosa J. C., Rodriguez-Perales S., Peinado M. A., Huang T. H, Esteller M. Genetic unmasking of epigenetically silenced tumor suppressor genes in colon cancer cells deficient in DNA methyltransferases. Hum Mol Genet, 2003, 12:2209-2219.
18. Ropero S, Setien F, Espada J, Fraga MF, Herranz M, Asp J, Benassi MS, Franchi A, Patino A., Ward L. S., Bovee J., Cigudosa J. C., Wim W., Esteller M. Epigenetic loss of the familial tumor-suppressor gene exostosin-1 (EXT1) disrupts heparan sulfate synthesis in cancer cells. Hum Mol Genet, 2004; 13:2753-2765.
19. Lin H., Huber R., Schlessinger D., Morin P. J. Frequent silencing of the GPC3 gene in ovarian cancer cell lines. Cancer Res, 1999; 59:807-810.
20. Miyamoto K., Asada K., Fukutomi T., Okochi E., Yagi Y., Hasegawa T., Asahara T., Sugimura T., Ushijima T. Methylation-associated silencing of heparan sulfate D-glucosaminyl 3-O-sulfotransferase-2 (3-OST-2) in human breast, colon, lung and pancreatic cancers. Oncogene, 2003; 22:274-280.
21. Bachman K. E., Herman J. G., Corn P. G., Merlo A., Costello J. F., Cavenee W. K., Baylin S. B., Graff J. R. Methylation-associated silencing of the tissue inhibitor of metalloproteinase-3 gene suggest a suppressor role in kidney, brain, and other human cancers. Cancer Res, 1999; 59:798-802.
22. Ivanova T., Vinokurova S., Petrenko A., Eshilev E., Solovyova N., Kisseljov F., Kisseljova N. Frequent hypermethylation of 5' flanking region of TIMP-2 gene in cervical cancer. Int J Cancer, 2004; 108:882-886.
23. Konduri S. D., Srivenugopal K S., Yanamandra N., Dinh D. H., Olivero W. C., Gujrati M., Foster D. C., Kisiel W., Ali-Osman F., Kondraganti S., Lakka S. S., Rao J. S. Promoter methylation and silencing of the tissue factor pathway inhibitor-2 (TFPI-2), a gene encoding an inhibitor of matrix metalloproteinases in human glioma cells. Oncogene, 2003; 22:4509-4516.
24. Sato N., Parker A. R., Fukushima N., Miyagi Y., Iacobuzio-Donahue C. A., Eshleman J. R., Goggins M. Epigenetic inactivation of TFPI-2 as a common mechanism associated with growth and invasion of pancreatic ductal adenocarcinoma. Oncogene, 2005; 24: 850-858.
25. Tomizawa Y., Sekido Y., Kondo M., Gao B., Yokota J., Roche J., Drabkin H., Lerman M. I., Gazdar A. F., Minna J. D. Inhibition of lung cancer cell growth and induction of apoptosis after reexpression of 3p21.3 candidate tumor suppressor gene SEMA3B. Proc Natl Acad Sci USA, 2001; 98:13954-13959.
26. Kuroki T., Trapasso F., Yendamuri S., Matsuyama A., Alder H., Williams N. N., Kaiser L. R., Croce C. M. Allelic loss on chromosome 3p21.3 and promoter hypermethylation of semaphorin 3B in non-small cell lung cancer. Cancer Res, 2003; 63:3352-3355.
27. Dickinson R. E., Dallol A., Bieche I., Krex D., Morton D., Maher E. R., Latif F. Epigenetic inactivation of SLIT3 and SLIT1 genes in human cancers. Br J Cancer, 2004; 91:2071-2078.
28. Li Q., Ahuja N., Burger P. C., Issa J. P. Methylation and silencing of the Thrombospondin-1 promoter in human cancer. Oncogene, 1999; 18:3284-3289.

29. Sathyanarayana U. G., Toyooka S., Padar A., Takahashi T., Brambilla E., Minna J. D., Gazdar A. F. Epigenetic inactivation of laminin-5-encoding genes in lung cancers. Clin Cancer Res, 2003; 9:2665-2672.
30. Villar-Garea A., Esteller M. DNA demethylating agents and chromatin-remodelling drugs: which, how and why? Curr Drug Metab. 2003; 4:11-31.
31. Villar-Garea A., Esteller M. Histone deacetylase inhibitors: understanding a new wave of anticancer agents. Int J Cancer, 2004; 112:171-178.

Chapter 2

A MOUSE SKIN MULTISTAGE CARCINOGENESIS MODEL THAT UNMASKS EPIGENETIC LESIONS RESPONSIBLE FOR METASTASIS

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Abstract: Although there is a wide range of accepted models of tumorigenesis involving genetic lesions, the timing and hierarchy of epigenetic alterations associated with tumor progression and metastasis are still poorly understood. In this regard, the best characterized mouse carcinogenesis system, the multistage skin cancer progression model, has recently been used to identify epigenetic alterations during tumor progression and to provide decisive information about how epigenetic lesions precede metastasis. This model reveals a progressive global loss of genomic methylcytosine that is associated with the degree of tumor aggressiveness and that occurs in the context of increasing numbers of hypermethylated CpG islands of tumor-suppressor genes during the most malignant stages of carcinogenesis. DNA microarrays coupled with demethylating drug treatments confirm the progressive establishment of hypermethylation events from the early stages to the most aggressive phenotypes. It is of particular interest that the transition from epithelial to spindle cell morphology with metastatic potential is associated with prominent epigenetic alterations: *E-cadherin* methylation, demethylation of the *Snail* promoter, and a profound decrease of global DNA methylation.

Key words: DNA methylation, tumour progression, metastasis, skin cancer, mouse models

The genesis and progression of cancer and metastasis involves the interplay of genetic and environmental factors. Epigenetics, understood as heritable states of gene expression in which the DNA sequence is not altered, is now beginning to be established as one of the important players in tumorigenesis and metastasis. Epigenetics predominantly involves DNA

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methylation and histone modifications. Aberrant DNA methylation is nowadays an accepted feature of human cancer and has two facets: CpG island hypermethylation-associated silencing of tumor-suppressor genes (*p16*^{INK4a}, *BRCA1*, *MLH*, etc.) and global genomic hypomethylation. Nevertheless, we are still in the early stages of understanding the timing and hierarchy of the epigenetic lesions and their cross-talk with genetic and environmental factors.

1. TUMOR PROGRESSION MODELS FOR STUDYING THE EPIGENETIC LESIONS PRECEDING METASTASIS

DNA methylation is the main epigenetic modification in humans. Aberrant DNA methylation has emerged in recent years as a common hallmark of all types of cancer. Two DNA methylation lesions coexist in human neoplasms: hypermethylation of the promoter region of specific tumor-suppressor genes in a genomic context of overall hypomethylation. Although for genetic lesions it is widely accepted that a cumulative chain of hits occur in oncogenes and tumor-suppressor genes, as represented in the elegant model of human colorectal tumorigenesis (1), very little is known about the precise timetable of epigenetic alterations occurring during the transition of a normal cell through intermediate tumorigenic stages to a tumor cell with invasive properties. To address this issue, the manner by which cumulative DNA methylation changes occur has recently been studied in the multistage skin tumor progression model (2-8) (Table1), which is the best known and one of the longest established systems of mouse carcinogenesis.

In this model, beginning with normal mouse skin, sequential topical application of different mutagens, such as the polycyclic aromatic hydrocarbon dimethylbenzanthracene (DMBA), and tumor promoting agents, such as 12-*O*-tetra-decanoylphorbol-13-acetate (TPA), gives rise to a complete spectrum of the stages of tumorigenesis, from premalignant papilloma to highly metastatic tumors with well defined genetic lesions in *H-ras* or *p53* (2-8). The cumulative appearance of DNA methylation aberrations has been widely demonstrated with this model, thus underlining its versatility as a tool for comparative studies of human cancer epigenetics.

Table 1. Origin and characteristics of the mouse skin cancer cell lines comprising the multistage skin cancer progression model.

Line	Tumorigenicity	Origin	Morphology	*H-ras*	*E-Cadherin*	*Snail*
MCA3D	-	DMBA Immortalized	Epithelial	Normal	++	-
PB	-	DMBA Papilloma	Epithelial	Normal	++	-
MSCP6	-	DMBA Papilloma	Epithelial	Mutation codon 61	++	-
PDV	+	DMBA *In vitro* transformed	Epithelial	Mutation codon 61	++	-
PAM212	+	Immortalized	Epithelial	Normal	++	-
MSC11B9	+	DMBA/TPA Carcinoma	Epithelial	Mutation codon 61	+	-
MSC11A5	++	DMBA/TPA Carcinoma	Spindle	Mutation codon 61	-	+
HaCa4	+++	HMSV/TPA Carcinoma	Epithelioid	Overexpression. Ras viral	-	+
CarB	++	DMBA/TPA Carcinoma	Spindle	Mutation codon 61	-	++
CarC	+++	DMBA/TPA Carcinoma	Spindle	Mutation codon 61	-	++

2. GLOBAL DNA METHYLATION ALTERATIONS DURING MOUSE SKIN TUMOR PROGRESSION AND METASTASIS

It is accepted that the majority of human tumors have a lower 5-methylcytosine DNA content than their normal tissue counterparts (9-11). These findings were obtained at separate times during the course of the tumor's natural history, but no information about their appearance or evolution is available. However, in the mouse progression model, alterations in the overall DNA methylation status have been studied using two complementary approaches: high-performance capillary electrophoresis (HPCE), an analytical technique that provides absolute measures of 5-methylcytosine content (12-14), and immunolocalization of 5-methylcytosine, which provides qualitative information regarding its nuclear distribution (15).

Interestingly, HPCE reveals that there is a continuous loss of 5-methylcytosine during tumor progression (Figure 1B), with strikingly sharp falls at two points. The first of these occurs in the transition from nontumorigenic (MCA3D cells) to benign papilloma cells (PB and MSCP6) and represents a drop of 25% of the total genomic content in 5-methylcytosine. This loss coincides with the mutational activation of *H-ras*, exemplifying the synergism between genetic and epigenetic processes on the road to metastasis. The second important hypomethylation event is a loss of around half (54%) of the total 5-methylcytosine genomic content, which, most importantly, occurs when the transformed cells undergo a phenotypic transition from MSC11B9 epithelial cells to MSC11A5 spindle cells. This morphological change is associated with a striking increase in their tumorigenicity and metastatic potential but the underlying cause is not fully understood.

The loss of 5-methylcytosine during the multistage carcinogenic process can also be revealed by immunolocalization experiments (Figure 1B, C). A strong correlation between the 5-methylcytosine levels determined by either HPCE or immunolocalization is also observed. Interestingly, 5-methylcytosine staining demonstrates that the loss of 5-methylcytosine is not homogeneous in the nucleus (Figures 1B and 1C). There are large local genomic regions almost completely devoid of 5-methylcytosine staining that may correspond to heterochromatic regions, as suggested by DAPI staining.

These findings highlight the value of using DNA hypomethylation levels as a biomarker of tumor aggressiveness, and confirm the role of global genomic hypomethylation as a dynamic characteristic, rather than as a static and fixed feature, of carcinogenesis. The data provided by the mouse model and those of recent experiments showing enhanced tumorigenicity and

chromosomal instability in the mouse with hypomorphic alleles of the DNMTs (16), strongly imply an important role for DNA hypomethylation in malignant transformation leading to metastasis.

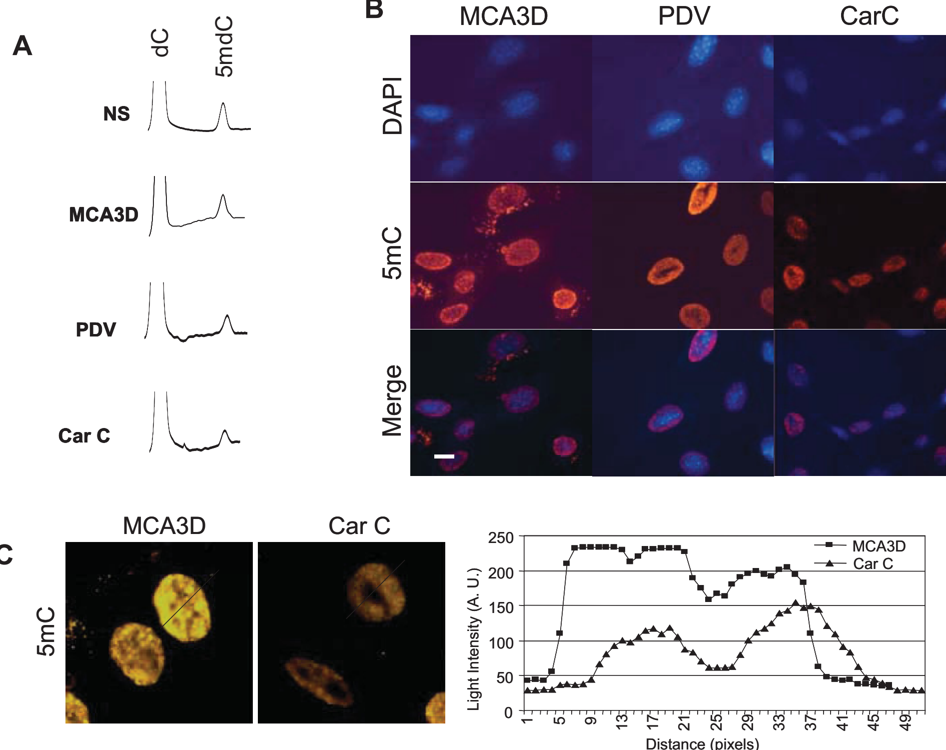


Figure 1. Global DNA methylation in a mouse skin tumor progression model. A. Representative partial electropherograms (cytosine and methylcytosine peaks) of enzymatically hydrolyzed genomic DNA extracted from normal skin (NS) and MDA3D, PDV and CarC cell lines. B. Double-staining of MCa3D, PDV and CarC cells with DAPI, a chromatin marker, and mouse monoclonal antibodies against 5mC show a clear nuclear redistribution of 5mC associated with cell tumorigenicity. Bar: 10 µm. C. Orthogonal densitometry through the annotated axis of MCA3D and CarC cells stained with antibodies against 5 mC reveal a strong diminution of light signal in CarC cells. Densitometric profiles were obtained using NIH Image software.

3. PROMOTER DNA METHYLATION OF CANDIDATE GENES IN TUMOR PROGRESSION AND METASTASIS

Cancer epigenetics studies cannot be fully understood if we do not appreciate the relevance of the silencing of tumor-suppressor genes associated with CpG island hypermethylation and their histone modifications and chromatin-linked changes. In such a context, the multistage mouse skin progression model is also demonstrably useful for defining the chronological inactivation pattern of tumor-suppressor genes with methylation-associated silencing that are known to be hypermethylated in human cancer (17, 18). Of these, there is information available about the DNA repair genes *MLH1, BRCA1* and adhesion-related genes *E-cadherin (CDH1),* the transcriptional repressor *Snail* and the Snail/Gfi-1 repressor family member (*MLT1*). The DNA methylation patterns of these genes during tumor progression are characterized by bisulfite genomic sequencing and by methylation-specific PCR (MSP). Four of these genes (*MGMT, Snail*, *E-cadherin* and *MLT1*) are methylated in the mouse skin carcinogenesis model (Figure 2). In most cases methylation of their promoter regions is associated with transcriptional silencing and is reactivated by the use of the demethylating agent 5-aza-2-deoxycytidine (Aza). Three out of the four genes (75%), (*MGMT, MLT1* and *Snail*), begin to be hypermethylated at the beginning of the carcinogenic process, when moving from fresh normal skin (NS) to immortalized nontumorigenic keratinocytes (MCA3D), while *E-cadherin* hypermethylation occurs at later stages coinciding with the loss of the epithelial phenotype (Figure 2A). Thus, CpG island hypermethylation of tumor-suppressor and related genes is an early event in tumorigenesis. In conjunction with the discovery of gene hypermethylation also in static studies of premalignant lesions, such as colorectal and gastric adenomas, uterine hyperplasias and ulcerative colitis (19), it becomes clear that the epigenetic silencing of tumor-suppressor genes is one of the earliest hits in tumorigenesis.

Figure 2. Promoter methylation of candidate genes in a mouse tumor progression. A. Summary of methylation-specific PCR (MSP) analyses of the CpG island methylation status of six genes that are known to undergo promoter hypermethylation in human cancer. Red and green indicate methylation and unmethylation, respectively. B. Schematic representation of the methylation status of the MGMT CpG island in several mouse skin cancer cell lines obtained by bisulfite genomic sequencing. Black and white dots indicate methylated and unmethylated CpGs, respectively. C. Example of the MSP analysis of the MLH1 and MLT11 genes. The presence of a PCR band under the "U" or "M" lane indicates unmethylated or methylated alleles, respectively. *In vitro*-methylated DNA (IVD), used as a positive methylated control, and normal skin (NS), used as an unmethylated control.

Gene by gene, the cases of *CDH1* deserve particular attention. The cross-talk between different DNA methylation events is represented by the epigenetic regulation of *E-cadherin* (*CDH1*) and its transcriptional repressor *Snail*. *CDH1* is a bona fide tumor-suppressor gene for which germline mutations confer an inherited high risk of developing gastric cancer (20) and codes for a cell-cell adhesion molecule (21). Hypermethylation-associated silencing of *CDH1* is found in human tumors (22), but loss of *CDH1* expression can also be due to the presence of the transcriptional repressor *Snail* (23, 24). Additionally, it was found that that the *Snail* gene undergoes epigenetic inactivation in an opposite fashion to that of the *CDH1* promoter (25). At early stages of tumorigenesis the *CDH1* and *Snail* CpG islands are unmethylated and hypermethylated, respectively, whilst when the transition

from an epithelial (MSC11B9) to a spindle (MSC11A5) or dedifferentiated (HaCa4) phenotype occurs the *CDH1* promoter becomes hypermethylated and the *Snail* island undergoes full demethylation (Figure 2A). For both genes, CpG island hypermethylation is associated with gene silencing and is released by 5-aza-2-deoxycytidine treatment. *E-cadherin* (*CDH1*) is a protein largely involved in cell-cell adhesion of epithelial tissues. *CDH1* loss has been causally linked to the acquisition of invasiveness (26), and *CDH1* methylation-associated silencing occurs in human cancer (22, 27). Interestingly, the subsequent growth of metastases in secondary organs has been related to its reexpression at these distal sites (28, 29) and CDH1 reactivation by demethylation occurs in cancer cells grown as spheroids, which requires homotypic cell adhesion (30). In the mouse skin carcinogenesis model the methylation of *CDH1* is the single locus event that signaled the drastic change from epithelial to dedifferentiated HaCa4 cells, and so is highly consistent with the previously described pattern of *E-cadherin* expression in this model and the acquisition of a metastatic phenotype (31). Furthermore, supporting the idea of epigenetic plasticity, *CDH1* hypermethylation in late stages of tumorigenesis is associated with the demethylation of its transcriptional repressor Snail, which features methylation-associated silencing in the early stages. The dynamics of Snail CpG island methylation is also strictly consistent with its expression pattern during mouse skin tumor progression (23). Furthermore, because *Snail* recruits histone deacetylases (HDACs) to the *CDH1* promoter (32), which themselves associate with DNMTs, the epigenetic reactivation of Snail in late stages of tumorigenesis may promote hypermethylation of the *CDH1* CpG island and its full silencing. This is a good example of cross-talk between epigenetic events.

Epigenetic changes are not isolated events in the molecular biology of the cancer cell, and a bi-directional communication with genetic events must exist. It is known that naturally occurring genetic defects in elements involved in the DNA methylation and chromatin machinery (*DNMT3b, ATRX, PML-RARβ*) cause DNA methylation changes at particular chromosomal loci (33). Similarly, the epigenetic silencing of DNA repair genes, such as *MLH1*, which controls mismatch repair, or the alkyl-group enzyme *MGMT*, is associated with the formation of certain gene mutations (19). Considering the latter gene, it is extremely intriguing that in the mouse skin tumor progression model the methylation-associated silencing of *MGMT* is almost exclusively observed in the cancer cell lines obtained by treatment with DMBA. The loss of *MGMT* activity has been associated with the generation of *K-ras* and *H-ras* transition mutations in mouse knockout models, cell lines and primary tumors (34-36), and, specifically, DMBA induces the adduct in the codon 61 of H-ras that, unless it is repaired by

MGMT, generates the transition mutation (2). Can a small population of normal cells with epigenetically inactivated *MGMT* preexist so that it expands clonally after DMBA induction? It is known, for example, that different intermediate states of methylation density in the CpG island of *MGMT* exist in normal diploid immortalized IMR90 cells (37). Again, the plasticity of methylation and the dynamism of the epigenetic changes could be a support for this hypothesis.

4. METHYLATION-ASSOCIATED SILENCING IN CANCER PROGRESSION INVOLVES ABNORMAL HISTONE MODIFICATIONS

It has been widely proposed that the hypermethylated CpG islands will attract methyl-DNA binding activities that will later recruit corepressor complexes that modify the structure of the chromatin to produce a transcriptionally silenced state (18, 38). The presence of different changes in the acetylation, methylation and phosphorylation status of histone, the denominated histone code (39), is fundamental to the determination of the active or silenced status of any given gene. Regarding tumor progression, chromatin immunoprecipitation (ChIP) assays using two antibodies against anti-acetylH4 and anti-dimethylK4 H3 that have been related with transcriptional activation in the mouse skin carcinogenesis model show a drastic loss of acetyl-H4 and dimethylK4-H3 in hypermethylated CpG island promoters, whilst there is significant enrichment in the unmethylated CpG islands in both modifications (25). For instance, the *MLH1* promoter that is unmethylated and actively transcribed in all cell lines has large amounts of both acetylH4 and dimethylK4H4 throughout tumoral progression. The scenario for *CDH1* and *Snail*, however, is more dynamic: in the PAM212 cell line an unmethylated CpG island for *CDH1* is associated with enhanced amounts of acetylH4 and dimethylK4-H3 and active transcription, while in CarB and CarC cells a dramatic fall of both histone modifications is associated with CpG island hypermethylation and silencing. For Snail, the opposite is the case: methylation of the island, loss of acetylH4 and dimethylK4-H3 and silencing in PAM212, and an unmethylated CpG island with increased acetylH4 and dimethylK4-H3 and active transcription in CarB and CarC cells (25).

5. ALTERED EXPRESSION OF DNA METHYLTRANSFERASES (DNMTS) DURING CANCER PROGRESSION AND METASTASIS

DNMTs are the enzymes directly responsible for hypermethylation of CpG islands (40) and are also able to recruit histone deacetylases to these methylated regions (41, 42). The expression of DNMT1 and DNMT3b in the mouse skin carcinogenesis model shows increased levels of both DNMTs from early to late stages of tumorigenesis. For example, the highly metastatic CarC cell line has levels of DNMT1 and DNMT3b four times that of the immortalized MDA3D cells, while intermediate stages (such as PAM212 cell lines) have intermediate values of DNMT1/3b activities (a two-fold increase with respect to MDA3D) (25). Thus, CpG island hypermethylation of tumor-suppressor genes is not an isolated phenomenon in the epigenetics of cancer progression and metastasis, since it occurs in a context of profound disturbance in the histone and DNA methylation machinery.

6. A GENOMIC OVERVIEW OF METHYLATION-ASSOCIATED SILENCING IN TUMOR PROGRESSION AND METASTASIS AND THE SEARCH FOR NEW TARGETS

To obtain a panoramic view of the number of genes that become epigenetically silent throughout the various stages of tumorigenesis, a mouse cDNA microarray has recently been used containing 15,000 clones with the RNA from each untreated cell line throughout the mouse skin carcinogenesis model, which is compared with that of the same cell line after treatment with the demethylating agent 5-aza-2-deoxycytidine. The efficiency of the demethylating treatment in this kind of experiment is determined by quantifying the 5-methylcytosine content by HPCE (Figure 3A) (14) and by reactivating the methylation-associated genes, as described above.

The cDNA microarray expression analysis demonstrates that an increasing number of genes undergo methylation-associated silencing in the course of tumorigenesis and metastasis. After treatment with the demethylating drug the cell lines representing early stages of carcinogenesis, such as MCA3D or PAM212, show a reactivation of 2.11% and 2.45% of their genes, respectively, while those representing metastatic stages, such as CarB and CarC, featured restored expression of 4.63% and 5.89% of their genes, respectively.

The reactivation expression microarray data allow the identification of a number of candidate sequences with more than three-fold upregulation after treatment with the demethylating agent (Figure 3B).

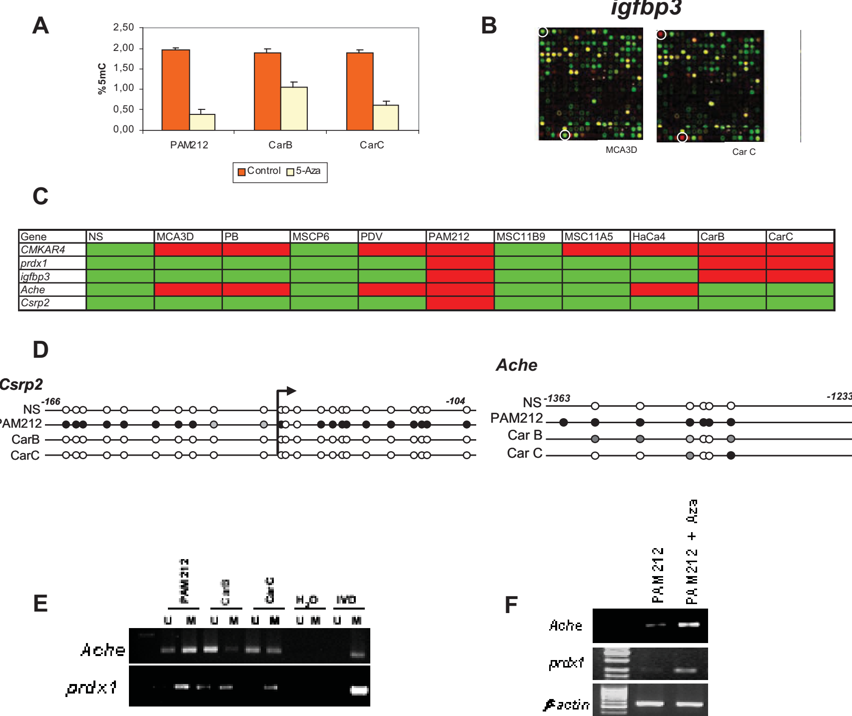


Figure 3. Searching for new methylated genes by combining cDNA microarray technology with 5-aza-2-deoxycytidine treatment. A. Measurement of 5-methylcytosine content by HPCE in mouse skin cancer cell lines treated and untreated with 5-aza-2-deoxycytidine. Results are expressed as the mean ± SD. B. representative blocks of a MouseChip array showing overexpression (red circles) of igfbp3 gene in CarC cell line relative to MCA3D. C. Summary of the methylation-specific PCR (MSP) and bisulfite sequencing analyses of the CpG island methylation status of five positive genes in the DNA microarray. D. Schematic representation of the methylation status of the CpG islands of some of the candidate genes in several mouse skin cancer cell lines obtained by bisulfite genomic sequencing. E. Example of the MSP analysis of some of the candidate genes identified. F. Example of the RT-PCR analysis of the Ache, and prdx1 genes. Restoration of gene expression is observed in PAM212 cell lines treated with Aza when compared with the untreated ones. β-actin expression is shown as an internal control.

The analysis of methylation-associated silencing of the candidate sequences permits the pharmacological unmasking of several genes involved in tumor progression and metastasis. Of these, the gene encoding the LIM domain protein CRP2 molecular adapter (*Csrp2*) (43), the insulin-like

growth factor binding protein-3 (*igfbp3*) (44, 45), the chemokine receptor *CMKAR4* (46), the enzyme acetylcholinesterase (*Ache*) (47), and the antioxidant enzyme peroxiredoxin-1 (*prdx1*) (48) (Figure 3B) is of particular relevance. The promoter of these genes is hypermethylation at different stages of the mouse skin carcinogenesis model (Figures 3D, 3E), and the presence of promoter hypermethylation is associated with transcriptional repression, which is released by the use of the demethylating drug (Figure 4F).

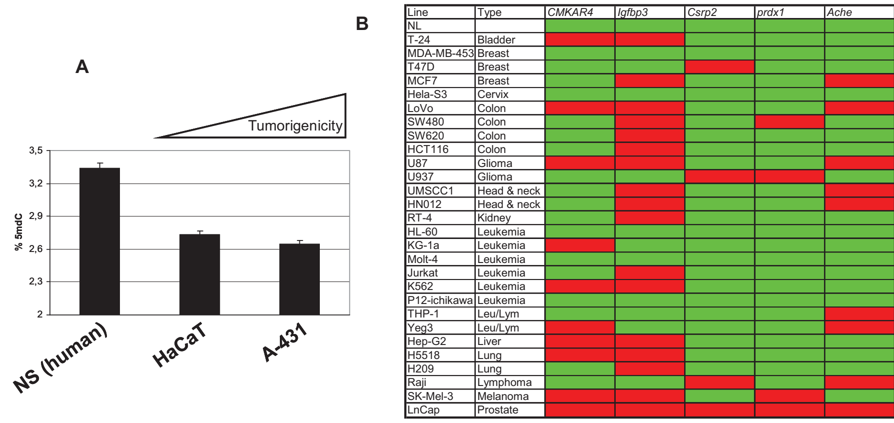
7. FROM MOUSE TO HUMANS: GENOMIC HYPOMETHYLATION AND HYPERMETHYLATION SILENCING OF GENES WITH GROWTH-INHIBITORY PROPERTIES IN HUMAN CANCER

The evaluation of the reliability of any mouse tumorigenic system as a model for human tumorigenesis with respect to the identified aberrant DNA methylation changes is essential for assessing its true worth.

The validation of the epigenetic alterations observed in the mouse skin multistage carcinogenesis model in humans was achieved by analyzing three different stages of nonmelanoma human skin cancer: normal skin (NS), immortal nontumorigenic human keratinocytes (HaCaT) (49) and high-grade malignant human epidermoid carcinoma cells (A431) (50). As observed in the mouse cells, an increased loss of 5-methylcytosine genomic content is associated with increased tumorigenic activity (Figure 4A). Furthermore, from the CpG island hypermethylation standpoint, three of the five (60%) CpG islands found to be hypermethylated in the mouse cells are also hypermethylated in the nonmelanoma skin cancer cell line A431. All five methylation-positive genes in the mouse model (*Csrp2*, *igfbp3*, *CMKAR4*, *Ache* and *prdx1*) also have methylation-associated silencing in an extensive panel of human cancer cell lines (n=28) (Figure 4B). CpG island promoter hypermethylation of *igfbp*3 is the most common event, being present in 61% (17/28) of cancer cell lines from different cell types (Figure 4B).

To gain further insight into the potential role and relevance of hypermethylated genes in tumor formation, they can be reintroduced into human cancer cell lines where they are inactivated by hypermethylation (51-53). After selection of drug-resistant colonies and the demonstration that each gene is reexpressed, the genetic restoration of any of these genes is found to be sufficient to decrease colony formation significantly.

Finally, all these genes are also hypermethylated in human primary tumors with a tumor-type-specific profile (Figure 4C). For example the chemokine inhibitor *CMKAR4* is methylated in leukemia but not in mammary or colon tumors. From a quantitative standpoint, *igfbp3* CpG island hypermethylation is the most common epigenetic alteration, similarly to what is observed in the cell lines, being found in 57% (37/65) of all primary malignancies. Thus, the aberrations in DNA methylation found in the mouse multistage skin carcinogenesis model are also mirrored in human tumorigenesis.



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	Breast	Lung	AML	Colon	Head & Neck	Glioma	Skin
Igfbp3	3/12 (25%)	10/12 (83%)	8/12 (67%)	9/14 (64%)	3/3 (100%)	4/12 (33%)	3/25 (12%)
CMKAR4	0/13	3/18 (16%)	2/14 (14%)	0/10	1/3 (33%)	3/12 (25%)	8/26 (31%)
prdx1	0/12	0/12	0/14	0/18	0/6	2/12 (17%)	14/24 (58%)
Csrp2	3/11 (27%)	0/12	2/13 (15%)	1/18 (6%)	1/3 (33%)	4/13 (13%)	6/24 (25%)
Ache	4/16 (25%)	1/15 (7%)	2/18 (11%)	0/18	-	0/12	1/25 (4%)

Figure 4. Promoter methylation analyses of the genes methylated in the mouse model in humans. A. Global DNA methylation in a human skin progression model quantified by high-performance capillary electrophoresis. B. Summary of the methylation-specific PCR (MSP) analyses of the CpG island methylation status of five candidate genes obtained by DNA microarray technology in several human cancer cell lines. C. Percentage of CpG island methylation at IGFBP3, CMKAR4, PRDX1, CSRP2 and AchE genes in breast, lung, colon, head and neck human primary tumors, acute myeloid leukemia and glioma.

The similar epigenetic behavior observed in the multistage skin tumor progression and in human cancer cell lines and primary tumors, may establish the mouse model as an excellent system for testing all current and newly produced anticancer drug targeting epigenetics (54). We are at an early stage in the use of DNA demethylating drugs and inhibitors of histone

deacetylases for treating cancer, although clinical studies using these agents have been initiated throughout the world. The versatility of the mouse carcinogenesis model now offers a safe basis for testing all these compounds for their clinical virtues and toxic side effects in a biological environment that has been carefully analyzed both genetically and epigenetically.

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REFERENCES

1. Fearon E. R., Vogelstein B. A genetic model for colorectal tumorigenesis. Cell, 1990; 61:759-767.
2. Quintanilla M., Brown K., Ramsden M., Balmain A. Carcinogen-specific mutation and amplification of Ha-ras during mouse skin carcinogenesis. Nature, 1986; 322:78-80.
3. Bizub D., Wood A. W., Skalka A. M. Mutagenesis of the Ha-ras oncogene in mouse skin tumors induced by polycyclic aromatic hydrocarbons. Proc Natl Acad Sci U S A, 1986; 83:6048-6052.
4. Yuspa S. H. The pathogenesis of squamous cell cancer: lessons learned from studies of skin carcinogenesis--Thirty-Third G. H. A. Clowes Memorial Award Lecture. Cancer Res, 1994; 54:1178-1189.
5. Quintanilla M., Haddow S., Jonas D., Jaffe D., Bowden G. T., Balmain A. Comparison of ras activation during epidermal carcinogenesis in vitro and in vivo. Carcinogenesis, 1991; 12:1875-1881.
6. Balmain A., Harris C. C. Carcinogenesis in mouse and human cells: parallels and paradoxes. Carcinogenesis, 2000; 21:371-377.
7. Hann B., Balmain A. Building 'validated' mouse models of human cancer. Curr Opin Cell Biol, 2001; 13:778-784.
8. Balmain A. Cancer as a complex genetic trait: tumor susceptibility in humans and mouse models. Cell, 2002; 108:145-152.
9. Feinberg A. P., Vogelstein B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature, 1983; 301:89-92.
10. Ehrlich M. “DNA hypomethylation in cancer.” In *DNA alterations in cancer: genetic and epigenetic changes*, M. Ehrlich ed.: Eaton Publishing, Natick, 2000.
11. Esteller M., Fraga M. F., Guo M., Garcia-Foncillas J., Hedenfalk I., Godwin A. K., Trojan J., Vaurs-Barriere C., Bignon Y. J., Ramus, S., et al. DNA methylation patterns in hereditary human cancers mimic sporadic tumorigenesis. Hum Mol Genet, 2001; 10:3001-3007.
12. Fraga M. F., Rodríguez R., Canal M. J. Rapid quantification of DNA methylation by high performance capillary electrophoresis. Electrophoresis, 2000; 21:2990-2994.

13. Fraga M. F., Uriel E., Borja Diego L., Berdasco M., Esteller M., Canal, M. J., Rodríguez R. High-performance capillary electrophoretic method for the quantification of 5-methyl 2'-deoxycytidine in genomic DNA: application to plant, animal and human cancer tissues. Electrophoresis, 2002; 23:1677-1681.
14. Paz M. F., Fraga M. F., Avila S., Guo M., Pollan M., Herman J. G., Esteller M. A systematic profile of DNA methylation in human cancer cell lines. Cancer Res, 2003; 63:1114-1121.
15. Habib M., Fares F., Bourgeois C. A., Bella C., Bernardino J., Hernandez-Blazquez F., de Capoa A., Niveleau A. DNA global hypomethylation in EBV-transformed interphase nuclei. Exp Cell Res, 1999; 249:46-53.
16. Gaudet F., Hodgson J. G., Eden A., Jackson-Grusby L., Dausman, J., Gray J. W., Leonhardt H., Jaenisch R. Induction of tumors in mice by genomic hypomethylation. Science, 2003; 300:489-492.
17. Esteller M., Risques R. A., Toyota M., Capella G., Moreno V., Peinado M. A., Baylin S. B., Herman J. G. Promoter hypermethylation of the DNA repair gene O(6)-methylguanine-DNA methyltransferase is associated with the presence of G:C to A:T transition mutations in p53 in human colorectal tumorigenesis. Cancer Res, 2001; 61:4689-4692.
18. Jones P. A., Baylin S. B. The fundamental role of epigenetic events in cancer. Nat Rev Genet, 2002; 3:415-428.
19. Esteller M. CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. Oncogene, 2002; 21:5427-5440.
20. Guilford P., Hopkins J., Harraway J., McLeod M., McLeod N., Harawira P., Taite H., Scoular R., Miller A., Reeve A. E. E-cadherin germline mutations in familial gastric cancer. Nature, 1998; 392:402-405.
21. Takeichi M. Morphogenetic roles of classic cadherins. Curr Opin Cell Biol, 1995; 7:619-627.
22. Graff J. R., Herman J. G., Lapidus R. G., Chopra H., Xu R., Jarrard D. F., Isaacs W. B., Pitha P. M., Davidson N. E., Baylin S. B. E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. Cancer Res, 1995; 55:5195-5199.
23. Cano A., Perez-Moreno M. A., Rodrigo I., Locascio A., Blanco M. J., del Barrio M. G., Portillo F., Nieto M. A. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat Cell Biol, 2000; 2:76-83.
24. Batlle E., Sancho E., Franci C., Dominguez D., Monfar M., Baulida J., Garcia de Herreros A. The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. Nat Cell Biol, 2000; 2:84-89.
25. Fraga M. F., Herranz M., Espada J., Ballestar E., Paz M. F., Ropero S., Erkek E., Bozdogan O., Peinado H., Niveleau A., Mao J. H., Balmain A., Cano A., Esteller M. A mouse skin multistage carcinogenesis model reflects the aberrant DNA methylation patterns of human tumors. Cancer Res, 2004, 64:5527-5534.
26. Perl A. K., Wilgenbus P., Dahl U., Semb H., Christofori G. A causal role for E-cadherin in the transition from adenoma to carcinoma. Nature, 1998; 392:190-193.
27. Yoshiura K., Kanai Y., Ochiai A., Shimoyama Y., Sugimura T., Hirohashi S. Silencing of the E-cadherin invasion-suppressor gene by CpG methylation in human carcinomas. Proc Natl Acad Sci U S A, 1995; 92:7416-7419.
28. Takeichi M. Cadherins in cancer: implications for invasion and metastasis. Curr Opin Cell Biol, 1989; 5:806-811.
29. Takeichi M. Cadherin cell adhesion receptors as a morphogenetic regulator. Science, 1991; 251:1451-1455.

30. Graff J. R., Gabrielson E., Fujii H., Baylin S. B., Herman J. G. Methylation patterns of the E-cadherin 5' CpG island are unstable and reflect the dynamic, heterogeneous loss of E-cadherin expression during metastatic progression. J Biol Chem, 2000; 275:2727-2732.
31. Navarro P., Gomez M., Pizarro A., Gamallo C., Quintanilla M., Cano A. A role for the E-cadherin cell-cell adhesion molecule during tumor progression of mouse epidermal carcinogenesis. J Cell Biol, 1991; 115:517-533.
32. Peinado H., Ballestar E., Esteller M., Cano A. The transcription factor Snail mediates E-cadherin repression by the recruitment of the Sin3A/Histone Deacetylase 1/2 complex. Mol. Cell Biol, 2004; 24:306-319.
33. Di Croce L., Raker V. A., Corsaro M., Fazi F., Fanelli M., Faretta M., Fuks F., Lo Coco F., Kouzarides T., Nervi C., et al. Methyltransferase recruitment and DNA hypermethylation of target promoters by an oncogenic transcription factor. Science, 2002; 295:1079-1082.
34. Pegg A. E. Repair of O(6)-alkylguanine by alkyltransferases. Mutat Res, 2000; 462:83-100.
35. Esteller M., Toyota M., Sanchez-Cespedes M., Capella G., Peinado M. A., Watkins D. N., Issa J. P., Sidransky D., Baylin S. B., Herman J. G. Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is associated with G to A mutations in K-ras in colorectal tumorigenesis. Cancer Res, 2000; 60:2368-2371.
36. Esteller M., Corn P.G., Baylin S.B., Herman J.G. A gene hypermethylation profile of human cancer. Cancer Res, 2001; 61:3225-3229.
37. Danam R. P., Howell S. R., Remack J. S., Brent T. P. Heterogeneous methylation of the O(6)-methylguanine-DNA methyltransferase promoter in immortalized IMR90 cell lines. Int J Oncol, 2001; 18:1187-1193.
38. Ballestar E., Esteller M. The impact of chromatin in human cancer: linking DNA methylation to gene silencing. Carcinogenesis, 2002; 23:1103-1109.
39. Turner B. M. Cellular memory and the histone code. Cell, 2002; 111:285-291.
40. Rhee I., Bachman K. E., Park B. H., Jair K. W., Yen R. W., Schuebel K. E., Cui H., Feinberg A. P., Lengauer C., Kinzler K. W., et al. DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. Nature, 2002; 416:552-556.
41. Fuks F., Burgers W. A., Brehm A., Hughes-Davies L., Kouzarides T. DNA methyltransferase Dnmt1 associates with histone deacetylase activity. Nat Genet, 2000; 24:88-91.
42. Deplus R., Brenner C., Burgers W. A., Putmans P., Kouzarides T., de Launoit Y., Fuks F. Dnmt3L is a transcriptional repressor that recruits histone deacetylase. Nucleic Acids Res, 2002; 30:3831-3838.
43. Weiskirchen R., Gunther, K. The CRP/MLP/TLP family of LIM domain proteins: acting by connecting. Bioessays, 2003; 25:152-162.
44. Baxter R. C. Signalling pathways involved in antiproliferative effects of IGFBP-3: a review. Mol Pathol, 2001; 54:145-148.
45. Furstenberger G., Senn H. J. Insulin-like growth factors and cancer. Lancet Oncol, 2002; 3:298-302.
46. Murakami T., Maki W., Cardones A .R., Fang H., Tun Kyi. A., Nestle F. O., Hwang S.T. Expression of CXC chemokine receptor-4 enhances the pulmonary metastatic potential of murine B16 melanoma cells. Cancer Res, 2002; 62:7328-7334.
47. Keller M., Robitzki A., Layer P. G. Heterologous expression of acetylcholinesterase affects proliferation and glial cytoskeleton of adherent chicken retinal cells. Cell Tissue Res, 2001; 306:187-198.

48. Manevich Y., Sweitzer T., Pak J. H., Feinstein S. I., Muzykantov V., Fisher A. B. 1-Cys peroxiredoxin overexpression protects cells against phospholipid peroxidation-mediated membrane damage. Proc Natl Acad Sci U S A, 2002; 99:11599-11604.
49. Boukamp P., Petrussevska R. T., Breitkreutz D., Hornung J., Markham A., Fusenig N. E. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J Cell Biol, 1998; 106:761-771.
50. Giard D. J., Aaronson S. A., Todaro G. J., Arnstein P., Kersey J. H., Dosik H., Parks W. P. *In vitro* cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. J Natl Cancer Inst, 1973; 51:1417-1423.
51. Tomizawa Y., Sekido Y., Kondo M., Gao B., Yokota J., Roche J., Drabkin H., Lerman M. I., Gazdar A. F., Minna J. D. Inhibition of lung cancer cell growth and induction of apoptosis after reexpression of 3p21.3 candidate tumor suppressor gene SEMA3B. Proc Natl Acad Sci U S A, 2001; 98:13954-13959.
52. Yoshikawa H., Matsubara K., Qian G. S., Jackson P., Groopman J. D., Manning J. E., Harris C. C., Herman J. G. SOCS-1, a negative regulator of the JAK/STAT pathway, is silenced by methylation in human hepatocellular carcinoma and shows growth-suppression activity. Nat Genet, 2001; 28:29-35.
53. Yamashita K., Upadhyay S., Osada M., Hoque M. O., Xiao Y., Mori M., Sato F., Meltzer S. J., Sidransky D. Pharmacologic unmasking of epigenetically silenced tumor suppressor genes in esophageal squamous cell carcinoma. Cancer Cell, 2002; 2:485-495.
54. Villar-Garea A., Fraga M. F., Espada J., Esteller M. Procaine is a DNA demethylating agent with growth-inhibitory effects in human cancer cells. Cancer Res, 2003; 63: 4984-4989.

Chapter 3

CPG ISLAND HYPERMETHYLATION AND LUNG CANCER INVASION AND METASTASIS

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Abstract: Invasion and metastasis are biological hallmarks of malignant tumors, and metastases are the major cause of cancer deaths. Invasion and destruction of BM is the earliest step in the multi-step process of metastases and it is the earliest morphological feature of invasive tumors. Disruption of organization or integrity of the basement membrane (BM) is a key histologic marker of the transition of a tumor from an *in situ* carcinoma to an invasive carcinoma. A fundamental and important question is what causes *in situ* cancers to become invasive even though cancer cells at the preinvasive and invasive stages are morphologically similar. One of the well-established mechanisms for invading and destroying BMs is by matrix metalloproteinases (MMPs), which are up regulated during invasion and metastasis. Developing molecular markers that mark the transition of *in situ* cancers to invasive cancer are very important because they may predict cancer for those who are at highest risk or those with early invasive cancers. It is logical to presume that disruption of all the homotypic and heterotypic cell adhesion junctions occurs in invasive cells, and that the loss of the involved protein components as well as loss of substances that inhibit tissue invasion may mark the transition from *in situ* to invasive cancers. This chapter reviews the different cell adhesion junctions and candidate invasion genes, which are inactivated by aberrant promoter methylation and their potential use as molecular markers.

Key words: invasion, metastasis, homotypic, heterotypic, cell adhesion, methylation, cancer

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1. INTRODUCTION

1.1 Lung Cancer Incidence and Subtypes

Lung cancer is the number-one cause of cancer-related deaths in industrialized countries. It is the leading cause of deaths in both men and women in the United States, killing more than 150,000 people every year (1, 2). These deaths are undoubtedly related to the causal relationship of cigarette smoking and bronchogenic carcinoma. The four major histologic types of brochogenic carcinomas are squamous cell carcinoma (SCC), adenocarcinoma, large cell undifferentiated carcinoma (LC), and small cell carcinoma (SCLC). It has become apparent that for most therapeutic decisions the first three can be lumped into a category termed non-small cell lung carcinoma (NSCLC) to distinguish them from SCLC which is one of the most virulent forms of human cancer, characterized by early dissemination and aggressive clinical evolution as compared to NSCLC. SCLC account for 25% of bronchogenic carcinomas, and the remaining tumors, NSCLC. In some cases there is a mixed histologic patterns, which account for 10% (3).

1.2 Origin, Evolution and Progression of Lung Cancer

Ninety-five per cent of primary lung tumors arise from the respiratory epithelium (bronchogenic or lungcarcinomas); the remaining 5% are a miscellaneous group that includes mesotheliomas, mesenchymal malignancies, lymphomas, and a few benign lesions (3). Several lines of evidence indicate that tumorigenesis in humans is a multi-step process and that these steps reflect genetic alterations that drive the progressive transformation of normal human cells into highly malignant derivatives (4). Molecular changes commence in histologically normal epithelium, and thus precede the onset of pathologically recognizable lesions (5, 6). Many defined patterns of epigenetic changes and genetic alterations characteristic of lung cancers can be detected in preneoplastic lesions, including deregulation of *p53*, *p16*, *FHIT*, and telomerase genes, and neoangiogenesis (7-12).

The pathology and molecular biology of lung cancer demonstrate that these tumors evolve through series of mutations, molecular abnormalities, and concomitant morphologic and histologic changes. Because multiple etiologic agents (13) cause occupational and environmental lung cancers, the integration of histology with cellular, biochemical, and molecular biomarker analysis may provide new approaches toward understanding lung cancer

pathogenesis. Hanahan and Weinberg (4) have suggested that the vast catalog of cancer cell genotypes is a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth: (a) self-sufficiency in growth signals, (b) insensitivity to growth-inhibitory (antigrowth) signals, (c) evasion of programmed cell death (apoptosis), (d) limitless replicative potential, (e) sustained angiogenesis and (f) tissue invasion and metastasis. "Each of these physiologic changes, novel capabilities acquired during tumor development, represents the successful breaching of an anticancer defense mechanism hardwired into cells and tissues". Hanahan and Weinberg proposed that these six capabilities are shared in common by most and perhaps all types of human tumors. This multiplicity of defenses may explain why cancers occur occasionally lifetime.

Lung cancers are believed to arise after a series of progressive pathological changes (preneoplastic or precursor lesions) in the respiratory mucosa (14). While the sequential preneoplastic changes have been defined for centrally arising squamous carcinoma, they have been poorly documented for LC, ADC and SCLC. Mucosal changes in the large airways that may precede or accompany invasive squamous cell carcinoma include hyperplasia, squamous metaplasia, squamous dysplasia and carcinoma *in situ*, still outlined by an intact basement membrane (BM) (14). Carcinoma *in situ* is a flat, superficial lesion and is the most common precursor to invasive cancers. Invasion and metastasis requires breakdown of cell-to-cell attachments, break down of BM and spread of cancer cells through ECM, which involves activation of matrix metalloproteinases MMPs. During invasion and metastasis, molecular changes induce local dissemination of carcinoma cells, possibly through an epithelial-mesenchymal transition (EMT), and the basement membrane becomes fragmented (15). The cells detachment from the primary tumor mass, migration through the extracellular matrix (ECM), degradation of the vascular endothelial BM and penetration into the vascular lumen – a process known as intravasation – survival within the circulation, which reflects resistance to both shear stress and immune surveillance, adhesion to and proliferation on distal vascular endothelia, and finally penetration into a new host tissue microenvironment and establishment of a relationship with the local stroma that is conducive to new tumor colony outgrowth. At secondary sites, extravasated solitary carcinoma cells either remain solitary micrometastasis or they can form a new macrometastasis through a mesenchymal-epithelial transition (MET) (15). Several if not all of these steps depend at least in part on matrix metalloproteinase (MMPs) activity. The MMPs are a family of zinc containing endopeptidases, which collectively are able to cleave most (if not all) ECM proteins (16). To date more than 21 human MMPs and

homologues from other species (17), and four endogenous tissue inhibitors (TIMPs) of MMPs have been characterized (16). MMPs are secreted from stroma while TIMPs are made from epithelial cells. TIMPs play important roles in normal physiology, as the ECM is a dynamic matrix of structural proteins, growth factors and latent enzymes that is constantly being remodeled. The TIMPs negatively regulate the proteolytic activity of MMPs during ECM turnover. TIMPs can suppress cell proliferation, invasion and reduce metastasis formation through inhibition of MMP activity and prevention of ECM turnover, i.e. TIMPs act as tumor suppressors (16).

Invasion and metastasis are biological hallmarks of malignant tumors, and metastases are the major cause of cancer deaths. Invasion and destruction of BM is the earliest step in the multi-step process of metastases and it is the earliest morphological feature of invasive tumors. Disruption of organization or integrity of the BM is a key histologic marker of the transition of a tumor from an *in situ* carcinoma to an invasive carcinoma. The most fundamental question is what causes in situ cancers to become invasive even though cancer cells at the preinvasive and invasive stages are morphologically similar. One of the well-established mechanisms for invading and destroying BMs is by MMPs, which are up regulated during invasion and metastasis. Developing molecular markers that mark the transition of in situ cancers to invasive cancer are very important because they may predict cancer for those who are at highest risk or those with early invasive cancers. Such markers should be normally expressed in epithelial cells, which are tethered to BM, and provide a barrier against invasion, by preinvasive cancers.

2. CELL ADHESION

Cell adhesion is a fundamental process influencing the life of most cells and may be divided into homotypic (cell-cell contacts) and heterotypic (cell-ECM) adhesions (Figure.1).

It is essential for tissue organization during development and for the maintenance of tissue integrity in adult organisms (18). Multicellular organisms have specialized cells, epithelial and endothelial that form selective barriers between tissues and different body compartments. Epithelial cells, except for cells in certain stratified multicellular surfaces such as skin, are polarized, i.e., they have an apical domain and a basolateral domain and they adhere to each other through complexes that form junctions between the neighboring cells.

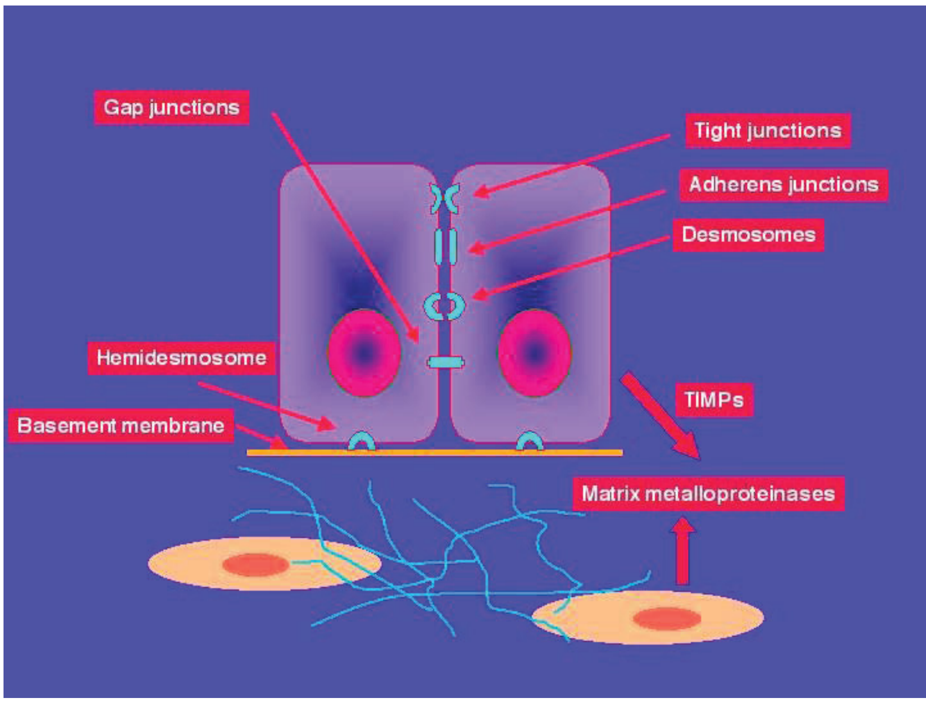

Figure 1. Types of cellular junctions and components of the extracellular matrix that play a role in invasion and metastasis. See text for details.

These intercellular junctions not only carry out adhesive functions but also contain crucial components of the signaling pathways that regulate epithelial proliferation and differentiation. In humans, while homotypic adhesion is mediated by a set of intercellular junctions that consists of gap junctions, desmosomes (maculae adhaerentes), adherens junctions (AJs or zonulae adhaerentes) and tight junctions (TJs or the zonulae occludentes), heterotypic adhesion is mediated by hemidesmosomes (HDs) located in the BM. Desmosomes, AJs and TJs are often referred to as the 'epithelial junctional complex' observed early in epithelia of many glands (18, 19). Betaig-H3 is TGF-beta-inducible cell adhesion molecule and its down regulation is linked to tumorigenic phenotype in asbestos treated immortalized human bronchial epithelial cells (20).

2.1 Cell - cell Adhesion

Gap junction channels are intercellular channels that allow the passage of ions and other small molecules between neighboring cells (permeant to molecules up to 1 kDa) (19). They are formed from two multimeric subunits

called hemichannels or connexons that reside in the plasma membranes of two closely opposed cells (21). Connexons are composed of six transmembrane protein subunits called connexins (Cx). The connexins belong to a multigene family composed of at least 19 human members. All of the connexins have four-membrane-spanning domains, and both the NH_2 and COOH terminals reside on the cytoplasmic side of the membrane. The regions of the proteins corresponding to the transmembrane-spanning and extracellular domains are highly conserved among connexins. In contrast, the regions corresponding to the central cytoplasmic loop and carboxy tail show much less homology (21).

Desmosomes are patch-like intercellular junctions found in vertebrate tissue, and are particularly abundant in tissues undergoing mechanical stress. The central plaque contains adhesion molecules such as desmocollins, desmogleins and is an anchorage point for cytoskeletal filaments of the intermediate filament type (19). Desmogleins and desmocollins, adhesive transmembrane glycoprtoeins, constitute the subgroup of desmosomal cadherins (18). They are connected to the intermediate filament network of the cytoskeleton via associated cytoplasmic proteins such as the common plaque protein plakoglobin and several other cell type specific proteins including desmoplakins and plakophilins. Desmosomes are not restricted to a particular site but are distributed along the entire lateral membrane. Three desmogleins (Dsg 1, 2 and 3) and three desmocollins (Dsc 1, 2 and 3) have been identified in humans; their genes locate to a single cluster on chromosome 18q21. Dsg 3 and Dsc 2 appear to be ubiquitously expressed (18). AJs mediate cell-cell adhesion via members of the cadherin family of transmembrane proteins and their connections to the cortical actin-based cytoskeleton. Depending on the epithelium, AJ components can be concentrated close to TJs and colocalize with a prominent actin belt (19), whereas in other types of epithelial cells, they can be distributed over the entire lateral membrane (for example, in cells of the mouse Trophectoderm) (22).

Tight junctions (TJs) with occludin, claudins and junctional adhesion molecules (JAMs) as integral membrane proteins act as important apical barriers, both regulating paracellular permeability and separating apical and basolateral membrane regions, thereby creating polarity (23, 24). Occludin is an integral membrane protein with four transmembrane domains, a long carboxy-terminal cytoplasmic domain and a short amino-terminal cytoplasmic domain. It seems to mediate cell-to-cell adhesion via its first extracellular domain and to form the paracellular permeability barrier via its second extracellular domain. The claudin superfamily consists of at least 18 homologous proteins in humans. These proteins are important structural and functional components of tight junctions in paracellular transport. These

proteins also have four transmembrane domains, but do not show any sequence similarity to occludins (24). There is accumulating evidence that claudins constitute the backbone of tight-junction strands (23). Claudins complexed with occludin and JAM, located in both epithelial and endothelial cells in all tight junction bearing tissues. Expression pattern of claudins varies depending on cell type. Claudins interact directly with tight junction-specific, membrane-associated guanylate kinase homologues, ZO-1 (TJP1), ZO-2 (TJP2) and ZO-3 (TJP3), and indirectly with AF-6 and the myosin-binding molecule cingulin (24). These protein-protein interactions promote scaffolding of the TJ transmembrane proteins and provide a link to the actin cytoskeleton for transducing regulatory signals to and from tight junctions. JAM, a novel member of the immunoglobulin (Ig) superfamily, is a type I transmembrane protein with two extracellular Ig-like domains and two consensus N-glycosylation sites. It is believed to mediate homotypic cell adhesion (24).

2.2 Extracellular Matrix and Basement Membrane

ECM is mainly composed of BM (type IV collagens, laminins, proteoglycons, fibronectin and tenascin-C) and interstitial matrix (fibronectin, tenascin-C, collagens and proteoglycons) (25). BMs are characteristic of multicellular organisms, and they are the first ECM component produced during embryogenesis (26). The BM is a thin (20-200 nm) carpet-like ECM structure that regulates cell attachment, differentiation, and growth. It is flat structure separating the epithelial cells from the underlying stromal tissues and forms the important barrier for invasion. Epithelial cells must be attached both to appropriate ECM components and to other similar epithelial cells to survive, a process termed anchorage dependence. The epithelium and underlying stroma function as a unit and constantly communicate. Two-way signaling occurs via extracellular proteins (laminins) and their transmembrane receptors, intergrins. ECM consists of several molecules; two of the most important are laminin 5 (LN5 secreted by overlying epithelial cells) and collagen 4 (secreted by stromal cells). Laminins, which are the major component of BM, contribute to the architecture of the basal lamina surrounding the epithelial cells and mediate cell adhesion, growth, migration, proliferation, and differentiation (27). They are heterotrimeric glycoproteins composed of three different polypeptide chains (α, β, and γ) arranged in a cruciform structure. A separate gene encodes each polypeptide chain, and different combinations of these chains have led to the identification of 13 different laminin isoforms. Laminins are ubiquitous in BM and represent the most abundant structural non-collagenous glycoproteins. Laminins are distributed in spatially and

temporally regulated manner in various epithelial and mesenchymal tissues (28-30).

2.3 Cell - Basement Membrane Adhesion

In BM there are specialized attachment units called hemidesmosomes, which mediate adhesion of epithelial cells to the underlying BM, and connect elements of the cytoskeleton to the ECM. HDs loss is prerequisite for cell motility. The key components of HDs are LN5, its ligand integrin $\alpha 6\beta 4$, and collagenous molecule bullous pemphigoid antigen 2 (BP180, also known as BPAG2 and collagen type XVII) (31-33). Integrin $\alpha 6\beta 4$ occurs basally and mediates association between cytoskeletal elements of the cell to proteins that constitute ECM. More than simple cell-ECM connectors, the $\alpha 6\beta 4$ integrin and BP180 are involved in hemidesmosome formation. HDs are implicated in signal transduction via the $\alpha 6\beta 4$ integrin. These HD-mediated signals are likely to influence cytoskeletal structure, cell differentiation and cell growth. LN5 is a heterotrimeric protein member of the laminin family, and it consists of three polypeptide chains, A3, B3 and C2, which are the products of three different genes, *LAMA3*, *LAMB3* and *LAMC2* (34). The chains are assembled in a coiled cruciate-like structure, which is deposited in the BM. The N-terminal globular domains of the β and γ chains are important in laminin polymerization (self-assembly). The N and C terminal globular domains of α chain are involved in interacting with integrins (25). LN5 functions as ligand for $\alpha 6\beta 4$, $\alpha 6\beta 1$ and $\alpha 3\beta 1$ integrins to regulate epithelial cell anchoring to BM, cell migration, morphogenesis and cell signaling. Integrin $\alpha 3\beta 1$ and $\alpha 6\beta 1$ occur primarily at cell-cell contact and implement cell migration. Cell adhesion promoting activity of LN5 is dependent on the coiled conformation of the molecule. LN5 is abundant in transitional epithelium, stratified squamous epithelia and other epithelial glands, all of which possess hemidesmosomes (25).

2.4 Oncogenes and Tumor Suppressor Genes

Deregulation of cell division leads to tumor growth. Protooncogenes code for proteins that stimulate cell division. Mutated forms, called oncogenes overexpress the stimulatory proteins that may be overactive resulting into excessive cell proliferation. On the other hand, tumor suppressor genes (TSGs) code for proteins that inhibit cell division. The activation of oncogenes is one of the known mechanisms in transforming the normal cell into cancerous cell. Now it has become evident that the inactivation of TSGs, which are brakes for cell growth, also are crucial for the development and progression of lung cancer. Multiple mechanisms of

gene silencing including loss of heterozygosity, point mutations, homozygous deletions, and aberrant promoter methylation have been reported in tumors (35). According to Knudson's two-hit hypothesis, loss of function of TSG requires inactivation of both the alleles. One allele is inactivated by mutation, methylation, deacetylation, or other changes that target the individual TSG. The other allele is usually inactivated as part of a chromosomal loss involving the gene of interest as well as many genetic markers in the vicinity.

2.5 CpG Island Hypermethylation

Aberrant promoter methylation is the major mechanism of silencing of genes in human tumors (36-38). Methylation of DNA occurs in vertebrates at cytosine residues of CpG dinucleotides. It is a reversible epigenetic change that does not modify the nucleotide structure and is passed on to daughter cells after cell division, but it is not heritable. CpG sites are non-randomly distributed in the human genome, and promoter regions of about half of the genes are rich in CpG sites ("CpG islands"). Normally, the islands are not methylated and methylation of these sites is associated with gene silencing. The methylation of cytosine residues in the newly replicated DNA is maintained by DNA cytosine methyltransferase (DNMT) enzymes, which transfer a methyl group from the methyl donor S-adenosylmethionine using the hemimethylated DNA template. Methylation plays a crucial role in the normal organism, and its role includes gene imprinting, X-chromosome inactivation, normal development and transcriptional silencing. Methylation of CpG sites at promoter may prevent binding of sequence specific transcription factors or recruit transcriptional co-repressors. Methyl CpG binding proteins frequently act synergistically with histone deacetylases in transcriptional repression (39). Deacetylation of lysine residues in histones results in compact nucleosome structure that inhibits transcription.

CpG island methylation is an epigenetic mechanism for the transcriptional silencing of tumor suppressor genes in many cancer types, and the number of genes methylated in individual cancers is estimated to be very high (40). In a global analysis of the methylation status of over 1000 human tumors, Costello *et al*. (40) estimated that an average of 600 CpG islands (0-4500 range) were aberrantly methylated in individual tumors. Patterns of CpG island methylation that were shared within each tumor type, together with patterns and targets that displayed distinct tumor-type specificity were identified. Lung tumors have a characteristic pattern of genes methylated and inactivated at frequencies greater than 20% (41-43). Methylation commences during preneoplasia and can be detected in the

bronchial epithelium of smokers (12). Treatment with demethylating agents such as 5-aza-2'-deoxycytidine restores gene expression. Histone deacetylation inhibitors such as trichostatin A in combination with demethylating agents enhance restoration of gene expression (36). The methylation pattern in the CpG islands is studied by methylation-specific PCR (MSP) assay (44). The DNA is modified by sodium bisulfite treatment. Treatment of genomic DNA with sodium bisulfite converts unmethylated cytosines (but not methylated cytosines) to uracil, which are then converted to thymidine during subsequent PCR. Thus, after bisulfite treatment, alleles that were originally methylated have DNA sequences different from those of their corresponding unmethylated alleles, and these differences can be used to design PCR primers that are specific for methylated or unmethylated alleles.

2.6 Methylation and Inactivation of Invasion Genes

An understanding of the mechanisms of tumor invasion is fundamental to understanding cancer pathogenesis and biology. A crucial step in the development of a tumor is its invasion through the BM into the underlying stroma or the parenchyma. Identification of the earliest steps involved in the transition of *in situ* to invasive cancers will be of critical importance in identifying individuals at increased risk and for use as surrogate markers for chemo -prevention trials. As mentioned above epithelial cells are anchored to each other and to the BM by hommotypic and heterotypic junctions respectively. During invasion and metastasis, migration of clustered cells through the ECM faces much more resistance than migration of single motile cells separated from the tumor mass. It is logical to presume that disruption of all the junctions occurs in invasive cells, and that the loss of the involved protein components as well as loss of substances that inhibit tissue invasion may mark the transition from *in situ* to invasive cancers. Several candidate genes involved in invasion, their expression pattern, cellular location, and methylation status in lung caner has been listed in Table 1. Invasion genes altered through methylation and their methylation frequencies in lung cancer has been listed in Table 2.

Table 1 shows homotypic (gap junction, desmosomes, adheren junction and tight junction) and heterotypic (hemidesmosome) cell adhesions and different cell adhesion molecules encoded by respective genes. Expression by RT-PCR and methylation pattern for some of the genes is also listed in Table 1. Table 2 presents some of the cell adhesion / invasion genes altered through methylation in lung cancer.

Table 1. Candidate Tumor Invasion Genes in Lung Cancer

Cellular gene Location	Gene	Gene name	Chromosomal Location	Down regulation Yes/No	Methylation Yes/No	CpG Yes / No	References
Gap junction	*Cx 43*	Connexin 43	6q21-q23.2	Yes	Yes	Yes	(56)
Desmosomes	*DSG1*	Desmoglein 1	18q12.1	ND	ND	No	(18)
	DSG2	Desmoglein 2	18q12.1	ND	ND	Yes	(18)
	DSG3	Desmoglein 3	18q12.1	ND	ND	No	(18)
	DSC1	Desmocollin 1	18q12.1	ND	ND	No	(18)
	DSC2	Desmocollin 2	18q12.1	ND	ND	Yes	(18)
	DSC3	Desmocollin 3	18q12.1	ND	ND	Yes	(18)
Adheren Junction	*CDH1*	E-cadherin	16q22.1	Yes	Yes	Yes	(43)
	CDH13	H-cadherin	16q24.2	Yes	Yes	Yes	(46)
	CDH3	P-cadherin	16q22.1	Yes	Yes	Yes	Sathyanarayana et al (unpublished)
Tight Junction	*CLDN7*	Claudin 7	17p13	Yes	Yes	Yes	Sathyanarayana et al (unpublished)
	TJP2a	Tight junction Protein 2a	9q13-q21	Yes	Yes	Yes	Sathyanarayana et al (unpublished)
Hemi-Desmosome	*LAMA3*	Laminin A3	18q11.2	Yes	Yes	Yes	(57)
	LAMB3	Laminin B3	1q32	Yes	Yes	Yes	(57)
	LAMC2	Laminin C2	1q25-q31	Yes	Yes	Yes	(57)
Others	*Betaig-H3*	TGF beta-induced protein IG-H3 precursor	5q31	Yes	Yes	Yes	Sathyanarayana et al (unpublished)
	TIMP3	Tissue inhibitor of Matrix metallo-proteinase 3	22q12.1-q13.2	Yes	Yes	Yes	(43)

ND = Not done

Table 2. Genes altered through methylation in lung cancer.

Gene	Tumor Type	Methylation Prevalence (%)	References
CDH1	NSCLC	15/90 (33)	(43)
CDH13	NSCLC	18/42 (43)	(46)
TIMP3	NSCLC	25/109 (23)	(43)
LAMA3	NSCLC	15/34 (44)	(57)
	SCLC	20/26 (77)	(57)
	Carcinoids	8/24 (32)	(57)
LAMC2	NSCLC	8/34 (24)	(57)
	SCLC	15/26 (58)	(57)
	Carcinoids	3/24 (13)	(57)

There were significant differences in methylation frequencies between NSCLC and SCLC tumors. SCLC is perhaps the most invasive/metastatic of all human cancers, and differences in methylation frequencies between SCLC and NSCLC have been described for other genes including *CDH1* (45), *CDH13* (46) and *CASP8* (47). It also suggests that the two major forms of lung cancer arise via different pathogenic pathways. There was co-ordinate methylation of LN5 genes in SCLC tumors while NSCLC tumors showed predominantly inactivation of any one gene. Theoretically only one of the three LN5 genes needs to be methylated and silenced for loss of function of the molecule. However, in some systems, such as the DISC components involved in TRAIL mediated apoptosis, multiple genes in the same pathway are inactivated in SCLC (48). It may be advantageous for tumor growth to knock out multiple seemingly redundant genes instead of only one. Carcinoid tumors, which are low-grade slow growing malignant tumors with a relatively low metastatic rate, had the lowest frequencies of methylation than other lung cancer types.

Theoretically, loss of any of the five component chains of this laminin-integrin complex could disrupt the hemidesmosome and lead to invasion (31, 49-51). LN5 can be down or up regulated depending on specific microenvironmental features, while its absence could favor disassembly or reduction in the number of hemidesmosomes with a consequent failure of cell anchoring leading to an invasive and metastatic phenotype. Thus, while loss of any chain results in loss of the functional molecule, unopposed expression of one or more chains (especially C2) may aid invasion (34). The loss of LN5 may cause perturbations of the ECM and integrin signaling affecting growth factors and cell-cycle regulators and apoptosis. As postulated, these gene expression changes may cause potential genetic instability in lung carcinoma due to loss of an ECM protein (4). Since LN5 is a component of BM, which is a major barrier for invasion of cancer, we presume that methylation of LN5 genes may help distinguish invasive from

non-invasive cancers. Indeed, our data indicates that methylation of LN5 encoding genes distinguishes invasive from non-invasive bladder cancers.

Our data satisfy the criteria required for the demonstration of biological significance of methylation (46, 52): 1) aberrant methylation is frequent in tumor type studied; 2) methylation is a rare event in non-malignant and control tissues; 3) loss of expression is frequent in tumors; 4) aberrant methylation and expression are concordant; 5) gene expression is restored after exposure to a demethylating agent. Our results strongly suggest that silencing of *LAMA3*, *LAMB3* and *LAMC2* genes by methylation plays an important role in pathogenesis of all types of lung cancers.

Higher frequencies of loss of LN5 chains in SCLC than in NSCLC have been observed (53). Our results confirm and extend these findings and demonstrate the mechanism of loss of the LN5 chains. While promoter methylation of LN5 encoding genes appears to be an important factor in the pathogenesis of invasive lung cancers, alternative mechanisms for disruption of the BM may exist. These include inactivation of LN5 encoding genes by mechanisms other than methylation; loss of $\alpha6\beta4$ encoding genes or over expression of matrix metalloproteinase 9 (17). Of interest, selective loss of integrin $\beta4$ has been reported in SCLC (54, 55).

We believe that development of molecular markers for identification of the earliest steps involved in the transition of *in situ* to invasive lung cancers will be of great utility in understanding tumor biology, identifying individuals at increased risk and for use in early diagnosis. Invasion as described earlier is a multi-step process and many factors influence them. There are multiple genes that have to be silenced for invasion / metastasis to proceed. Methylation plays a key role in silencing of many of these gene products. However alternative methods exist. In addition the interplay between methylation and histone deacetylation needs to be studied.

REFERENCES

1. Jemal A., Thomas A., Murray T. and Thun M. Cancer statistics, 2002. CA. Cancer J Clin, 2002; 52: 23-47.
2. Virmani A. K. and Gazdar A. F. Tumor suppressor genes in lung cancer, in Methods in Molecular Biology, Tumor suppressor genes: pathways and isolation strategies (El-Deiry, W. S., ed.), Humana Press inc., Totowa, NJ. 2003; pp. 97-115.
3. Travis W. D., Colby T. V., Corrin B., Shimosato Y. and Brambilla E. Histological typing of lung and pleural tumours, 1999; p. 1-156. Berlin: Springer.
4. Hanahan D. and Weinberg R. A. The hallmarks of cancer. Cell, 2000; 100: 57-70.
5. Mao L., Hruban R. H., Boyle J. O., Tockman M. and Sidransky D. Detection of oncogene mutations in sputum precedes diagnosis of lung cancer. Cancer Res, 1994; 54: 1634-1637.

6. Wistuba I. I., Berry J., Behrens C., Maitra A., Shivapurkar N., Milchgrub S., Mackay B., Minna J. D. and Gazdar A. F. Molecular changes in the bronchial epithelium of patients with small cell lung cancer. Clin Cancer Res, 2000; 6: 2604-2610.
7. Sozzi G., Sard L., De Gregorio L., Marchetti A., Musso K., Buttitta F., Tornielli S., Pellegrini S., Veronese M. L., Manenti G., Incarbone M., Chella A., Angeletti C. A., Pastorino U., Huebner K., Bevilaqua G., Pilotti S., Croce C. M. and Pierotti M. A. Association between cigarette smoking and FHIT gene alterations in lung cancer. Cancer Res, 1197; 57: 2121-2123.
8. Franklin W. A., Gazdar A. F., Haney J., Wistuba II, La Rosa F. G., Kennedy T., Ritchey D. M. and Miller Y. E. Widely dispersed p53 mutation in respiratory epithelium. A novel mechanism for field carcinogenesis. J Clin Invest, 1997; 100: 2133-2137.
9. Keith R. L., Miller Y. E., Gemmill R. M., Drabkin H. A., Dempsey E. C., Kennedy T. C., Prindiville S. and Franklin W. A. Angiogenic squamous dysplasia in bronchi of individuals at high risk for lung cancer. Clin Cancer Res, 2000; 6: 1616-1625.
10. Hirsch F. R., Franklin W. A., Gazdar A. F. and Bunn P. A., Jr. Early detection of lung cancer: clinical perspectives of recent advances in biology and radiology. Clin Cancer Res, 2001; 7: 5-22.
11. Yashima K., Litzky L. A., Kaiser L., Rogers T., Lam S., Wistuba I. I., Milchgrub S., Srivastava S., Piatyszek M. A., Shay J. W. and Gazdar, A. F. Telomerase expression in respiratory epithelium during the multistage pathogenesis of lung carcinomas. Cancer Res., 1197; 57: 2373-2377.
12. Palisano W. A., Divine K. K., Saccomanno G., Gilliland F. D., Baylin S. B., Herman J. G., and Belinsky, S. A. Predicting lung cancer by detecting aberrant promoter methylation in sputum. Cancer Res, 2000; 60: 5954-5958.
13. Ginsberg R. J., Kris M. G. and Armstrong, J. G. Cancer of the lung, in Cancer: Priniciples and Practice of Oncology, 3rd ed. (DeVita, V. T. J., Hellman, S., and Rosenberg, S. A., eds.), Lippin cott, Philadelphia, 1993; pp. 673-758.
14. Wistuba I. I. and Gazdar A. F. Molecular abnormalities in the sequential development of lung carcinoma, in Molecular Pathology of Early Cancer (Srivastava, S., Henson, D. E., and Gazdar, A. F., eds.), IOS Press, Amsterdam, 1999; pp. 265-276.
15. Thiery J. P. Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer, 2002; 2: 442-454.
16. Stetler-Stevenson W. G. and Yu A. E. Proteases in invasion: matrix metalloproteinases. Semin. Cancer Biol, 2001; 11: 143-152.
17. Egeblad M. and Werb Z. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer, 2002; 2: 161-174.
18. Potter E., Bergwitz C., and Brabant G. The cadherin-catenin system: implications for growth and differentiation of endocrine tissues. Endocr Rev, 1999; 20: 207-239.
19. Matter K. and Balda M. S. Signalling to and from tight junctions. Nat Rev Mol Cell Biol, 2003; 4: 225-236.
20. Zhao Y. L., Piao C. Q., and Hei T. K. Downregulation of Betaig-h3 gene is causally linked to tumorigenic phenotype in asbestos treated immortalized human bronchial epithelial cells. Oncogene, 2002; 21: 7471-7477.
21. Ebihara L. New roles for connexons. News Physiol Sci, 2003; 18: 100-103.
22. Fleming T. P., Sheth B. and Fesenko I. Cell adhesion in the preimplantation mammalian embryo and its role in trophectoderm differentiation and blastocyst morphogenesis. Front Biosci, 2001; 6: D1000-1007.
23. Tsukita S., Furuse M. and Itoh M. Multifunctional strands in tight junctions. Nat. Rev. Mol. Cell Biol, 2001; 2: 285-293.

24. Heiskala M., Peterson P. A. and Yang Y. The roles of claudin superfamily proteins in paracellular transport. Traffic, 2001; 2: 93-98.
25. Teller I. C. and Beaulieu J. F. Interactions between laminin and epithelial cells in intestinal health and disease. Expert Rev Mol Med, 2001; 2001: 1-18.
26. Hood J. D. and Cheresh D. A. Role of integrins in cell invasion and migration. Nat Rev Cancer, 2002; 2: 91-100.
27. Colognato H. and Yurchenco P. D. Form and function: the laminin family of heterotrimers. Dev Dyn, 2000; 218: 213-234.
28. Hao J., Jackson L., Calaluce R., McDaniel K., Dalkin B. L. and Nagle R. B. Investigation into the mechanism of the loss of laminin 5 (alpha3beta3gamma2) expression in prostate cancer. Am J Pathol, 2001; 158: 1129-1135.
29. Calaluce R., Kunkel M. W., Watts G. S., Schmelz M., Hao J., Barrera J., Gleason-Guzman M., Isett R., Fitchmun M., Bowden G. T., Cress A. E., Futscher B. W. and Nagle R. B. Laminin-5-mediated gene expression in human prostate carcinoma cells. Mol Carcinog, 2001; 30: 119-129.
30. Patarroyo M., Tryggvason K. and Virtanen I. Laminin isoforms in tumor invasion, angiogenesis and metastasis. Semin Cancer Biol, 2002; 12: 197-207.
31. Baker S. E., Hopkinson S. B., Fitchmun M., Andreason G. L., Frasier F., Plopper G., Quaranta V. and Jones J. C. Laminin-5 and hemidesmosomes: role of the alpha 3 chain subunit in hemidesmosome stability and assembly. J Cell Sci, 1996; 109 (Pt 10): 2509-2520.
32. Michelson P. H., Tigue M. and Jones J. C. Human bronchial epithelial cells secrete laminin 5, express hemidesmosomal proteins, and assemble hemidesmosomes. J. Histochem. Cytochem, 2000; 48: 535-544.
33. Borradori L. and Sonnenberg A. Hemidesmosomes: roles in adhesion, signaling and human diseases. Curr Opin Cell Biol, 1996; 8: 647-656.
34. Giannelli G. and Antonaci S. Biological and clinical relevance of Laminin-5 in cancer. Clin Exp Metastasis, 2000; 18: 439-443.
35. Shapiro G. I., Park J. E., Edwards C. D., Mao L., Merlo A., Sidransky D., Ewen M. E., and Rollins B. J. Multiple mechanisms of p16INK4A inactivation in non-small cell lung cancer cell lines. Cancer Res, 1995; 55: 6200-6209.
36. Baylin S. B., Esteller M., Rountree M. R., Bachman K. E., Schuebel K. and Herman J. G. Aberrant patterns of DNA methylation, chromatin formation and gene expression in cancer. Hum Mol Genet, 2001; 10: 687-692.
37. Baylin S. B., Herman J. G., Graff J. R., Vertino P. M. and Issa J. P. Alterations in DNA methylation: a fundamental aspect of neoplasia. Adv Cancer Res, 72: 141-196, 1998.
38. Belinsky, S. A. Gene-promoter hypermethylation as a biomarker in lung cancer. Nat. Rev Cancer, 2004; 4: 707-717.
39. Cameron E. E., Bachman K. E., Myohanen S., Herma, J. G, and Bayli, S. B. Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nat Genet, 1999; 21: 103-107.
40. Costello J. F., Fruhwald M. C., Smiraglia D. J., Rush L. J., Robertson G. P., Gao X., Wright F. A., Feramisco J. D., Peltomaki P., Lang J. C., Schuller D. E., Yu L., Bloomfield C. D., Caligiuri M. A., Yates A., Nishikawa R., Su Huang H., Petrelli N. J., Zhang X., O'Dorisio M. S., Held W. A., Cavenee W. K. and Plass C. Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. Nat Genet, 2000; 24: 132-138.
41. Esteller M., Sanchez-Cespedes M., Rosell R., Sidransky D., Baylin S. B. and Herman J. G. Detection of aberrant promoter hypermethylation of tumor suppressor genes in serum DNA from non-small cell lung cancer patients. Cancer Res, 1999; 59: 67-70.

42. Esteller M., Corn P. G., Baylin S. B. and Herman J. G. A gene hypermethylation profile of human cancer. Cancer Res, 2001; 61: 3225-3229.
43. Zochbauer-Muller S., Fong K. M., Virmani A. K., Geradts J., Gazdar A. F. and Minna J. D. Aberrant promoter methylation of multiple genes in non-small cell lung cancers. Cancer Res, 2001; 61: 249-255.
44. Herman J. G., Graff J. R., Myohanen S., Nelkin B. D. and Baylin S. B. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci U S A, 1996; 93: 9821-9826.
45. Toyooka S., Toyooka K. O., Maruyama R., Virmani A. K., Girard L., Miyajima K., Harada K., Ariyoshi Y., Takahashi T., Sugio K., Brambilla E., Gilcrease M., Minna J. D., and Gazdar A. F. DNA methylation profiles of lung tumors. Mol Cancer Ther, 2001; 1: 61-67.
46. Toyooka K. O., Toyooka S., Virmani A. K., Sathyanarayana U. G., Euhus D. M., Gilcrease M., Minna J. D. and Gazdar A. F. Loss of expression and aberrant methylation of the CDH13 (H-cadherin) gene in breast and lung carcinomas. Cancer Res, 2001; 61: 4556-4560.
47. Shivapurkar N., Toyooka S., Eby M. T., Huang C. X., Sathyanarayana U. G., Cunningham H. T., Reddy J. L., Brambilla E., Takahashi T., Minna J. D., Chaudhary P. M. and Gazdar A. F. Differential inactivation of caspase-8 in lung cancers. Cancer Biol Ther, 2002; 1: 65-69.
48. Shivapurkar N., Reddy J., Matta H., Sathyanarayana U. G., Huang C. X., Toyooka S., Minna J. D., Chaudhary P. M. and Gazdar A. F. Loss of expression of death-inducing signaling complex (DISC) components in lung cancer cell lines and the influence of MYC amplification. Oncogene, 2002; 21: 8510-8514.
49. Bergstraesser L. M., Srinivasan G., Jones J. C., Stahl S. and Weitzman S. A. Expression of hemidesmosomes and component proteins is lost by invasive breast cancer cells. Am J Pathol, 1995; 147: 1823-1839.
50. Davis T. L., Cress A. E., Dalkin B. L. and Nagle R. B. Unique expression pattern of the alpha6beta4 integrin and laminin-5 in human prostate carcinoma. Prostate, 2001; 46: 240-248.
51. Falk-Marzillier J., Domanico S. Z., Pelletier A., Mullen L. and Quaranta V. Characterization of a tight molecular complex between integrin alpha 6 beta 4 and laminin-5 extracellular matrix. Biochem Biophys Res Commun, 1998; 251: 49-55.
52. Fearon E. R. BRCA1 and E-cadherin promoter hypermethylation and gene inactivation in cancer-association or mechanism? J Natl Cancer Inst, 2000; 92: 515-517.
53. Manda R., Kohno T., Niki T., Yamada T., Takenoshita S., Kuwano H. and Yokota J. Differential expression of the LAMB3 and LAMC2 genes between small cell and non-small cell lung carcinomas. Biochem Biophys Res Commun, 2000; 275: 440-445.
54. Costantini R. M., Falcioni R., Battista P., Zupi G., Kennel S. J., Colasante A., Venturo I., Curio C. G. and Sacchi A. Integrin (alpha 6/beta 4) expression in human lung cancer as monitored by specific monoclonal antibodies. Cancer Res, 1990; 50: 6107-6112.
55. Guo N., Templeton N. S., Al-Barazi H., Cashel J. A., Sipes J. M., Krutzsch H. C. and Roberts D. D. Thrombospondin-1 promotes alpha3beta1 integrin-mediated adhesion and neurite-like outgrowth and inhibits proliferation of small cell lung carcinoma cells. Cancer Res, 2000; 60: 457-466.
56. Chen J. T., Cheng Y. W., Chou M. C., Sen-Lin T., Lai W. W., Ho W. L. and Lee H. The correlation between aberrant connexin 43 mRNA expression induced by promoter methylation and nodal micrometastasis in non-small cell lung cancer. Clin Cancer Res, 2003; 9: 4200-4204.

57. Sathyanarayana U. G., Toyooka S., Padar A., Takahashi T., Brambilla E., Minna J. D. and Gazdar A. F. Epigenetic inactivation of laminin-5-encoding genes in lung cancers. Clin Cancer Res, 2003; 9: 2665-2672.

Chapter 4

CPG ISLAND HYPERMETHYLATION CHANGES DURING PROSTATE CANCER PROGRESSION AND METASTASIS

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Abstract: Recent studies have implicated the dysregulation or maladaptation of epigenetic mechanisms to be a central feature of prostate carcinogenesis. Hypermethylation of CpG islands (CGI), clusters of CpG dinucleotides frequently found at gene regulatory regions, has been demonstrated to be one of the most frequent somatic genome alterations associated with prostate carcinogenesis. A few recent studies have explored the role of CGI hypermethylation during prostate cancer progression from the early precursor lesions to distant metastases. This chapter will focus on the time course of CGI hypermethylation changes that occur at each step during the development and progression of prostate cancer in an effort to understand how these epigenetic changes contribute to the formation of prostate cancer metastases. We will begin by giving an overview of the epidemiology, natural progression, and pathogenesis of prostate cancer, then detail the CGI hypermethylation changes that occur at each step along the progression, then postulate the molecular mechanisms that may be involved in generating and propagating these changes, and finally, use the pattern and timing of DNA methylation changes during the natural progression of prostate cancer to derive models that describe how prostate cancer metastases may form.

Key words: CpG island hypermethylation, proliferative inflammatory atrophy, prostatic intraepithelial neoplasia, prostate cancer, prostate cancer metastasis, GSTP1, RASSF1A, APC, COX2, MDR1, EDNRB, DNA methyltransferase

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1. EPIDEMIOLOGY, PATHOGENESIS AND NATURAL PROGRESSION OF PROSTATE CANCER

In 2004, there will be an estimated 230,110 new cases of prostate cancer and 29,900 deaths from prostate cancer in the United States alone. Prostate cancer is the most commonly diagnosed primary cancer and is the second highest cause of cancer related deaths among men in the United States. Indeed, approximately one in six American men will be diagnosed with prostate cancer in their lifetime and one in 30 American men will die from prostate cancer. (1)

As with many other solid organ malignancies, prostate cancer deaths result from progression of the primary lesion to metastatic disease. One challenge with prostate cancer management has been the identification of the subset of men with prostate cancer that will progress to symptomatic and/or metastatic disease. This issue is highlighted by autopsy studies, which suggest that 29% of men between the age of 30 and 40 and 64% of men between the age 60 and 70 harbor small prostate cancers (2). Clearly, only a minority of these men will develop symptomatic or metastatic disease. Consequently, there may be a danger in over-diagnosis and unnecessary treatment of prostate cancer because many men seem to die *with* but not *from* prostate cancer. This has become a rising controversy in the era of PSA screening for prostate cancer (3). Yet, there have been several favorable trends during the PSA screening era. Since the widespread use of serum PSA screening began about one decade ago, mortality from prostate cancer has gradually and consistently decreased in the United States (1). Another important and related trend during the last decade has been the shift to lower stage and grade at the time of diagnosis of prostate cancer (1).

In addition to allowing treatment options to be administered earlier during the disease progression, at a stage when treatment can still achieve cures, this shift has allowed researchers to study prostate cancer at the earliest stages and to follow these lesions throughout their progression to metastatic disease. Such studies are crucial to the understanding of the mechanisms involved in the pathogenesis and ultimate progression of prostate cancer to metastatic disease.

In recent years, studies have demonstrated that one of the earliest lesions that occur during this progression from benign prostate to invasive prostate cancer and metastasis is characterized by inflammation in the prostate. Chronic inflammation is present in almost all radical prostatectomy specimens from men with prostate cancer (4). De Marzo *et al.* provided a compelling link between prostate cancer and inflammation by characterizing a prostate inflammatory lesion, termed proliferative inflammatory atrophy

(PIA), that might be a precursor to prostatic intraepithelial neoplasia (PIN) and to prostate cancer (5). Specifically, PIA lesions contain regions of highly proliferative, but blunted and dysfunctional prostatic secretory cells, that are present in a backdrop of chronic inflammatory leukocytes (5). PIA lesions are often found adjacent to and directly merging into regions of PIN and prostate cancer (6), giving rise to the notion that PIA lesions may be direct precursors to PIN and prostate cancer. The epithelial cells in these PIA lesions appear to be under tremendous stress as they express high levels of stress response proteins such as COX2, GSTA1, and GSTP1 (5, 7, 8). Indeed, this setting of inflammation, proliferation, and stress found in PIA lesions may provide the initial selection pressures for prostate epithelial cells to accumulate the somatic genome alterations necessary for carcinogenesis.

PIN lesions, which are often contiguous with regions of PIA, are characterized by the presence of malignant-appearing, proliferating prostate epithelial cells contained within a normal glandular architecture. PIN lesions have been considered to be immediate precursors of adenocarcinoma of the prostate because they are more frequently observed in prostates that also contain prostate cancers, and they often directly merge into regions of prostate adenocarcinoma (9). The atypical, malignant-appearing cells in these lesions may have already begun accumulating the somatic genome alterations characteristic of prostate cancer.

Like precursor PIN lesions, primary prostate cancers are most frequently observed in the peripheral zone of the prostate (9). Patients with adenocarcinoma of the prostate can harbor multiple, often heterogeneous, cancerous lesions. The aggressiveness, extent, and long-term treatment outcomes of prostate cancers can be estimated by nomograms that take into account the histological grade of the lesions, the clinical stage, and the serum PSA (10-12). Histological grading of prostate adenocarcinomas is specified by the Gleason score. The Gleason grade, or pattern, describes the differentiation and architectural patterns of prostate cancer, and is scaled from one, referring to well-differentiated lesions, to five, referring to poorly-differentiated lesions. In order to account for the often heterogeneous nature of prostate adenocarcinomas, the combined Gleason score is a sum of the two most prevalent Gleason patterns observed in any given prostate cancer lesion. Staging of prostate cancer follows the revised American Joint Commission on Cancer TNM conventions (13-15). One nomogram suggests that men undergoing radical prostatectomy for treatment of primary prostate cancer can be stratified into three groups: a low risk group (Gleason score < 6, AND stage of T1c to T2a, AND serum PSA < 10 ng/ml) with 83% 10-year recurrence free survival, an intermediate risk group (Gleason score = 7, or stage of T2b, or serum PSA between 10 and 20 ng/ml) with a 46% 10-year recurrence free survival, and a high risk group (Gleason score > 8, or

stage T2c or higher, or serum PSA > 20 ng/ml) with a 26% 10-year recurrence free survival(12). Unfortunately, the use of these nomograms is limited by the variability in the combined Gleason score assigned by different pathologists evaluating the same prostate cancer lesion (16, 17). This remains a significant challenge despite the development of educational texts and tools on the internet by expert pathologists (18, 19). Another potential approach to prognostication would be the identification of molecular markers for prostate cancer risk stratification, either to directly predict post-treatment outcomes, or to predict prostate cancer stage and grade and therefore indirectly predict for post-treatment outcomes. Additionally, as we shall discuss in this chapter, identification of such markers may also provide insight into the molecular pathogenesis of prostate cancer progression and metastasis development.

Since survival and mortality from prostate cancer is directly related to the development of progressive metastatic disease, the prognostic markers for risk stratification of prostate cancer are often indirect measures of metastatic potential. Of note, since Gleason scores stratify prostate cancers based on architecture, they cannot be applied to characterize metastatic prostate cancer. One direct measure for the development of metastases is post-prostatectomy rise of serum PSA, termed PSA recurrence. In fact, PSA recurrence is so predictive for prostate cancer recurrence and development of metastases, that it is commonly used for follow-up after radical prostatectomy for treatment of primary prostate cancer. When metastases do develop, they can occur locally at pelvic lymph nodes, or involve distant organs, typically the axial or appendicular skeleton, and less commonly, the liver, lungs, and brain. Metastases to pelvic lymph nodes can lead to compression of iliac veins and edema of lower extremeties. Approximately 90% of advanced stage prostate cancer patients develop skeletal metastases, most typically at the lumbar spines or pelvic bones, leading to significant morbidity including severe bone pain, spinal cord compression, and pancytopenia due to invasion of the bone marrow (20, 21). Liver metastases can produce abdominal pain and jaundice in some rare cases. Lung metastases can lead to chest pain, coughing, as well as paraneoplastic syndromes due to ectopic hormone production from small cell forms of metastatic prostate cancers. Other uncommon manifestations of metastatic prostate cancer include malignant retroperitoneal fibrosis due to metastasis into the periureteral lymphatics, and disseminated intravascular coagulation (DIC) (9). The mechanisms guiding prostate cancer cells to metastasize so commonly to a specific set of tissues, such as bone, are largely unknown and the subject of intense research. In this chapter, we will examine how DNA methylation changes in metastatic prostate cancer lesions may help us understand how these metastases develop.

There is mounting evidence in the literature to view prostate cancer progression as a continuum from normal prostate, to PIA, to PIN, to primary prostate cancer, and finally to metastatic and androgen-independent metastatic prostate cancer (Figure 1). Likewise, within primary prostate cancers, there is a continuum of aggressiveness as measured by the combined Gleason score and tumor stage. Several recent studies have tracked the molecular changes at each step along this progression. A host of somatic genome changes and biochemical alterations have been implicated at each step along the progression, and have provided several clues to the pathogenesis of symptomatic, life-threatening, metastatic prostate cancer (Figure 1). These changes are often quite heterogeneous between different patients with prostate cancer, different cancer lesions within the same patient, and even different regions within the same cancer (22). One of the earliest and most frequent genome alterations in prostate cancer is the shortening of telomere repeat sequences at the ends of chromosomes in prostate cancers. Telomeres, which are repetitive sequences at the ends of chromosomes that protect against inappropriate loss and recombination of chromosomes during replication, are significantly shortened in PIN and prostate cancer lesions (23, 24). These shortened telomeres may allow illegitimate chromosomal recombination and genetic instability early in prostate carcinogenesis, leading to prostate cancer progression. Chromosomal gains at 7p, 7q, 8q, and Xq, and losses at 8p, 10q, 13q, and 16q are among the most commonly reported chromosomal abnormalities in prostate cancer. Somatic genome alterations and expression changes at specific genes within these chromosomal regions have been implicated in prostate cancer progression (22). For instance, *NKX3.1*, a prostate specific homeobox gene required for normal prostate development, is located on 8p21 (25-27). Loss of 8p21 and absence of *NKX3.1* expression appear to be frequent changes early during prostate cancer progression, occurring as early as the precursor PIN lesions (28, 29). *PTEN*, a tumor suppressor gene located at chromosome 10q that encodes a phosphatase inhibitor of the phosphatidylinositol 3'-kinase/protein kinase B (PI3K/Akt) signaling pathway that is needed for cell cycle progression and cell survival, frequently contains somatic alterations in prostate cancer (30-34). Though it is expressed in normal prostates and PIN lesions, PTEN is under-expressed and contains somatic alterations in primary prostate cancer, and even more so in metastatic prostate cancer lesions (35-37). Additionally, somatic gene alterations at the androgen receptor (AR), such as gene amplifications and ligand-specificity altering mutations, have been documented in prostate cancer cells (38-41). These alterations may account for the inevitable development of androgen-insensitive cancers when patients with prostate cancer metastases are treated with androgen deprivation and/or anti-

androgen therapy (42-45). Metastatic prostate cancers can also acquire androgen-insensitivity by biochemical modifications, such as post-translational phosphorylation, on wild type AR causing constitutive activation even in the absence of androgen (46, 47). Development of hormone refractory metastatic prostate cancer carries an ominous prognosis as these patients have a median survival of only 9 – 12 months (48). Other biochemical alterations implicated in prostate cancers include gene expression changes. For instance, gene expression microarray experiments consistently document the over-expression of Hepsin and AMACR in prostate cancers, as well as EZH2 in metastatic androgen-independent prostate cancers (9). The precise role of these genes in prostate cancer progression has not yet been determined.

While the changes detailed above are strongly associated with prostate cancer, hypermethylation of CGI sequences is perhaps the earliest and most frequent somatic genome alteration in prostate carcinogenesis and progression. The remainder of this chapter will detail the specific DNA methylation changes that occur during each step of prostate cancer progression and use this information to build a potential model for metastasis development.

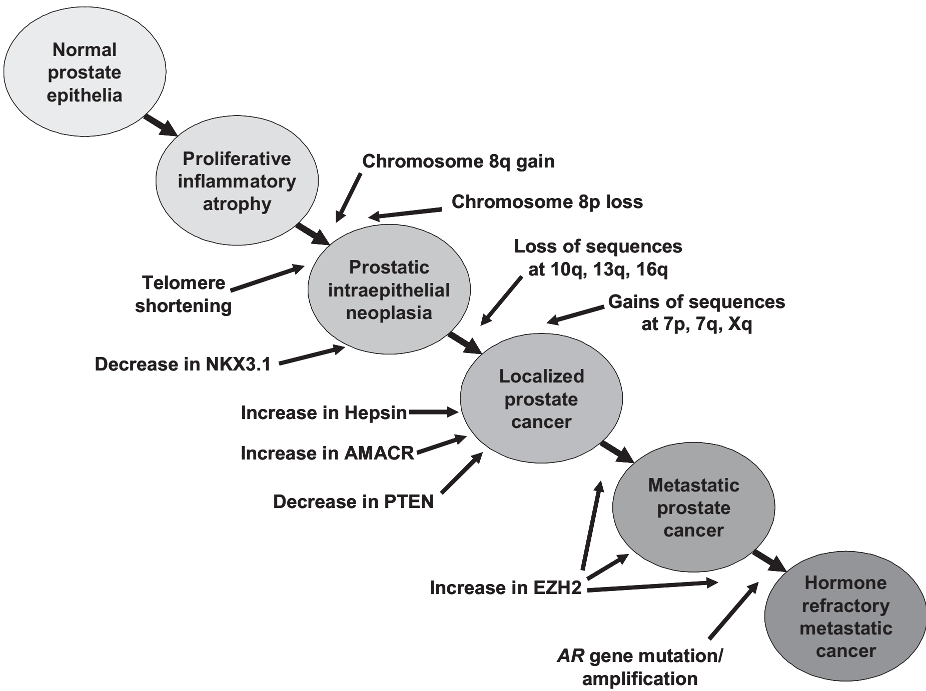

Figure 1. Summary of somatic genome changes occurring during prostate cancer progression.

2. CPG ISLAND HYPERMETHYLATION CHANGES DURING PROSTATE CANCER INITIATION AND PROGRESSION

In 1994, Lee *et al.* demonstrated that hypermethylation of CGI sequences within the regulatory region of *GSTP1*, which encodes the pi-class glutathione S-transferase (GST) enzyme, is an extremely frequent feature of human prostate cancer (49, 50). Since that initial study, numerous groups have independently corroborated these findings using a wide array of techniques applied to numerous prostate cancer DNA sources, including prostatectomy specimens, prostate autopsy specimens, prostate biopsy specimens, prostate secretions, and various bodily fluids from prostate cancer patients. Furthermore, *GSTP1* CGI hypermethylation appears to be an extremely specific finding for prostate cancer as it is not characteristic of normal prostates or benign prostatic hyperplasia. The GST enzymes catalyze the detoxification of carcinogens and reactive chemical species via the conjugation of glutathione. It has been hypothesized that loss of this detoxification agent in prostate cells might make them susceptible to genome damaging agents, leading to carcinogenesis. Mice deficient for the GSTP1 enzyme are more susceptible to developing skin cancers after treatment with the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) (51). Additionally, GSTP1 deficient human prostate cancer cells cultivated *in vitro* accumulate genome alterations at high levels when exposed to 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-b]pyridine (*N*-OH-PhIP), an agent known to have toxicity in the prostate (52-54). Futhermore, loss of *GSTP1* expression closely follows hypermethylation of CGI sequences at the regulatory region of the *GSTP1* gene (49, 55). *GSTP1* is expressed in normal prostate epithelium at the basal cells but is absent in the columnar secretory cells. However, *GSTP1* expression can be induced in the columnar epithelial cells by inflammation and other genome damaging stresses. Prostate cancer epithelia, in contrast, almost never express *GSTP1*. For more than 90% of prostate cancer cases, this absence of *GSTP1* expression is due to repression of *GSTP1* transcription by hypermethylation of the CGI at the *GSTP1* promoter region (55).

Other recent studies have demonstrated that CGIs at the *GSTP1* regulatory regions are only one of several CGIs to become hypermethylated in prostate cancer progression. In the following sections, we will examine the timing and extent of these DNA hypermethylation changes at each step in the progression of prostate cancer to metastatic disease (Figure 2).

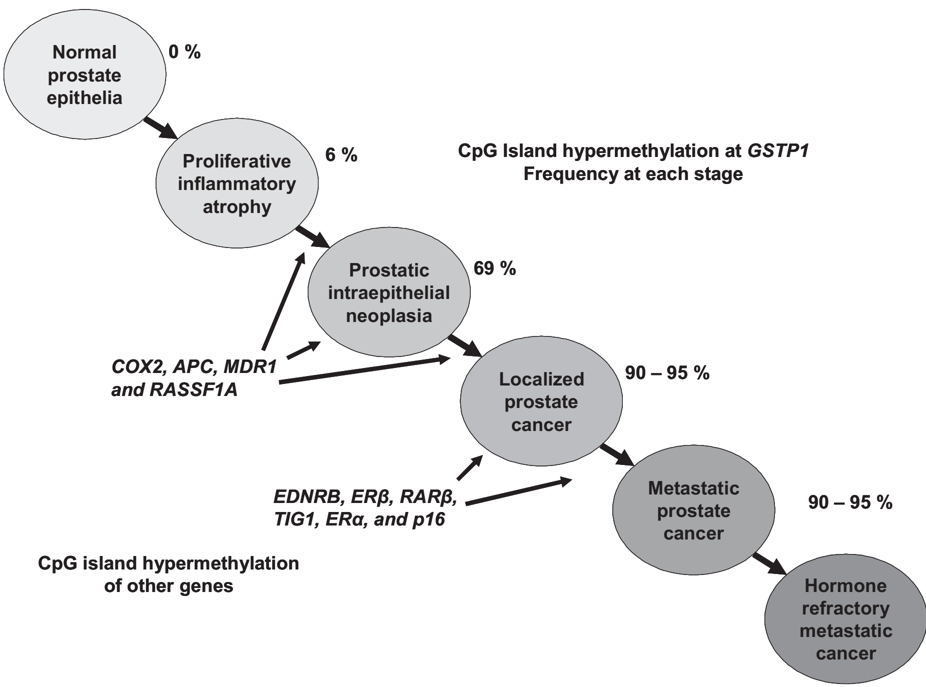

Figure 2. CpG island hypermethylation changes during prostate cancer progression. CpG islands at GSTP1 become hypermethylated very early during prostate cancer progression, as early as the PIA and PIN lesions, and are subsequently observed in greater than 90% of primary prostate cancers and prostate cancer metastases. CpG islands at other genes such as COX2, APC, MDR1 and RASSF1A also become hypermethylated as part of an early wave of gene hypermethylation in prostate cancer progression. A subsequent wave of hypermethylation at genes such as EDNRB, ERβ, RARβ, and TIG1 occurs as the primary prostate cancer progresses to higher stage and grade. As hypermethylation changes develop, they appear to be maintained and propagated during the subsequent progression.

2.1 Normal Prostate and Benign Prostatic Hyperplasia

Extensive methylation of CGIs in the 5' upstream regulatory regions of cancer related genes seldom occurs in entirely normal or benign prostate tissues. However, extensive methylation of CpG dinucleotides within non-CGI sequences and within the coding sequences of genes is the norm in benign tissues. The term "hypermethylation", then, usually refers to extensive methylation of CGIs in DNA from cancer tissues but not from DNA from the corresponding normal tissues. Consequently, most studies compare the methylation pattern in cancer tissues with the methylation pattern in normal tissues. One challenge in detecting the pattern of hypermethylation in prostate cancer is that it is quite difficult to obtain

tissues that are devoid of prostate cancer lesions and their precursors. Most studies use tissues from men undergoing prostate resection for treatment of symptomatic benign prostatic hyperplasia (BPH). However, these men typically are of advanced age and a large percentage of them will harbor small, asymptomatic, undiagnosed prostate cancer foci, as demonstrated by autopsy studies (2). It is not surprising then, that many of these studies often find a low level of methylation at the CGIs in regulatory regions of genes such as *GSTP1* even in these normal tissues (56-58). In order to test whether truly benign prostate tissues contain methylated CGI DNA, we collected benign prostates from 13 brain-dead transplant tissue donors, ages 4-52, immediately following organ harvest and cardiac arrest (59). Tissue sections from these specimens were histologically examined to ensure the absence of malignant and pre-malignant lesions. DNA from these prostate tissues did not exhibit any significant level of CGI hypermethylation at 15 different cancer related genes, including *GSTP1, APC, RASSF1a, PTGS2,* and *MDR1*. Only CGI sequences at *HIC1* were consistently methylated in these tissues. Interestingly, extensive methylation of this gene may be a marker for epithelial cells as it was found to be entirely unmethylated in benign prostate stromal cells and normal white blood cells (59). Thus, when examining the extent of hypermethylation in prostate cancers, it is very important to use appropriate normal controls.

Because multiple heterogeneous foci of prostate cancer occur within the same prostate, some researchers have suggested that a "field effect" might be at the root of prostate carcinogenesis (60). One version of the "field effect" hypothesis maintains that abnormalities in all of the stromal and epithelial cells in the prostate gland cause changes in the microenvironment of the prostate that lead to the development of malignant epithelia. This hypothesis also suggests that many of the earliest changes found in prostate cancers would also be found in the surrounding normal appearing prostate cells. Since hypermethylation of CGIs, particularly the ones in the regulatory region of *GSTP1*, appear to be one of the earliest changes in prostate cancer, the field effect hypothesis would suggest that normal prostate glands and stroma surrounding prostate cancer cells and PIN cells should also contain extensive *GSTP1* CGI hypermethylation. We tested this hypothesis by microdissecting tumor-adjacent benign prostate epithelia and stroma from the prostates of 12 patients with varying stages and grades of prostate cancer (59). Two of these 12 specimens contained large areas of high grade PIN. Of all the twelve tumor-adjacent benign tissues, only the two sections containing large regions of high grade PIN demonstrated significant levels of CGI hypermethylation (59). All of the other tumor-adjacent benign specimens exhibited a complete lack of CGI hypermethylation at all 15 genes, including *GSTP1, RASSF1a, APC, COX2,* and *MDR1* (59). Another

study, using more rigorous laser capture microdissection techniques to harvest tumor-adjacent, histologically normal epithelia, also demonstrated a complete absence of *GSTP1* CGI hypermethylation in these normal regions (61).

Indeed, it appears that normal prostate epithelia, regardless of whether they are within a completely normal gland or within one containing prostate cancer foci, do not show extensive hypermethylation at CGIs in the regulatory regions of cancer related genes. Therefore, at least from a CGI hypermethylation point of view, the "field effect" hypothesis is not strongly supported for prostate cancer.

2.2 Proliferative Inflammatory Atrophy of the Prostate

Another, more attractive, hypothesis maintains that widespread chronic inflammation, atrophy, and regeneration in the prostate might provide the fertile ground of stress and genome damage that lead to the development of multiple prostate cancer lesions that are heterogeneous in their age and aggressiveness. The identification of highly prevalent PIA lesions lends much support to this hypothesis, and offers direct evidence for a link between prostate inflammation and carcinogenesis. As mentioned previously, the blunted, dysfunctional luminal epithelia in these PIA glands generally express high levels of stress response and caretaker genes such as *GSTP1, GSTA1* and *COX2* (5, 7, 8). The induction of these genes may be a response to electrophile and oxidative stress. However, a minority of epithelial cells within PIA lesions lose *GSTP1* expression (5, 61). This observation led to the hypothesis that a subset of PIA cells may have already begun to repress *GSTP1* expression by hypermethylation of regulatory CGI sequences. To test this hypothesis, Nakayama *et al*. undertook laser capture microdissection of epithelial cells from PIA lesions and assessed the methylation status of CGI sequences at the regulatory region of the GSTP1 gene. They discovered that approximately 6% of these lesions harbored hypermethylated GSTP1 CGI sequences (61). Though this frequency appears rather low, because there can be hundreds of PIA lesions in a single prostate gland, the absolute number of PIA lesions harboring hypermethylated GSTP1 CGIs can easily account for the typical number of prostate cancer foci found in prostate cancer patients. The mechanisms involved in causing the hypermethylation of CGI sequences in the regulatory region of GSTP1 have yet to be determined. However, the idea that chronic inflammation and the resultant oxidative stresses may directly dysregulate the epigenetic and DNA methylation machinery is very attractive. Regardless, it can be hypothesized that within this hotbed of inflammation and proliferation, loss of GSTP1 expression in rare PIA lesions might lead to

enhanced susceptibility to oxidant and electrophile damage, leading to increased genomic instability and carcinogenesis (62).

2.3 Prostatic Intraepithelial Neoplasia

While PIA lesions usually express high levels of stress response proteins such as GSTP1 and COX2, PIN lesions are most often characterized by an absence of GSTP1 and COX2 expression. Despite this stark contrast, PIA lesions can be considered as precursors to PIN lesions for the following reasons: first, like PIN and primary prostate cancer lesions, PIA is most often found within the peripheral zone of the prostate; second, the secretory cells in PIA are highly dysfunctional, like PIN and prostate cancer epithelia; finally, and perhaps most compellingly, PIA regions can often be observed to merge directly into regions of PIN, often within the same secretory gland (5, 6). One possible explanation for these paradoxical observations is that PIN lesions may develop from PIA epithelia that repress or lose expression of genes such as *GSTP1* and *COX2*. Consistent with this possible explanation, PIN lesions exhibit a much higher frequency of hypermethylation at CGI sequences at the regulatory region of *GSTP1* than their PIA counterparts. One study found that seven out of ten PIN lesions harbored hypermethylated *GSTP1* CGIs (63). More recently, Nakayama *et al*. corroborated this finding by assessing *GSTP1* CGI hypermethylation in DNA from 32 laser capture microdissected regions of PIN, and observed a 68.8% rate of *GSTP1* CGI hypermethylation in these specimens (61). This pattern may be true for other genes as well. We recently showed that *COX2* and *APC* CGI hypermethylation was found in two out of five high grade PIN lesions (59). Another recent study found CGI hypermethylation of *RASSF1a* and *RARβ* in three out of ten high grade PIN lesions (64). However, the frequency of hypermethylation of these genes in PIA has yet to be determined. Nevertheless, it is clear that CGIs at several genes that are often found to be hypermethylated in primary prostate cancers are already hypermethylated in PIN lesions. These findings give even more support to the widely held idea that PIN lesions are premalignant precursor lesions to primary adenocarcinoma of the prostate.

2.4 Primary Prostate Cancer

Accumulating evidence suggests that the CGIs at several genes are hypermethylated very early in the development of primary prostate cancer, many of these as early as the precursor PIN and PIA lesions (as discussed above). Since the initial discovery that *GSTP1* CGI hypermethylation is a nearly universal event in primary prostate cancer (49, 50), several studies

have demonstrated that the CGIs adjacent to numerous other cancer related genes are also hypermethylated very frequently in primary prostate cancer (57, 59, 65-68). Using a quantitative real-time methylation-specific PCR (RT-MSP) technique, we recently showed that CGIs at *GSTP1, APC, RASSF1a, COX2* and *MDR1* were each hypermethylated in greater than 85% of primary prostate cancers (59). Additionally, greater than 25% of input DNA, on average, showed hypermethylation at each of these CGIs (59). This large prevalence of hypermethylated CGIs at these genes was present even in the primary prostate cancers with the lowest pathological stages and Gleason scores (Figure 3). In fact, the percentage of input DNA that was hypermethylated at these genes was in general rather high, and was not found to be correlated with pathological stage or Gleason score (Figure 3). This lack of correlation suggests that CGIs at these particular genes are probably coordinately hypermethylated extremely early in carcinogenesis, perhaps even at the stage of the precursor lesions. These early hypermethylation changes are tightly maintained as the cancers grow and become more aggressive. The genes that become hypermethylated are likely specific to prostate cancers, since many other cancer related genes that are known to be hypermethylated in other human primary cancers were not hypermethylated in these prostate cancers. As such, it appears that prostate cancers develop a hypermethylation fingerprint that is distinct form other human cancers (59).

The reason that a specific set of genes are potentially targeted very early in prostate carcinogenesis for CGI hypermethylation is unclear. The known functions of the proteins encoded by these genes may provide some clues. The function of GSTP1 as a caretaker enzyme involved in detoxification of carcinogens and scavenging electrophiles and oxidants has already been described above. CGI hypermethylation at *GSTP1* and the subsequent repression of its expression may be one of the key initiating factors in prostate carcinogenesis, as its absence would make prostate epithelia significantly more susceptible to genome damaging agents. The importance of the COX2 enzyme in prostate carcinogenesis has also been discussed above briefly. *COX2* encodes cyclooxygenase 2 (COX2), the inducible isoform of the cyclooxygenase enzymes, which are the rate-limiting enzymes in the conversion of arachidonic acid to various proinflammatory prostaglandins (69). These cyclooxygenase enzymes are the primary targets for nonsteroidal anti-inflammatory drugs (69). COX2 has been reported to be unexpressed in the normal prostate, highly expressed in many proliferative inflammatory atrophy lesions of the prostate, but generally unexpressed in high grade PIN and prostate adenocarcinoma lesions at both the protein and transcript levels (7). One mechanism by which *COX2* is silenced in the PIN and prostate cancer lesions may be CGI

hypermethylation. Given the similar pattern of expression and CGI hypermethylation between *COX2* and *GSTP1*, it is possible that, like *GSTP1*, early inactivation of *COX2* by CGI hypermethyaltion may lead to carcinogenesis. It should be noted that Kaplan-Meier and multivariate Cox proportional hazards survival analyses demonstrated that the quantity of alleles with *COX2* CGI hypermethylation was strongly correlated with PSA recurrence after treatment (hazards ratio = 4.26, p = 0.01), and that this correlation was independent of Gleason score and pathological stage (59).

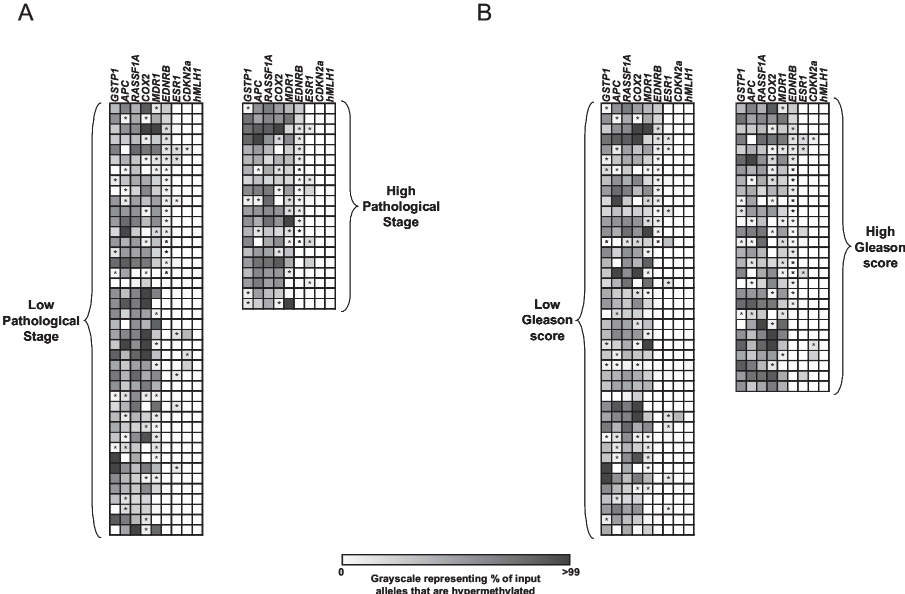

Figure 3. Hypermethylation of CpG islands in primary prostate cancer. Panel A. CpG islands at GSTP1, APC, RASSF1A, COX2 and MDR1 become hypermethylated very early in prostate cancer progression. Therefore, hypermethylation at these genes occurs at similar frequencies in low pathological stage (organ confined) primary prostate cancers as in high pathological stage (involvement of seminal vesicles and/or pelvic lymph nodes) primary prostate cancers. On the other hand, CpG island hypermethylation at EDNRB occurs later during prostate cancer progression and is thus predictive of high pathological stage (odds ratio = 3.193, p = 0.04). Panel B. Because GSTP1, APC, RASSF1A, COX2 and MDR1 CpG islands are already highly hypermethylated very early in prostate cancer initiation, hypermethylation at these sequences is not correlated with high Gleason score. However, EDNRB CpG island sequences, which presumably become hypermethylated as the primary prostate cancer becomes more advanced, predicts strongly for high Gleason score (4+3, 4+4, 4+5, 5+4) with an odds ratio of 4.615 (p = 0.005).

This finding suggests that prostate cancers accumulate cells with DNA containing *COX2* CGI hypermethylation as they progress to metastatic disease, in a manner that is entirely independent of advancing Gleason score

and pathological stage. Therefore, though *COX2* CGI hypermethylation initially occurs very early in prostate carcinogenesis, perhaps even as early as PIN and PIA precursor lesions, there is a gradual increase in the prevalence of *COX2* CGI hypermethylation as the cancer develops increasing metastatic potential. *RASSF1A*, located on chromosome 3p21.3 in a region that is frequently lost in human cancers, is a putative tumor-supressor gene and is thought to be a negative effector of Ras GTPase signaling pathways, leading to decreased proliferation and increased apoptosis (70). It has been reported to be epigenetically silenced by CGI hypermethylation in a wide array of human cancers including prostate cancer (66, 70). However, the precise role of this gene in physiological processes and its role in prostate carcinogenesis and progression are largely unknown.

APC is a well characterized tumor suppressor gene that has been found to be inactivated by genetic and epigenetic mechanisms in many other human neoplasms (71-74). The APC protein, required for binding and degradation of β-catenin, is a part of the WNT signaling pathway, a key regulator of cell growth and proliferation (75). In the absence of functional APC, β-catenin does not get degraded, accumulates in the nucleus, and constitutively activates expression of proliferation promoting genes such as *c-myc*. More than 80% of sporadic colon cancers harbor mutations in the APC gene that affect its ability to bind β-catenin (75). Furthermore, putative activating mutations in β-catenin have been documented in primary prostate cancer and prostate cancer metastases (76). Repression of APC expression via CGI hypermethylation, and the constitutive activation of the Wnt signaling pathway may be one of the early events leading to prostate carcinogenesis. However further studies would have to be performed to dissect this association in greater detail.

The *MDR1* gene encodes the P-glycoprotein (P-gp), an ATP dependent efflux pump implicated in resistance to the cytotoxic actions of several antineoplastic drugs (77, 78). Another important function of P-gp is the ATP dependent extrusion of toxins and their metabolites from normal cells (79, 80). An inverse correlation between methylation at CpG dinucleotides at the *MDR1* promoter and its expression levels has been found in many human neoplasms and thus, demethylation of CpG dinucleotides at the promoter region of *MDR1* is thought to underlie one mechanism of acquired drug resistance (81-84). However, loss of P-gp expression very early in prostate cancer initiation, during a period of exposure to inflammatory stresses, may lead to susceptibility of the prostate epithelia to oxidant and electrophilic toxins, leading to genome damage and carcinogenesis. Still, the precise role of *MDR1* in prostate carcinogenesis and drug resistance has not yet been determined.

Hypermethylation of CGIs at *GSTP1, APC, RASSF1a, COX2* and MDR1 occur during a coordinate wave of CGI hypermethylation that occurs extremely early in prostate carcinogenesis. As prostate cancer progresses through higher stages and Gleason grades, other genes also appear to get targeted for CGI hypermethylation and repression. CGIs at these genes are more frequently and prevalently hypermethylated in primary prostate cancers with higher stage and Gleason grade. For instance, increasing quantities of *EDNRB* CGI hypermethylation was shown to be strongly correlated with extraprostatic involvement of the primary prostate cancer (odds ratio = 3.193, p = 0.04) and with Gleason grade > 7 (odds ratio = 4.615, p = 0.005) (59) (Figure 3). Hypermethylation of CGIs at the *ERβ* gene has also been shown to correlate with prostate cancer stage and grade (67). Similarly, the quantity of *RARβ* CGI hypermethylation was shown to be correlated with increased pathological stage (85), while *TIG1* CGI hypermethylation was demonstrated to predict increasing Gleason grade (86). Since stage and grade are known predictors of prostate cancer recurrence and metastatic disease, hypermethylation at these genes may lead to increased metastatic protential. A small percentage of prostate cancers also appear to become hypermethylated at the *ERα* and *p14/INK4a* genes. However, hypermethylation at these genes does not correlate with stage or grade (59).

Hypermethylation of CGIs in prostate cancer intiation and progression appears to occur in at least two waves. The first, and possibly most significant of these, occurs very early in prostate carcinogenesis, perhaps even at the level of the PIA and PIN premalignant lesions. A subsequent wave likely occurs as primary prostate cancer progresses through higher stage and grade. After each of these waves, the pattern of CGI hypermethylation appears to be largely maintained and reinforced during the subsequent progression of the lesion. In the following section, we will examine in greater detail the mechanisms by which hypermethylation and silencing of genes late in the progression of primary prostate cancer may contribute to the development of cells that are capable of metastatic invasion.

2.5 Metastatic Prostate Cancer

Very few studies have described CGI hypermethylation patterns in metastatic prostate cancers, due to the difficulty in obtaining metastatic cancer tissue. Surgical resection of metastatic deposits of prostate cancer does not enhance survival from the disease significantly. Therefore, most patients with refractory, metastatic prostate cancer are not candidates for further surgical intervention. Therefore, the few studies that have examined

CGI hypermethylation patterns in metastases obtained these specimens from autopsy cases of patients who died from refractory prostate cancer or from the small group of patients undergoing surgical resection of bone metastases to alleviate symptoms or monitor for response to novel therapies. Over a 7 year period, we systematically collected metastatic prostate cancer specimens at autopsy from 28 men who died of refractory prostate cancer. One to six anatomically distinct metastases from a wide array of sites, including bone, lymph node, liver, adrenal gland, intracranial subdural, and intraprostatic, were obtained from each patient (59).

Using this collection of tissues, we recently demonstrated that the pattern of CGI hypermethylation in prostate cancer metastases is extremely similar to the pattern seen in primary prostate cancer (59) (Figure 4). That is, CGIs at genes such as *GSTP1, RASSF1a, APC, COX2,* and *MDR1* remain hypermethylated in the DNA from metastatic prostate cancer deposits at high frequencies, while very few new genes are hypermethylated (59). This would suggest that these methylation patterns are maintained even through the process of metastatic invasion and occupation of distant sites. Indeed, the pattern of CGI hypermethylation is largely maintained in a clonal manner and appears homogeneous across all sites from any given patient (59) (Figure 4). For instance, case 22 exhibits a significant degree of hypermethylation at the *hMLH1* CGI in his intraprostatic lesion, and this hypermethylation is observed at every one of the other three metastatic deposits. This observation is particularly noteworthy considering *hMLH1* hypermethylation is very infrequent in prostate cancer (59) (Figure 4). Similarly, in case 32, the *MDR1* CGI is not hypermethylated in the intraprostatic lesion and does not become hypermethylated at the other four metastatic deposits. Considering *MDR1* CGI sequences are methylated in greater than 85% of primary prostate cancers, it is especially telling that *MDR1* CGIs were not hypermethylated in any of the five metastastatic prostate cancer specimens taken from this patient (59) (Figure 4). Additionally, a significant degree of heterogeneity in the CGI hypermethylation pattern is observed when metastatic specimens are grouped by category (lymph node, bone, liver, etc.) of anatomical site involvement. These observations suggest that the CGI hypermethylation pattern does not appear to be affected by the site of metastasis (Figure 4). We tested this hypothesis quantitatively by using a novel biostatistical method called analysis of molecular variance (AMOVA) (59) to assess whether the heterogeneity of the hypermethylation pattern within sites was greater than the heterogeneity of the hypermethylation pattern in the entire collection of metastases. We found that there was a 5-fold greater variability in the hypermethylation profile within each site category than there was

across all specimens ($p < 0.0001$), providing strong statistical evidence that the hypermethylation pattern is not site-specific (59) (Figure 4).

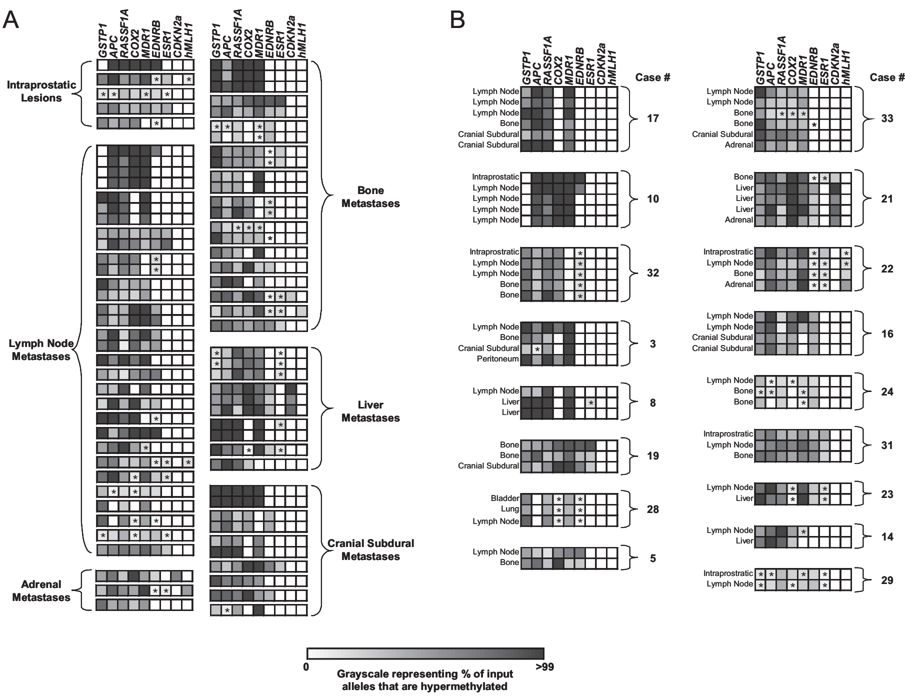

Figure 4. Hypermethylation of CpG islands in metastatic prostate cancer. Panel A. There is a relatively high degree of variability in the quantitative CpG island hypermethylation pattern in any given site of metastatic deposit. Panel B. Conversely, the CpG island hypermethylation pattern is highly homogeneous across all sites of metastatic deposit within any given patient. The variability in the quantitative CpG island hypermethylation pattern was 5-fold higher ($p < 0.0001$) when metastases were grouped by site of anatomical involvement than when all metastases were pooled by patient and sites. This suggests that there is no site-specificity in the CpG island hypermethylation pattern.

Despite the striking similarities between the CGI hypermethylation patterns between the primary and metastatic prostate cancers, there are a few important differences. First, it appears that, on average, the normalized quantity of hypermethylated CGIs at each gene in the metastatic tissues is greater than in the primary cancers (59). However, this difference may simply be due to an artifact resulting from the increased purity of tumor cells in the metastatic tissues compared to the primary prostate cancer tissues.

Second, CGIs at a few genes that are rarely hypermethylated in the primary cancers have been observed to be hypermethylated in a small

percentage of metastatic prostate cancer specimens. For instance, the *hMLH1* CGI was hypermethylated in all four metastatic deposits, including the intraprostatic cancer, from one out of 28 autopsy subjects, while it was never found to be hypermethylated in the primary prostate cancer specimens (59). *hMLH1* encodes the MutL homolog 1 mismatch repair protein, which is frequently inactivated by mutations and CGI hypermethylation in a wide variety of human cancers (73, 87, 88). Loss of this protein by hypermethylation may occur very late in a small percentage of primary and metastatic prostate cancers, leading to increased genomic instability and further cancer progression.

Additionally, it has been shown that there is a modest, but significant increase in the frequency and quantity of CGI hypermethylation of the *ERα* gene, which encodes the alpha isoform of the estrogen receptor, in metastatic prostate cancers as compared to primary prostate cancers (59). In the meanwhile, Zhu *et al*. demonstrated that the opposite trend occurs for the *ERβ* gene, which codes for the beta isoform of the estrogen receptor (67). While they are hypermethylated in many high Gleason grade prostate cancers, CGIs at *ERβ* appear to lose their hypermethylation in metastatic prostate cancer tissues from lymph node and bone (67). Although estrogens have been widely used historically for the treatment of advanced prostate cancer, the mechanisms by which they act are still unclear. Though both ERα and ERβ bind estrogens, their roles in the regulation of downstream gene expression may be quite divergent (89). While both ERα and ERβ are expressed in the normal prostate, their roles in the prostate are largely unknown. However, it has been suggested that ERβ may be more important as a negative regulator of prostate growth and proliferation as supported by the observations that: *i*) mice carrying targeted disruptions in *ERβ* develop prostatic hyperplasia (90), and *ii*) though ERβ expression is common in normal prostate epithelia, ERβ expression is lost in approximately 75% of high grade (Gleason pattern 4 or 5) primary prostate cancers (91). However, this view is complicated by the finding that CGIs at *ERβ* lose their methylation and the gene is re-expressed in metastatic prostate cancers (67). Additionally, Horvath *et al.* found that the subset of primary prostate cancers that retain expression of ERβ are significantly more likely to recur after treatment (92). Taken together, these observations suggest that loss of ERβ expression is important for the progression of primary prostate cancer to higher grade, but lesions that retain ERβ expression very late in the primary prostate cancer may metastasize or recur. However, the precise role of the estrogen receptors in the development of primary and metastatic prostate cancer still requires much clarification.

In summary, CGI hypermethylation patterns in metastatic prostate cancers are largely similar to those in primary prostate cancers. Indeed, it

appears that the CGI hypermethylation changes found in metastases are already present in a large portion of primary prostate cancer cells. That is, at least from a CGI hypermethylation perspective, the predilection for developing metastasis is already coded for in the primary prostate cancer lesions. These patterns are then maintained in an almost clonal manner even through the process of invasion and metastasis. We will now examine how these observations may be integrated to understand how these CGI hypermethylation changes are initiated, maintained and propagated during the process of prostate carcinogenesis and cancer progression.

3. MOLECULAR MECHANISMS IN THE GENERATION AND PROPAGATION OF ABERRANT DNA METHYLATION PATTERNS IN PROSTATE CANCER INITIATION AND PROGRESSION

In our discussions thus far, we have been continually alluding to a fundamental paradox concerning CGI hypermethylation in prostate cancer initiation and propagation: DNA methylation processes appear to be dysregulated enough to cause hypermethylation of CGIs at multiple genes; yet, the same DNA methylation processes have high enough fidelity that they can maintain the acquired changes in CGI hypermethylation through every step of prostate cancer initiation and progression. This observed paradox would suggest that the CGI hypermethylation changes in prostate cancer are not due to a total dysregulation of the DNA methylation machinery, with subsequent loss of discrimination and fidelity in which CGI sequences are stochastically hypermethylated. Rather, it appears that certain CGI sequences are targeted for hypermethylation resulting in silencing of the corresponding genes.

One possibility is that targeting these genes for CGI hypermethylation provides a growth advantage for these cells early in prostate carcinogenesis. This hypothesis is indirectly supported by studies with GSTP1 overexpression in the LNCaP prostate cancer cell line. This cell line contains hypermethylated CGIs at all copies of *GSTP1*, and does not express this gene (55, 59, 93). When exposed to oxidizing stress from low dose ionizing radiation, LNCaP cells genetically modified to constitutively express GSTP1 exhibited significantly decreased amounts of oxidized DNA bases compared to unmodified LNCaPs and control transfectants (94). Presumably, GSTP1 scavenges the oxidant and electrophilic species before they can damage DNA. This would lead to an increase in glutathione-

conjugated oxidant species that would then have to be transported out of the cell or metabolized further into inert compounds. Surprisingly, however, the GSTP1 expressing LNCaP clones exhibited less clonogenic survival than the control LNCaP cells (94). In a similar experiment, Diah *et al.* overexpressed GSTP1 and/or MRP1, a glutathione-conjugated toxin active transporter, in MCF7 breast cancer cells, which normally do not express either of these proteins. They then treated these cells and MCF7 wild type controls with the toxin 1-chloro-2,4-dinitrobenzene (CDNB), and found that: a) MCF7 cells overexpressing MRP1 alone had three to four fold increased resistance to CDNB cytotoxicity associated with a 10 fold increase in efflux of the glutathione conjugate of CDNB as compared to wild type MCF7 cells, b) MCF7 cells overexpressing both MRP1 and GSTP1 showed increased formation and a commensurate increase in efflux of the glutathione conjugate of CDNB and increased resistance to short-term (10 min) CDNB exposure but not to longer exposures (1 hr) compared to wild type MCF7 cells, c) MCF7 cells overexpressing GSTP1 alone had increased intracellular levels of the glutathione conjugate of CDNB but *decreased* resistance to CDNB cytotoxicity compared to wild type MCF7 cells, d) inhibition of glutathione conjugated CDNB by depletion of intracellular glutathione prior to CDNB treatment in wild type MCF7 cells confers increased resistance to CDNB cytotoxicity compared to undepleted wild type MCF7 cells. In yet another study, when compared to Gstp1/2 +/+ mice, Gstp1/2 -/- mice experienced considerably less liver toxicity after being gavaged with toxic doses of acetaminophen, a compound whose toxic metabolite is known to be detoxified by pi class glutathione in rats and humans (95). The data from these experiments suggest that accumulation of glutathione-conjugated toxins and/or depletion of reduced glutathione in the intracellular compartment beyond a certain threshold may lead to decreased proliferation and/or increased cell death (96).

The pattern of GSTP1 expression in PIA lesions parallels the GSTP1 expression pattern engineered in the experiments detailed above. In the context of inflammation and oxidative stress, PIA epithelia express high levels of GSTP1 and GSTA1, presumably to detoxify oxidative inflammatory and/or environmental toxins by conjugating them to glutathione. These glutathione-conjugated species are in turn transported out of the cells by ATP binding cassette (ABC) transport proteins such as MDR1 and MRP1, which have been shown to actively transport glutathione-conjugated toxins out of epithelial cells in an ATP dependent fashion (96-99). When PIA cells are exposed to toxins for prolonged periods, the GST enzymes may produce accumulating amounts of glutathione-conjugated toxins, overwhelming the ABC transport proteins, and leading to rising levels of glutathione-conjugated toxins inside cells. The increasing

intracellular concentrations of glutathione-conjugated toxins and/or the corresponding depletion of reduced glutathione stores might lead to an increase in cytotoxicity as described above. In this setting, a few cells that repress expression of GSTP1 by CGI hypermethylation, as a result of adaptation or selection, may acquire a significant survival advantage. However, the resulting unchecked exposure to intracellular toxins would result in accumulating genome damage, and ultimately, malignant transformation. This model, though still largely speculative, offers a potential mechanism by which CpG hypermethylation at genes such as *GSTP1* can be targeted by selection and/or adaptation in these cells.

There is significant evidence in other experimental models that suggest that CGI hypermethylation changes can result from adaptation or selection when a survival pressure is imposed. For example, by long term exposure to doxorubicin and other antineoplastic drugs, it is possible to select for rare variants of MCF7 breast cancer cells that stably express high levels of the P-glycoprotein and GSTP1 due primarily to loss of hypermethylation at the corresponding regulatory CGIs (100, 101). In another experiment, using Luria Delbruck fluctuation analysis, Holst *et al.* have shown that a small minority of normal human mammary epithelial cells (HMECs) develop hypermethylation and silencing of the p16/INK4a cell cycle regulatory protein, and that these cells are highly selected for during the passaging of HMECs in culture (102). This study also showed that almost all of the cells that escape senescence and continue growing for an indefinite number of passages harbored hypermethylated and silenced p16/INK4a alleles (102). These experiments offer firm evidence to support the hypothesis that DNA methylation changes can occur during exposure to survival pressures.

Regardless of whether these changes occur as a result of selection or adaptation to a survival pressure, the mechanism by which prostate cells acquire *de novo* CGI hypermethylation changes, and then maintain them through the subsequent initiation and progression of prostate cancer to metastatic, hormone-insensitive disease remains largely unknown. The mammalian DNA methyltransferases (DNMTs), which include DNMT1, DNMT3a, and DNMT3b, are likely to be central to these processes. Based on their propensity to modify cytosine to 5-methylcytosine in unmethylated versus hemimethylated double stranded DNA oligonucleotides *in vitro*, the mammalian DNMTs have been classified as primarily "*de novo"* (DNMT3a and DNMT3b) or "maintenance" (DNMT1) methyltransferases (103-106). Under this paradigm, the *de novo* methyltransferases would be capable of initiating new CpG methylation patterns, while the maintenance methyltransferase would be responsible for maintaining and propagating established CpG methylation patterns during replication. The idea that DNMT3a and DNMT3b are *de novo* methyltransferases was further

supported by data showing that targeted disruption of these genes in mice results in a blockage of *de novo* methylation in ES cells and early embryos without hampering maintenance of pre-existing imprinted methylation patterns (107). However, it is clear that the *de novo* and maintenance human DNA methyltransferases may not strictly adhere to this paradigm and may cooperate and/or complement each other. For example, HCT-116 human colon cancer cells carrying targeted homozygous disruptions of the DNMT1 or DNMT3b genes lose only 20% and 3% of their genomic methylation levels respectively (108, 109). However, HCT-116 colon cancer cells carrying targeted homozygous disruptions of both DNMT1 and DNMT3b lose approximately 95% of their genomic methylation levels, suggesting that DNMT3b may cooperate with and/or complement DNMT1 in maintaining genomic methylation patterns (109).

How these enzymes produce the CGI hypermethylation changes observed in prostate cancer initiation and progression is largely unknown and is the focus of intense research. It is possible that exposure of prostate epithelia to inflammatory stresses and oxidative agents may directly or indirectly activate these enzymes to hypermethylate CGIs *de novo*. One study has shown that IL-1β, an inflammatory cytokine, can induce CGI hypermethylation and silencing of *FMR1* and *HPRT* in cells (110). Furthermore, this study showed that this effect is likely to be mediated by a modulation of DNA methyltransferase activity by nitric oxide, a potent oxidative agent, as evidenced by the following observations: *i*) IL-1β often leads to induction of the inducible form of nitric oxide synthase (iNOS), *ii*) the silencing of *FMR1* and *HPRT* could be prevented by iNOS inhibitors, *iii*) the silencing of *FMR1* and *HPRT* could be reversed by treatment with DNA demethylating agents, *iv*) treatment of nuclear protein extract with nitric oxide donors increased overall DNA methyltransferase activity, but treatment of whole cells with nitric oxide donors did not cause an increase in DNMT expression (110). This provides preliminary evidence that the inflammatory cytokines and oxidative species found in PIA lesions may directly enhance the activity of DNA methyltransferases and lead to increased *de novo* CGI hypermethylation of specific genes in prostate epithelia. These hypermethylation changes would then be tightly maintained when survival pressures lead to selection of cells with the appropriate CGI hypermethylation changes. However, this paradigm is still hypothetical, and must be further explored by rigorous *in vitro* studies examining the mechanism by which inflammatory cytokines and nitric oxide can enhance DNA methyltransferase activity, and *in vivo* studies examining how this enhanced activity may contribute to CGI hypermethylation and carcinogenesis.

4. MODELS FOR THE DEVELOPMENT OF PROSTATE CANCER METASTASES

The mechanisms underlying the dissemination of primary prostate cancer and establishment of metastatic deposits is a topic of great interest since these lesions are ultimately responsible for the vast majority of prostate cancer deaths. It is clear from clinical observations that prostate cancers have a predilection to metastasize to a distinct set of anatomical organ systems, such as lymph node, bone and liver. The first formal hypothesis suggesting an explanation for the non-random distribution of sites to which primary cancers metastasize was proposed by Stephen Paget in 1889 (111, 112). His "seed and soil" hypothesis suggested that factors in the target site environment promoted the growth of cancer cells there, much like fertile soil would promote the growth of seeds. A modern view of this hypothesis would suggest two possibilities: *i*) that the target site microenvironment would either promote cancer cells to change and adapt when they reach the target site and then establish a metastatic neoplasm, or *ii*) that the microenvironment in the target sites would select for cells that already possess the ability to grow there and form a metastatic neoplasm (Figure 5).

We have already observed that the pattern of CGI hypermethylation in prostate cancer metastases is extremely similar to the pattern seen in the primary prostate cancers. Moreover, the CGI hypermethylation patterns in anatomically distinct metastatic deposits appear to be very similar to each other, showing almost no site specificity. Taken together, these two observations support the second of the two possibilities stated above; that is, some subset of primary prostate cancer cells already have the ability to grow and thrive in a specific set of distant sites but not others. When these prostate cancer cells disseminate and reach various compatible target sites, they can invade those sites, and make modifications to the microenvironment in that site if need be, and develop into a metastatic prostate cancer focus. Indeed, the potential to metastasize to a specific set of organs may be programmed in the primary prostate cancer itself. There is some evidence for this hypothesis in the CGI hypermethylation patterns seen in primary prostate cancer. For instance, accumulation of cells that are hypermethylated at the *EDNRB* and the *PTGS2* CGIs appear to increase the metastatic potential of primary prostate cancers, since a high degree of hypermethylation at these loci correlate directly with increasing disease severity and increased risk of recurrence respectively (59). Interestingly, prostate cancer bone metastases produce an osteoblastic invasion that may be facilitated by loss of *EDNRB* expression (113). The *EDNRB* gene encodes one of two endothelin receptors, which are G-protein coupled receptors that bind their endothelin (ET) ligands and mediate a wide range of functions including

vasoconstriction, angiogenesis, differentiation and bone remodeling (113). The two known isoforms, endothelin receptor type A (ETA) and B (ETB), appear to have diverse, at times opposing downstream functions after binding endothelin-1 (ET-1) (113). For instance, ETB is thought to aid in ET clearance, providing a check on ligand-dependent ETA mediated mitogenic and angiogenic processes (113). Normal prostate epithelia express both isoforms of the endothelin receptor as well as high levels of ET-1 (114).

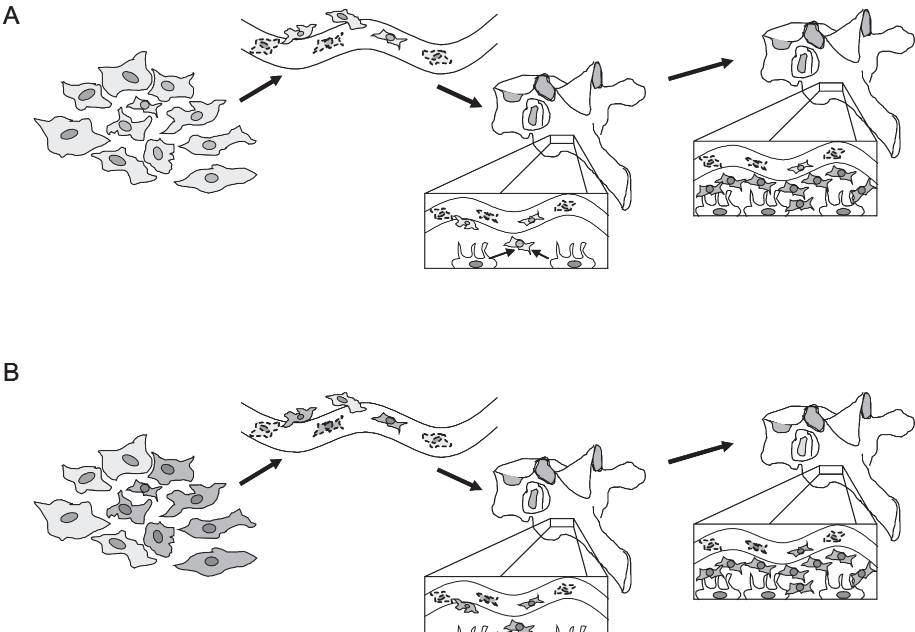

Figure 5. Two potential versions of the “seed and soil” hypothesis with regards to the development of prostate cancer metastases. Panel A. The primary prostate cancer consists of a predominantly homogeneous population of malignant cells. Some of these cells can invade the vasculature, survive and extravasate into distant sites such as the lumbar spines. The microenvironment at these sites can then cause the malignant cancer cells to adapt, acquiring the ability to establish a metastatic neoplasm at the distant site. Panel B. The primary prostate cancer contains many subsets of cells, some of which already have the ability to proliferate at distant sites. Malignant prostate cancer cells from multiple subsets may intravasate. However, the prostate cancer cells with high metastative potential (dark gray) selectively survive in the vasculature and invade distant sites such as the lumbar spines. These cells can then signal changes in the microenvironment of the distant site and proliferate into a metastatic neoplasm. The high degree of similarity in the CpG island hypermethylation pattern between the primary and metastatic prostate cancers and the lack of site-specificity in the CpG island hypermethylation pattern lends support to this second version of the “seed and soil” hypothesis.

However, during prostate cancer progression and metastasis, ETB expression is lost, likely due to CGI hypermethylation, while ETA and ET-1 expression is maintained (114). The osteoblastic processes observed in prostate cancer metastasis to bone are likely mediated by unchecked ETA stimulation of osteoblastic activity in the absence of ETB mediated ET-1 clearance (113). This osteoblastic activity may represent an interplay between the prostate cancer cells and the surrounding bone that may be required for the sustenance and proliferation of the metastatic neoplasm. These notions are further supported by studies showing that: *i*) ETA selective inhibitors decreased prostate cancer growth but ETB selective inhibitors did not (114), *ii*) ETB selective agonists did not stimulate prostate cancer growth (114), *iii*) ET-1 could stimulate osteoblastic activity in cultured mouse calvaria growing in tumor conditioned media and that this stimulation could be inhibited by ETA specific blockers, but not ETB specific blockers (115), *iv*) treatment with ETA specific blockers in mice inoculated with ZR-75-1 cells, which produce radiographic evidence of bone metastases within 3 - 6 months in untreated mice, causes dramatically decreased bone metastases and tumor burden (116), and *v*) patients with hormone-refractory asymptomatic prostate cancer receiving atrasentan, an ETA receptor antagonist, in a multi-center phase II clinical trial had prolonged time to prostate cancer and PSA progression, with corresponding decreases in other serum markers of disease burden (117). These observations provide support to the hypothesis that alterations in gene expression and CGI hypermethylation that were already acquired by cells in the primary prostate cancer allow these cells to metastasize and survive in distant sites such as bone.

Whether prostate cancer metastases arise from a rare variant in the primary cancer or from a highly prevalent population of cells prone to metastasis is also an interesting question. If prostate cancer metastases arose from rare cells in the primary prostate cancer that acquired the requisite changes to invade and metastasize, we would expect that the distant metastases would contain somatic genome changes that are not found in the primary prostate cancer lesion. However, we observe that the CGI hypermethylation changes in the metastatic lesions were already detectable and highly prevalent in the primary prostate cancer lesions, suggesting that cells that accumulated the necessary derangements proliferate in the primary prostate cancer to form a highly prevalent subset of cells and, in some cases, invade and metastasize to distant sites.

The primary prostate cancer cells that acquire the potential to metastasize must still overcome tremendous barriers before they can form distant metastases. In order to successfully metastasize, prostate cancer cells must presumably: a) escape the confines of the local prostate tumor architecture

either by direct extension or migration into lymphatic or blood vessels, b) survive in the lymphatic or blood circulation by withstanding the various sheer forces and evading immune surveillance, c) adhere to endothelial cells in blood vessels at distant sites or epithelial cells in lymphatic vessels in lymph nodes, d) invade into the target site, and e) grow into a metastatic neoplasm. It is still unclear which of these steps is rate limiting in the overall formation of prostate cancer metastases. However, the rate limiting step is likely to occur after prostate cancer cells invade blood vessels since numerous studies have detected copies of hypermethylated *GSTP1* CGIs in the blood of patients with primary prostate cancer even though these patients had no evidence of metastatic disease (118-122). Since hypermethylated GSTP1 CGIs do not occur in normal prostates, these hypermethylated GSTP1 CGIs are likely to be from prostate cancer cells that intravasated and ruptured in the systemic circulation. A more recent study has also demonstrated that the quantity of hypermethylated GSTP1 CGIs in the serum of patients with localized prostate cancer directly correlates with Gleason grade, pathologic stage and the risk of PSA recurrence after radical prostatectomy (121). The accumulation of prostate cancer DNA in the circulation before the development of metastatic disease suggests that the rate limiting step in metastasis formation occurs after prostate cancer cells intravasate.

The CGI hypermethylation patterns seen during prostate cancer initiation and progression suggest a model in which there is an early epigenetic catastrophe, in which several CGIs become hypermethylated very early in the progression of prostate cancer. Indeed, this wave of CGI hypermethylation may begin as early as the PIA lesions and continue during the development of PIN lesions and early stage prostate cancers. The epigenetic DNA methylation changes that occur during this catastrophe are maintained throughout the disease progression, even during the development of metastatic disease. A subsequent wave of CGI hypermethylation begins during the progression of primary prostate cancer through increasing stage and grade. We postulate that when a cell with the requisite somatic genome alterations has proliferated to form a significant subset of cells in the primary prostate cancer lesion, some of these proliferating cells can invade and metastasize to distant sites. These cells are already competent to seed and develop into a metastatic neoplasm at a specific set of organ systems, without much need for additional CGI hypermethylation changes and adaptation at the distant site. This would explain the lack of site-specificity in the CGI hypermethylation pattern among the metastatic prostate cancers (59). Thus, in some ways, the DNA hypermethylation changes necessary for prostate cancer metastases had already formed very early in the disease progression. Much of the models presented in this chapter are somewhat

speculative and hypothetical, reflecting the fact that the study of DNA methylation and prostate cancer metastasis is still in its infancy. As this field matures, many of the unanswered questions posed in this chapter will come to light, leading to novel therapeutic targets and a better understanding of the processes involved in prostate cancer metastasis.

REFERENCES

1. Jemal A., Tiwari R. C., Murray T., Ghafoor A., Samuels A., Ward E., Feuer E. J. and Thun M. J. Cancer statistics, 2004. CA Cancer J Clin, 2004; 54: 8-29.
2. Sakr W. A., Grignon D. J., Crissman J. D., Heilbrun L. K., Cassin B. J., Pontes J. J. and Haas G. P. High grade prostatic intraepithelial neoplasia (HGPIN) and prostatic adenocarcinoma between the ages of 20-69: an autopsy study of 249 cases. In Vivo, 1994; 8: 439-443.
3. Etzioni R., Penson D. F., Legler J. M., di Tommaso D., Boer R., Gann P. H. and Feuer E. J. Overdiagnosis due to prostate-specific antigen screening: lessons from U.S. prostate cancer incidence trends. J Natl Cancer Inst, 2002; 94: 981-990.
4. Platz E. A. and De Marzo A. M. Epidemiology of inflammation and prostate cancer. J Urol, 2004; 171: S36-40.
5. De Marzo A. M., Marchi V. L., Epstein J. I. and Nelson W. G. Proliferative inflammatory atrophy of the prostate: implications for prostatic carcinogenesis. Am J Pathol, 1999; 155: 1985-1992.
6. Putzi M. J. and De Marzo A. M. Morphologic transitions between proliferative inflammatory atrophy and high-grade prostatic intraepithelial neoplasia. Urology, 2000; 56: 828-832.
7. Zha S., Gage W. R., Sauvageot J., Saria E. A., Putzi M. J., Ewing C. M., Faith D. A., Nelson W. G., De Marzo A. M. and Isaacs W. B. Cyclooxygenase-2 is up-regulated in proliferative inflammatory atrophy of the prostate, but not in prostate carcinoma. Cancer Res, 2001; 61: 8617-8623.
8. Parsons J. K., Nelson C. P., Gage W. R., Nelson W. G., Kensler T. W. and De Marzo A. M. GSTA1 expression in normal, preneoplastic, and neoplastic human prostate tissue. Prostate, 2001; 49: 30-37.
9. Nelson W. G., Carter H. B., DeWeese T. L., Bajaj G., Thompson T. L. and Eisenberger M. A. Prostate Cancer. In: M. D. Abeloff (ed.), Clinical Oncology, 2004; 3rd edition, pp. 2085-2148. Philadelphia, Pa.: Elsevier Churchill Livingstone.
10. Partin A. W., Kattan M. W., Subong E. N., Walsh P. C., Wojno K. J., Oesterling J. E., Scardino P. T. and Pearson J. D. Combination of prostate-specific antigen, clinical stage, and Gleason score to predict pathological stage of localized prostate cancer. A multi-institutional update. Jama, 1997; 277: 1445-1451.
11. Han M., Partin A. W., Zahurak M., Piantadosi S., Epstein J. I. and Walsh P. C. Biochemical (prostate specific antigen) recurrence probability following radical prostatectomy for clinically localized prostate cancer. J Urol, 2003; 169: 517-523.
12. D'Amico A. V., Whittington R., Malkowicz S. B., Weinstein M., Tomaszewski J. E., Schultz D., Rhude M., Rocha S., Wein A. and Richie J. P. Predicting prostate specific antigen outcome preoperatively in the prostate specific antigen era. J Urol, 2001; 166: 2185-2188.

13. Fleming I. D. American Joint Committee on Cancer., American Cancer Society., and American College of Surgeons. AJCC cancer staging manual, 1997; 5th ed / edition, p. xv, 294 p. Philadelphia: Lippincott-Raven.
14. Hermanek P., Sobin L. H. and International Union against Cancer. TNM classification of malignant tumours, 1992; 4th edition, p. xiii, 217 p. Berlin; New York: Springer-Verlag.
15. Greene F. L., American Joint Committee on Cancer., and American Cancer Society. AJCC cancer staging manual, 2002; 6th edition, p. xiv, 421 p. New York: Springer-Verlag.
16. Allsbrook W. C., Jr., Mangold K. A., Johnson M. H., Lane R. B., Lane C. G., Amin M. B., Bostwick D. G., Humphrey P. A., Jones E. C., Reuter V. E., Sakr W., Sesterhenn I. A., Troncoso P., Wheeler T. M. and Epstein J. I. Interobserver reproducibility of Gleason grading of prostatic carcinoma: urologic pathologists. Hum Pathol, 2001; 32: 74-80.
17. Allsbrook W. C., Jr., Mangold K. A., Johnson M. H., Lane R. B., Lane C. G. and Epstein J. I. Interobserver reproducibility of Gleason grading of prostatic carcinoma: general pathologist. Hum Pathol, 2001; 32: 81-88.
18. Kronz J. D., Silberman M. A., Allsbrook W. C., Jr., Bastacky S. I., Burks R. T., Cina S. J., Mills S. E., Ross J. S., Sakr W. A., Tomaszewski J. E., True L. D., Ulbright T. M., Weinstein M. W., Yantiss R. K., Young R. H. and Epstein J. I. Pathology residents' use of a Web-based tutorial to improve Gleason grading of prostate carcinoma on needle biopsies. Hum Pathol, 2000; 31: 1044-1050.
19. Kronz J. D., Silberman M. A., Allsbrook W. C. and Epstein J. I. A web-based tutorial improves practicing pathologists' Gleason grading of images of prostate carcinoma specimens obtained by needle biopsy: validation of a new medical education paradigm. Cancer, 2000; 89: 1818-1823.
20. Bubendorf L., Schopfer A., Wagner U., Sauter G., Moch H., Willi N., Gasser T. C. and Mihatsch M. J. Metastatic patterns of prostate cancer: an autopsy study of 1,589 patients. Hum Pathol, 2000; 31: 578-583.
21. Rubin M. A., Putzi M., Mucci N., Smith D. C., Wojno K., Korenchuk S. and Pienta K. J. Rapid ("warm") autopsy study for procurement of metastatic prostate cancer. Clin Cancer Res, 2000; 6: 1038-1045.
22. Nelson W. G., De Marzo A. M. and Isaacs W. B. Prostate cancer. N Engl J Med, 2003; 349: 366-381.
23. Meeker A. K., Gage W. R., Hicks J. L., Simon I., Coffman J. R., Platz E. A., March G. E. and De Marzo A. M. Telomere length assessment in human archival tissues: combined telomere fluorescence in situ hybridization and immunostaining. Am J Pathol, 2002; 160: 1259-1268.
24. Meeker A. K., Hicks J. L., Platz E. A., March G. E., Bennett C. J., Delannoy M. J. and De Marzo A. M. Telomere shortening is an early somatic DNA alteration in human prostate tumorigenesis. Cancer Res, 2002; 62: 6405-6409.
25. Bieberich C. J., Fujita K., He W. W. and Jay G. Prostate-specific and androgen-dependent expression of a novel homeobox gene. J Biol Chem, 1996; 271: 31779-31782.
26. Bhatia-Gaur R., Donjacour A. A., Sciavolino P. J., Kim M., Desai N., Young P., Norton C. R., Gridley T., Cardiff R. D., Cunha G. R., Abate-Shen C. and Shen M. M. Roles for Nkx3.1 in prostate development and cancer. Genes Dev, 1999; 13: 966-977.
27. He W. W., Sciavolino P. J., Wing J., Augustus M., Hudson P., Meissner P. S., Curtis R. T., Shell B. K., Bostwick D. G., Tindal, D. J., Gelmann E. P., Abate-Shen C. and Carter K. C. A novel human prostate-specific, androgen-regulated homeobox gene (NKX3.1)

that maps to 8p21, a region frequently deleted in prostate cancer. Genomics, 1997; 43: 69-77.

28. Ornstein D. K., Cinquanta M., Weiler S., Duray P. H., Emmert-Buck M. R., Vocke C. D., Linehan W. M. and Ferretti J. A. Expression studies and mutational analysis of the androgen regulated homeobox gene NKX3.1 in benign and malignant prostate epithelium. J Urol, 2011; 165: 1329-1334.
29. Bowen C., Bubendorf L., Voeller H. J., Slack R., Willi N., Sauter G., Gasser T. C., Koivisto P., Lack E. E., Kononen J., Kallioniemi O. P. and Gelmann E. P. Loss of NKX3.1 expression in human prostate cancers correlates with tumor progression. Cancer Res, 2000; 60: 6111-6115.
30. Wu X., Senechal K., Neshat M. S., Whang Y. E. and Sawyer, C. L. The PTEN/MMAC1 tumor suppressor phosphatase functions as a negative regulator of the phosphoinositide 3-kinase/Akt pathway. Proc Natl Acad Sci U S A, 1998; 95: 15587-15591.
31. Myers M. P., Stolarov J. P., Eng C., Li J., Wang S. I., Wigler M. H., Parsons R. and Tonks N. K. P-TEN, the tumor suppressor from human chromosome 10q23, is a dual-specificity phosphatase. Proc Natl Acad Sci U S A, 1997; 94: 9052-9057.
32. Myers M. P., Pass I., Batty I. H., Van der Kaay J., Stolarov J. P., Hemmings B. A., Wigler M. H., Downes C. P. and Tonks N. K. The lipid phosphatase activity of PTEN is critical for its tumor supressor function. Proc Natl Acad Sci U S A, 1998; 95: 13513-13518.
33. Ramaswamy S., Nakamura N., Vazquez F., Batt D. B., Perera S., Roberts T. M. and Sellers W. R. Regulation of G1 progression by the PTEN tumor suppressor protein is linked to inhibition of the phosphatidylinositol 3-kinase/Akt pathway. Proc Natl Acad Sci U S A, 1999; 96: 2110-2115.
34. Sun H., Lesche R., Li D. M., Liliental J., Zhang H., Gao J., Gavrilova N., Mueller B., Liu X. and Wu H. PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3,4,5,-trisphosphate and Akt/protein kinase B signaling pathway. Proc Natl Acad Sci U S A, 1999; 96: 6199-6204.
35. Li J., Yen C., Liaw D., Podsypanina K., Bose S., Wang S. I., Puc J., Miliaresis C., Rodgers L., McCombie R., Bigner S. H., Giovanella B. C., Ittmann M., Tycko B., Hibshoosh H., Wigler M. H. and Parsons R. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science, 1997; 275: 1943-1947.
36. McMenamin M. E., Soung P., Perera S., Kaplan I., Loda M.. and Sellers W. R. Loss of PTEN expression in paraffin-embedded primary prostate cancer correlates with high Gleason score and advanced stage. Cancer Res, 1999; 59: 4291-4296.
37. Suzuki H., Freije D., Nusskern D. R., Okami K., Cairns P., Sidransky D., Isaacs W. B., and Bova, G. S. Interfocal heterogeneity of PTEN/MMAC1 gene alterations in multiple metastatic prostate cancer tissues. Cancer Res, 1998; 58: 204-209.
38. Shi X. B., Ma A. H., Xia L., Kung H. J. and de Vere White, R. W. Functional analysis of 44 mutant androgen receptors from human prostate cancer. Cancer Res, 2002;62: 1496-1502.
39. Marcelli M., Ittmann M., Mariani S., Sutherland R., Nigam R., Murthy L., Zhao Y., DiConcini D., Puxeddu E., Esen A., Eastham J., Weigel N. L. and Lamb D. J. Androgen receptor mutations in prostate cancer. Cancer Res, 2000; 60: 944-949.
40. Visakorpi T., Hyytinen E., Koivisto P., Tanner M., Keinanen R., Palmberg C., Palotie A., Tammela T., Isola J. and Kallioniemi O. P. In vivo amplification of the androgen receptor gene and progression of human prostate cancer. Nat Genet, 1995; 9: 401-406.

41. Veldscholte J., Ris-Stalpers C., Kuiper G. G., Jenster G., Berrevoets C., Claassen E., van Rooij H. C., Trapman J., Brinkmann A. O. and Mulder E. A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. Biochem Biophys Res Commun, 1990; 173: 534-540.
42. Laufer M., Denmeade S. R., Sinibaldi V. J., Carducci M. A. and Eisenberger M. A. Complete androgen blockade for prostate cancer: what went wrong? J Urol, 2000; 164: 3-9.
43. Koivisto P., Kononen J., Palmberg C., Tammela T., Hyytinen E., Isola J., Trapman J., Cleutjens K., Noordzij A., Visakorpi T. and Kallioniemi O. P. Androgen receptor gene amplification: a possible molecular mechanism for androgen deprivation therapy failure in prostate cancer. Cancer Res, 1997; 57: 314-319.
44. Taplin M. E., Bubley G. J., Shuster T. D., Frantz M. E., Spooner A. E., Ogata G. K., Keer H. N. and Balk S. P. Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer. N Engl J Med, 1995; 332: 1393-1398.
45. Culig Z., Hobisch A., Cronauer M. V., Cato A. C., Hittmair A., Radmayr C., Eberle J., Bartsch G., and Klocker H. Mutant androgen receptor detected in an advanced-stage prostatic carcinoma is activated by adrenal androgens and progesterone. Mol Endocrinol, 1993; 7: 1541-1550.
46. Craft N., Shostak Y., Carey M., and Sawyers C. L. A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase. Nat Med, 1999; 5: 280-285.
47. Nazareth L. V. and Weigel N. L. Activation of the human androgen receptor through a protein kinase A signaling pathway. J Biol Chem, 1996; 271: 19900-19907.
48. Cheville J. C., Tindall D., Boelter C., Jenkins R., Lohse C. M., Pankratz V. S., Sebo T. J., Davis B. and Blute M. L. Metastatic prostate carcinoma to bone: clinical and pathologic features associated with cancer-specific survival. Cancer, 2002; 95: 1028-1036.
49. Lee W. H., Morton R. A., Epstein J. I., Brooks J. D., Campbell P. A., Bova G. S., Hsieh W. S., Isaacs W. B. and Nelson W. G. Cytidine methylation of regulatory sequences near the pi-class glutathione S-transferase gene accompanies human prostatic carcinogenesis. Proc Natl Acad Sci U S A, 1994; 91: 11733-11737.
50. Lee W. H., Isaacs W. B., Bova G. S., and Nelson W. G. CG island methylation changes near the GSTP1 gene in prostatic carcinoma cells detected using the polymerase chain reaction: a new prostate cancer biomarker. Cancer Epidemiol Biomarkers Prev, 1997; 6: 443-450.
51. Henderson C. J., Smith A. G., Ure J., Brown K., Bacon E. J. and Wolf C. R. Increased skin tumorigenesis in mice lacking pi class glutathione S-transferases. Proc Natl Acad Sci U S A, 1998; 95: 5275-5280.
52. Nelson C. P., Kidd, L. C., Sauvageot, J., Isaacs, W. B., De Marzo, A. M., Groopman, J. D., Nelson, W. G. and Kensler T. W. Protection against 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-b]pyridine cytotoxicity and DNA adduct formation in human prostate by glutathione S-transferase P1. Cancer Res, 2001; 61: 103-109.
53. Shirai T., Sano M., Tamano S., Takahashi S., Hirose M., Futakuchi M., Hasegawa R., Imaida K., Matsumoto K., Wakabayashi K., Sugimura T. and Ito N. The prostate: a target for carcinogenicity of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) derived from cooked foods. Cancer Res, 1997; 57: 195-198.
54. Stuart G. R., Holcroft J., de Boer J. G. and Glickman B. W. Prostate mutations in rats induced by the suspected human carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. Cancer Res, 2000; 60: 266-268.

55. Lin X., Tascilar M., Lee W. H., Vles W. J., Lee B. H., Veeraswamy R., Asgari K., Freije D., van Rees B., Gage W. R., Bova G. S., Isaacs W. B., Brooks J. D., DeWeese T. L., De Marzo A. M. and Nelson W. G. GSTP1 CpG island hypermethylation is responsible for the absence of GSTP1 expression in human prostate cancer cells. Am J Pathol, 2001; 159: 1815-1826.
56. Jeronimo C., Usadel H., Henrique R., Oliveira J., Lopes C., Nelson W. G. and Sidransky D. Quantitation of GSTP1 methylation in non-neoplastic prostatic tissue and organ-confined prostate adenocarcinoma. J Natl Cancer Inst, 2001; 93: 1747-1752.
57. Florl A. R., Steinhoff C., Muller M., Seifert H. H., Hader C., Engers R., Ackermann R. and Schulz, W. A. Coordinate hypermethylation at specific genes in prostate carcinoma precedes LINE-1 hypomethylation. Br J Cancer, 2004; 91: 985-994.
58. Yamanaka M., Watanabe M., Yamada Y., Takagi A., Murata T., Takahashi, H., Suzuki H., Ito H., Tsukino H., Katoh T., Sugimura Y. and Shiraishi T. Altered methylation of multiple genes in carcinogenesis of the prostate. Int J Cancer, 2003; 106: 382-387.
59. Yegnasubramanian S., Kowalski J., Gonzalgo M. L., Zahurak M., Piantadosi S., Walsh P. C., Bova G. S., De Marzo A. M., Isaacs W. B. and Nelson W. G. Hypermethylation of CpG islands in primary and metastatic human prostate cancer. Cancer Res, 2004; 64: 1975-1986.
60. Foster C. S., Bostwick D. G., Bonkhoff H., Damber J. E. van der Kwast T., Montironi R. and Sakr W. A. Cellular and molecular pathology of prostate cancer precursors. Scand J Urol Nephrol Suppl 2000; 19-43.
61. Nakayama M., Bennett C. J., Hicks J. L., Epstein J. I., Platz E. A., Nelson W. G. and De Marzo A. M. Hypermethylation of the human glutathione S-transferase-pi gene (GSTP1) CpG island is present in a subset of proliferative inflammatory atrophy lesions but not in normal or hyperplastic epithelium of the prostate: a detailed study using laser-capture microdissection. Am J Pathol, 2003; 163: 923-933.
62. Nelson W. G., De Marzo A. M., Deweese T. L., Lin X., Brooks J. D., Putzi M. J., Nelson C. P., Groopman J. D. and Kensler T. W. Preneoplastic prostate lesions: an opportunity for prostate cancer prevention. Ann N Y Acad Sci, 2001; 952: 135-144.
63. Brooks J. D., Weinstein M., Lin X., Sun Y., Pin S. S., Bova G. S., Epstein J. I., Isaacs W. B. and Nelson W. G. CG island methylation changes near the GSTP1 gene in prostatic intraepithelial neoplasia. Cancer Epidemiol Biomarkers Prev, 1998; 7: 531-536.
64. Woodson K., Gillespie J., Hanson J., Emmert-Buck M., Phillips J. M., Linehan W. M. and Tangrea J. A. Heterogeneous gene methylation patterns among pre-invasive and cancerous lesions of the prostate: a histopathologic study of whole mount prostate specimens. Prostate, 20044; 60: 25-31.
65. Konishi N., Nakamura M., Kishi M., Nishimine M., Ishida E. and Shimada K. DNA hypermethylation status of multiple genes in prostate adenocarcinomas. Jpn J Cancer Res, 2002; 93: 767-773.
66. Liu L., Yoon J. H., Dammann R., and Pfeifer G. P. Frequent hypermethylation of the RASSF1A gene in prostate cancer. Oncogene, 2002; 21: 6835-6840.
67. Zhu X., Leav I., Leung Y. K., Wu M., Liu Q., Gao Y., McNeal J. E. and Ho S. M. Dynamic regulation of estrogen receptor-beta expression by DNA methylation during prostate cancer development and metastasis. Am J Pathol, 2004; 164: 2003-2012.
68. Maruyama R., Toyooka S., Toyooka K. O., Virmani A. K., Zochbauer-Muller S., Farinas A. J., Minna J. D., McConnell J., Frenkel E. P. and Gazdar A. F. Aberrant promoter methylation profile of prostate cancers and its relationship to clinicopathological features. Clin Cancer Res, 2002; 8: 514-519.

69. Smith W. L., DeWitt D. L. and Garavito R. M. Cyclooxygenases: structural, cellular, and molecular biology. Annu Rev Biochem, 2000; 69: 145-182.
70. Dammann R., Schagdarsurengin U., Strunnikova M., Rastetter M., Seidel C., Liu L., Tommasi S. and Pfeifer G. P. Epigenetic inactivation of the Ras-association domain family 1 (RASSF1A) gene and its function in human carcinogenesis. Histol Histopathol, 2003; 18: 665-677.
71. Nagase H. and Nakamura Y. Mutations of the APC (adenomatous polyposis coli) gene. Hum Mutat, 1993; 2: 425-434.
72. Esteller M., Sparks A., Toyota M., Sanchez-Cespedes M., Capella G., Peinado M. A., Gonzalez S., Tarafa G., Sidransky D., Meltze, S. J., Baylin S. B. and Herman J. G. Analysis of adenomatous polyposis coli promoter hypermethylation in human cancer. 2000; Cancer Res, 60: 4366-4371.
73. Esteller M., Corn P. G., Baylin S. B. and Herman J. G. A gene hypermethylation profile of human cancer. Cancer Res, 2001; 61: 3225-3229.
74. Virmani A. K., Rathi A., Sathyanarayana U. G., Padar A., Huang C. X., Cunnigham H. T., Farinas A. J., Milchgrub S., Euhus D. M., Gilcrease M., Herman J., Minna J. D. and Gazdar A. F. Aberrant methylation of the adenomatous polyposis coli (APC) gene promoter 1A in breast and lung carcinomas. Clin Cancer Res, 2001; 7: 1998-2004.
75. Alberts B. Molecular biology of the cell, 2002, 4th edition. New York: Garland Science.
76. Chesire D. R., Ewing C. M., Sauvageot J., Bova G. S. and Isaacs W. B. Detection and analysis of beta-catenin mutations in prostate cancer. Prostate, 2000; 45: 323-334.
77. Ling V. Multidrug resistance: molecular mechanisms and clinical relevance. Cancer Chemother Pharmacol, 1997; 40 Suppl: S3-8.
78. Campain J. A., Padmanabhan R., Hwang J., Gottesman M. M. and Pastan I. Characterization of an unusual mutant of human melanoma cells resistant to anticancer drugs that inhibit topoisomerase II. J Cell Physiol, 1993; 155: 414-425.
79. Abu-Qare A. W., Elmasry E. and Abou-Donia M. B. A role for P-glycoprotein in environmental toxicology. J Toxicol Environ Health B Crit Rev, 2003; 6: 279-288.
80. van Tellingen O. The importance of drug-transporting P-glycoproteins in toxicology. Toxicol Lett, 2001; 120: 31-41.
81. Tada Y., Wada M., Kuroiwa K., Kinugawa N., Harada T., Nagayama J., Nakagawa M., Naito S. and Kuwano M. MDR1 gene overexpression and altered degree of methylation at the promoter region in bladder cancer during chemotherapeutic treatment. Clin Cancer Res, 2000; 6: 4618-4627.
82. Nakayama M., Wada M., Harada T., Nagayama J., Kusaba H., Ohshima K., Kozuru M., Komatsu H., Ueda R. and Kuwano M. Hypomethylation status of CpG sites at the promoter region and overexpression of the human MDR1 gene in acute myeloid leukemias. Blood, 1998; 92: 4296-4307.
83. Kantharidis P., El-Osta A., deSilva M., Wall D. M., Hu X. F., Slater A., Nadalin G., Parkin J. D. and Zalcberg J. R. Altered methylation of the human MDR1 promoter is associated with acquired multidrug resistance. Clin Cancer Res, 1997; 3: 2025-2032.
84. Desiderato L., Davey M. W. and Piper A. A. Demethylation of the human MDR1 5' region accompanies activation of P-glycoprotein expression in a HL60 multidrug resistant subline. Somat Cell Mol Genet, 1997; 23: 391-400.
85. Jeronimo C., Henrique R., Hoque M. O., Ribeiro F. R., Oliveira J., Fonseca D., Teixeira M. R., Lopes, C. and Sidransky D. Quantitative RARbeta2 hypermethylation: a promising prostate cancer marker. Clin Cancer Res, 2004; 10: 4010-4014.

86. Zhang J., Liu L. and Pfeifer G. P. Methylation of the retinoid response gene TIG1 in prostate cancer correlates with methylation of the retinoic acid receptor beta gene. Oncogene, 23: 2004; 2241-2249.
87. Bronner C. E., Baker S. M., Morrison P. T., Warren G., Smith L. G., Lescoe M. K., Kane M., Earabino C., Lipford J., Lindblom A. and et al. Mutation in the DNA mismatch repair gene homologue hMLH1 is associated with hereditary non-polyposis colon cancer. Nature, 1994; 368: 258-261.
88. Li A. J. and Karlan B. Y. Genetic factors in ovarian carcinoma. Curr Oncol Rep, 2001; 3: 27-32.
89. Paech K., Webb P., Kuiper G. G., Nilsson S., Gustafsson J., Kushner P. J. and Scanlan, T. S. Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. Science, 1997; 277: 1508-1510.
90. Krege J. H., Hodgin J. B., Couse J. F., Enmark E., Warner M., Mahler J. F., Sar M., Korach K. S., Gustafsson J. A. and Smithies, O. Generation and reproductive phenotypes of mice lacking estrogen receptor beta. Proc Natl Acad Sci U S A, 1998; 95: 15677-15682.
91. Leav I., Lau K. M., Adams J. Y., McNeal J. E., Taplin M. E., Wang J., Singh H. and Ho S. M. Comparative studies of the estrogen receptors beta and alpha and the androgen receptor in normal human prostate glands, dysplasia, and in primary and metastatic carcinoma. Am J Pathol, 2001; 159: 79-92.
92. Horvath L. G., Henshall S. M., Lee C. S., Head D. R., Quinn D. I., Makela S., Delprado W., Golovsky D., Brenner P. C., O'Neill G., Kooner R., Stricker P. D., Grygiel J. J., Gustafsson J. A. and Sutherland R. L. Frequent loss of estrogen receptor-beta expression in prostate cancer. Cancer Res, 2001; 61: 5331-5335.
93. Lin X., Asgari K., Putzi M. J., Gage W. R., Yu X., Cornblatt B. S., Kumar A., Piantadosi S., DeWeese T. L., De Marzo A. M. and Nelson W. G. Reversal of GSTP1 CpG island hypermethylation and reactivation of pi-class glutathione S-transferase (GSTP1) expression in human prostate cancer cells by treatment with procainamide. Cancer Res, 2001; 61: 8611-8616.
94. De Weese T. L. and Nelson W. G. Unpublished Data. 2004.
95. Henderson C. J., Wolf C. R., Kitteringham N., Powell H., Otto D. and Park B. K. Increased resistance to acetaminophen hepatotoxicity in mice lacking glutathione S-transferase Pi. Proc Natl Acad Sci U S A, 2000; 97: 12741-12745.
96. Morrow C. S., Diah S., Smitherman P. K., Schneider E. and Townsend A. J. Multidrug resistance protein and glutathione S-transferase P1-1 act in synergy to confer protection from 4-nitroquinoline 1-oxide toxicity. Carcinogenesis, 1998; 19: 109-115,.
97. Lorico A., Nesland J., Emilsen E., Fodstad O. and Rappa G. Role of the multidrug resistance protein 1 gene in the carcinogenicity of aflatoxin B1: investigations using mrp1-null mice. Toxicology, 2002; 171: 201-205.
98. Loe D. W., Stewart R. K., Massey T. E., Deeley R. G. and Cole S. P. ATP-dependent transport of aflatoxin B1 and its glutathione conjugates by the product of the multidrug resistance protein (MRP) gene. Mol Pharmacol, 1997; 51: 1034-1041.
99. Diah S. K., Smitherman P. K., Townsend A. J. and Morrow C. S. Detoxification of 1-chloro-2,4-dinitrobenzene in MCF7 breast cancer cells expressing glutathione S-transferase P1-1 and/or multidrug resistance protein 1. Toxicol Appl Pharmacol, 1999; 157: 85-93.
100. Lin X. and Nelson W. G. Methyl-CpG-binding domain protein-2 mediates transcriptional repression associated with hypermethylated GSTP1 CpG islands in MCF-7 breast cancer cells. Cancer Res, 2003; 63: 498-504.

101. David G. L., Yegnasubramanian S., Kumar A., Marchi V. L., De Marzo A. M., Lin X. and Nelson W. G. MDR1 Promoter Hypermethylation in MCF-7 Human Breast Cancer Cells: Changes in Chromatin Structure Induced by Treatment with 5-Aza-Cytidine. Cancer Biol Ther, 2004; 3: 540-548.
102. Holst C. R., Nuovo G. J., Esteller M., Chew K., Baylin S. B., Herman J. G. and Tlsty T. D. Methylation of p16(INK4a) promoters occurs in vivo in histologically normal human mammary epithelia. Cancer Res, 2003; 63: 1596-1601.
103. Pradhan S., Bacolla A., Wells R. D. and Roberts R. J. Recombinant human DNA (cytosine-5) methyltransferase. I. Expression, purification, and comparison of de novo and maintenance methylation. J Biol Chem, 1999; 274: 33002-33010.
104. Gowher H. and Jeltsch A. Enzymatic properties of recombinant Dnmt3a DNA methyltransferase from mouse: the enzyme modifies DNA in a non-processive manner and also methylates non-CpG [correction of non-CpA] sites. J Mol Biol, 2001; 309: 1201-1208.
105. Okano M., Xie S., and Li E. Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. Nat Genet, 1998; 19: 219-220.
106. Jeltsch A. Beyond Watson and Crick: DNA methylation and molecular enzymology of DNA methyltransferases. Chembiochem, 2002; 3: 274-293.
107. Okano M., Bell D. W., Haber D. A. and Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell, 1999; 99: 247-257.
108. Rhee I., Jair K. W., Yen R. W., Lengauer C., Herman J. G., Kinzler K. W., Vogelstein B., Baylin S. B. and Schuebel K. E. CpG methylation is maintained in human cancer cells lacking DNMT1. Nature, 2000; 404: 1003-1007.
109. Rhee I., Bachman K. E., Park B. H., Jair K. W., Yen R. W., Schuebel K. E., Cui H., Feinberg A. P., Lengauer C., Kinzler K. W., Baylin S. B. and Vogelstein B. DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. Nature, 2002; 416: 552-556.
110. Hmadcha, A., Bedoya, F. J., Sobrino, F., and Pintado, E. Methylation-dependent gene silencing induced by interleukin 1beta via nitric oxide production. J Exp Med, 190: 1595-1604, 1999.
111. Paget S. The distribution of secondary growths in cancer of the breast. Lancet, 1889; 1: 571.
112. Tantivejkul K., Kalikin L. M. and Pienta K. J. Dynamic process of prostate cancer metastasis to bone. J Cell Biochem, 2004; 91: 706-717.
113. Nelson J., Bagnato A., Battistini B. and Nisen P. The endothelin axis: emerging role in cancer. Nat Rev Cancer, 2003; 3: 110-116.
114. Nelson J. B., Chan-Tack K., Hedican S. P., Magnuson S. R., Opgenorth T. J., Bova G. S. and Simons J. W. Endothelin-1 production and decreased endothelin B receptor expression in advanced prostate cancer. Cancer Res, 1996; 56: 663-668.
115. Mohammad K. S. and Guise T. A. Mechanisms of osteoblastic metastases: role of endothelin-1. Clin Orthop 2003; S67-74.
116. Yin J. J., Mohammad K. S., Kakonen S. M., Harris S., Wu-Wong J. R., Wessale J. L., Padley R. J., Garrett I. R., Chirgwin J. M. and Guise T. A. A causal role for endothelin-1 in the pathogenesis of osteoblastic bone metastases. Proc Natl Acad Sci U S A, 2003; 100: 10954-10959.
117. Carducci M. A., Padley R. J., Breul J., Vogelzang N. J., Zonnenberg B. A., Daliani D. D., Schulman C. C., Nabulsi A. A., Humerickhouse R. A., Weinberg M. A., Schmitt J. L. and Nelson J. B. Effect of endothelin-A receptor blockade with atrasentan on tumor

progression in men with hormone-refractory prostate cancer: a randomized, phase II, placebo-controlled trial. J Clin Oncol, 2003; 21: 679-689.

118. Goessl C., Muller M., Heicappell R., Krause H. and Miller K. DNA-based detection of prostate cancer in blood, urine, and ejaculates. Ann N Y Acad Sci, 2001; 945: 51-58.
119. Goessl C., Krause H., Muller M., Heicappell R., Schrader M., Sachsinger J. and Miller K. Fluorescent methylation-specific polymerase chain reaction for DNA-based detection of prostate cancer in bodily fluids. Cancer Res, 2000; 60: 5941-5945.
120. Jeronimo C., Usadel H., Henrique R., Silva C., Oliveira J., Lopes C. and Sidransky D. Quantitative GSTP1 hypermethylation in bodily fluids of patients with prostate cancer. Urology, 2002; 60: 1131-1135.
121. Bastian P. J., Palapattu G. S., Lin X. S., Yegnasubramanian S., Mangold L. A., Trock B., Eisenberger C. F., Partin A. W. and Nelson W. G. Preoperative Serum DNA GSTP1 CpG Island Hypermethylation and the Risk of Early PSA Recurrence Following Radical Prostatectomy. Unpublished Data, 2004.
122. Goessl C., Muller M. and Miller K. Methylation-specific PCR (MSP) for detection of tumour DNA in the blood plasma and serum of patients with prostate cancer. Prostate Cancer Prostatic Dis, 2000; 3: S17.

Chapter 5

CPG ISLAND HYPERMETHYLATION IN BREAST CANCER PROGRESSION AND METASTASIS

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Abstract: Breast cancer is the most common malignancy in women and comprises 18% of all female cancers. The incidence of breast cancer increases with age and in the western countries the disease is the single most common cause of death among women aged 40-50, accounting for about a fifth of all deaths in this age group. The advent of mammography screening has led to an increased detection of pre-invasive mammary lesions and a better elucidation of the pathological events that precede the development of invasive breast carcinoma. Invasive breast cancer is classified in two main morphological subtypes ductal carcinoma representing about 80% of breast malignancy, and lobular carcinoma that accounts for approximately 10% of breast cancers. Among pre-invasive breast lesions, the hyperplasia of the usual type (HUT) is morphologically and phenotypically heterogeneous, whereas atypical ductal hyperplasia (ADH) and ductal carcinoma in situ (DCIS) are homogenous in cell type and marker expression. On the basis of epidemiological and clinical data ADH at the present is seen as a risk factor and not as a direct precursor of DCIS or invasive lesions. Thus the only proliferative lesion that can be considered as a true precursor of invasive breast cancer is DCIS. This model of pathological progression is partially corroborated by genetic studies. In recent years progresses were made in defining some of the critical processes involved in breast cancer development and progression, and CpG island hypermethylation is emerging as one of the main mechanisms for inactivation of cancer related genes in breast tumorigenesis. Three types of genes are involved in carcinogenesis: oncogenes, tumor suppressor genes (TSGs) and stability (caretaker) genes. They encode for proteins involved in a series of pathways that control the basic functions of the cell: proliferation, communication with neighboring cells and with extra cellular matrix, senescence and programmed cell death (apoptosis). Epigenetic mechanism can modulate these pathways by acting directly on tumor suppressor genes and

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stability genes and indirectly on oncogenes through their regulators. Studies on several tumor types indicate changes in the number of methylated genes as well as an increase in methylation density during tumor progression, but only few studies have investigated changes in promoter hypermethylation during breast cancer progression. This is mainly due to the intrinsic difficulties to collect lesions that might be representative of all stages of the diseases. An increase in promoter hypermethylation was demonstrated for CCND2, ESR1, CDH1, RASSF1A, AP2α, Twist and maspin from DCIS to invasive tumor. In distant metastases from bone, brain and lung the frequency of methylation for CCND2, RASSF1A, Twist, RARβ2 and HIN1 was statistically significant different as compared with the primary tumor. The analysis of six cases of paired primary tumors and lymph node metastasis showed same methylation patterns for all but one case. The identification of changes in methylation distribution during breast cancer progression is fundamental not only for a better comprehension of the mechanisms involved in breast carcinogenesis, but because such alterations may represent potential markers for early cancer detection and for a better definition of the prognosis.

Key words: breast cancer, progression, metastasis, estrogen receptor, cpg methylation

Breast Cancer is the most common neoplastic disease in women with approximately 1 million new cases diagnosed each year worldwide. In spite of earlier detection and better management, mainly related to recent advances in technology, breast cancer is still the primary cause of cancer deaths among women, responsible for about 375,000 deaths in the year 2000 (1). In recent years progresses were made in defining some of the critical processes involved in breast cancer development and progression. However, the specific molecular mechanisms underlying many of these events still remain to be elucidated. CpG island hypermethylation is emerging as one of the main mechanism for inactivation of cancer related genes in breast tumorigenesis, moreover there are evidences that the number of hypermethylated genes increases during tumor progression representing an interesting target for the development of new molecular markers for cancer detection as well as for novel therapeutical approaches. In the effort to catalogue all these accumulating evidences the chapter provides: 1) an overview of our current knowledge of breast cancer progression from normal cell to pre-invasive, invasive and metastatic lesions; 2) a detailed description of genes found hypermethylated in breast cancer; 3) an attempt to establish a temporal correlation among gene promoter methylation and breast cancer progression and metastasis; 4) the potential application of CpG hypermethylation analysis to breast cancer diagnosis and treatment.

1. BREAST CANCER PROGRESSION MODEL

1.1 Normal Mammary Gland Development

The adult breast consists of a tree-like network of ducts embedded in a stroma of connective and adipose tissues. Terminal duct lobuloaveolar units (TDLU) are the functional milk-producing structure, and are lined by an inner layer of luminal epithelial cells, and an outer layer of contractile mioepithelial cells (2). Unlike most of the organs, which develop during embryogenesis and maintain their architecture during adult life, the breast undergoes two major changes in morphology throughout distinct developmental stages (3). The network of ducts originate before birth by branching and invading the surrounding fat pad and TDLUs exists initially as alveolar buds (3, 4). At puberty under hormonal stimulation ductal outgrow rapidly increases resulting in side branching. The final differentiation is reached during pregnancy and lactation, when lobulo-acinar structures are formed by extensive proliferation followed by terminal differentiation of the milk secreting alveolar cells. Cessation of lactation is followed by massive apoptosis and tissue remodeling with reversion to structure resembling those present in the non-pregnant gland (3, 4). However proliferation and apoptosis occur during each menstrual cycle as demonstrated by thymidine labeling, thus adult breast can never be considered as completely "resting"(5).

1.2 From the Normal Breast to Cancer: the Concept of "Breast Cancer Stem Cell"

It is now well established that breast cancer originates from the TDLU, but it is not clear which are the cells targeted by tumorigenesis (6-10). A recent interesting hypothesis based on experimental evidence from tumor subpopulation transplantation and animal models suggests that mammary tumors may derive from adult breast stem cells (2, 11). The involvement of stem cells in carcinogenesis was suggested more than 30 years ago (10, 12-14), but only recently the tools of stem cell biology were applied to the study of carcinogenesis (14). Adult stem cells are defined by their ability for self-renewal and differentiation into cell lineages present in the specific tissue. Self-renewal ensures propagation of the stem cell compartment, which sustains morphogenesis, tissue repair and maintenance, whereas differentiation generates the specialized cells that constitute each organ (7, 15-17) (Figure 1A). The adult mammary gland requires stem cells or stem

cells like activity to fulfill the developmental changes during pregnancy and lactation (11, 18), and recent studies indicates that stem (or stem like) cell may in effect exist in the human adult breast (19-25). The experimental evidences point to an undifferentiated, suprabasal cell, able to differentiate toward luminal or mioepithelial phenotypes (24). In *in vitro* three-dimensional studies and in xerografts this cell was also able to generate TDLU-like structures (2). According to the classical progression model, breast cancer arises as consequence of the accumulation of genetic and epigenetic alterations. The "cancer stem cell" hypothesis suggests that adult mammary stem (or stem-like) cells are the targets of these changes. Multiple mutation and epigenetic deregulation will affect the balance between self-renewal and differentiation leading ultimately to tumor development and progression. Al-Hajj *et al.* (14) have provided the first direct evidence of the existence of a human breast cancer stem cell. By flow cytometry was identified a subpopulation of tumor cells defined by the presence of the surface marker CD44, absent or low expression of CD24 and the lack of mammary cell specific lineage markers ($CD44^{+}/CD24^{-/low}/lineage^{-}$). This population was 10-50 fold more tumorigenic in xerografts compared with the bulk of the tumor, and was able to generate both tumorigenic cells and non-tumorigenic cells with distinct molecular phenotype. These behaviors suggest that the population identified can undergo to both self-renewal and differentiation albeit abnormal, properties typical of a stem cell (6) (Figure 1B). The presence of such a population may have important implications for cancer therapies. Current anticancer treatments target mostly proliferating cells that may eliminate the mass of the tumor but not the relative quiescent tumor stem cells. On the other hand novel therapeutic strategies based on molecular specific abnormalities (i.e. Herceptin in Her-2neu positive breast cancer) might be ineffective if they are absent in the stem-like cells. In both cases the mass of the tumor will be reduced but the tumor will continue to grow (26).

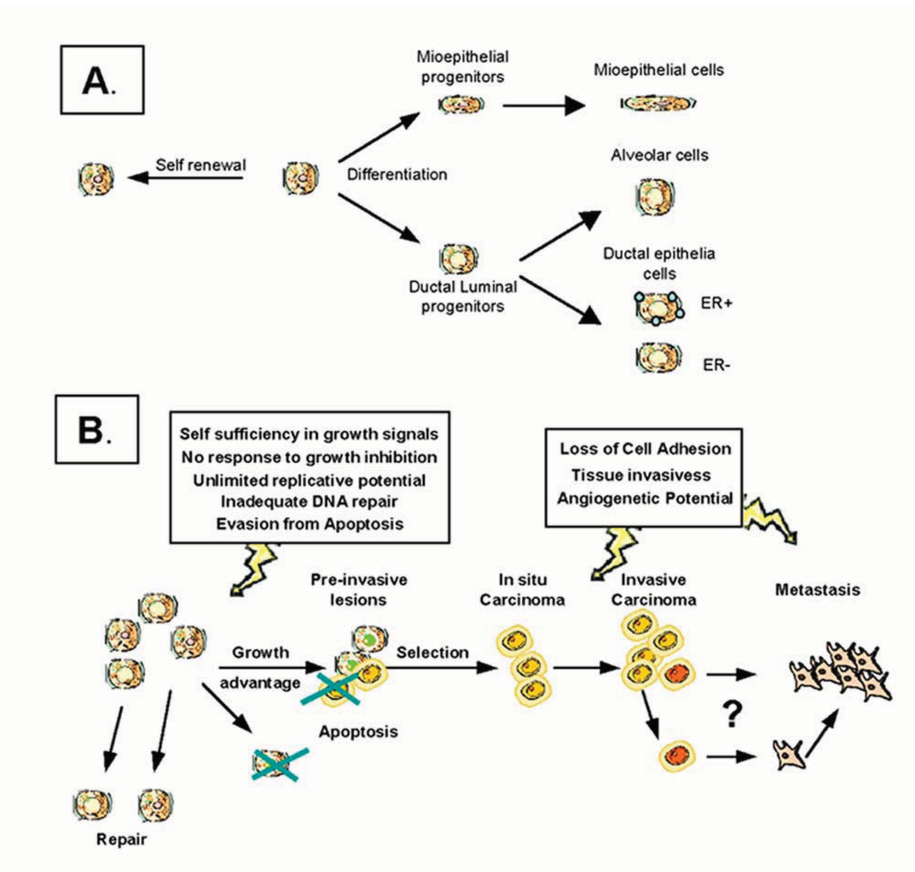

Figure 1. Mammary gland normal development and carcinogenesis. A) During normal development mammary stem cells undergo to self-renewal, and differentiation. Committed progenitors will give rise to mioepithelial, ductal luminal and alveolar cells forming terminal duct lobuloaveolar units structures. B) Carcinogenesis is the result of genetic and epigenetic "hits" affecting any of the progenitors cells, determining an initial growth advantage that, through clonal selection processes lead to the development of *in situ* carcinoma, invasive breast cancer and metastatic disease.

1.3 Stages on the Way to Breast Cancer

The advent of mammography screening has led to an increased detection of pre-invasive breast lesions and a better elucidation of the multistep events leading to invasive carcinoma (Figure 2). This model is partially corroborated by genetic studies based on Comparative Genomic Hybridization (CGH) and Loss of Heterozigosity (LOH) analysis. Similar chromosomal abnormalities were found in Atypical Ductal Hyperplasia

(ADH) and Ductal Carcinoma *in situ* (DCIS), whereas the majority of Hyperplasia of Usual Type (HUT) does not seems to be precursors of DCIS and invasive breast carcinoma (27).

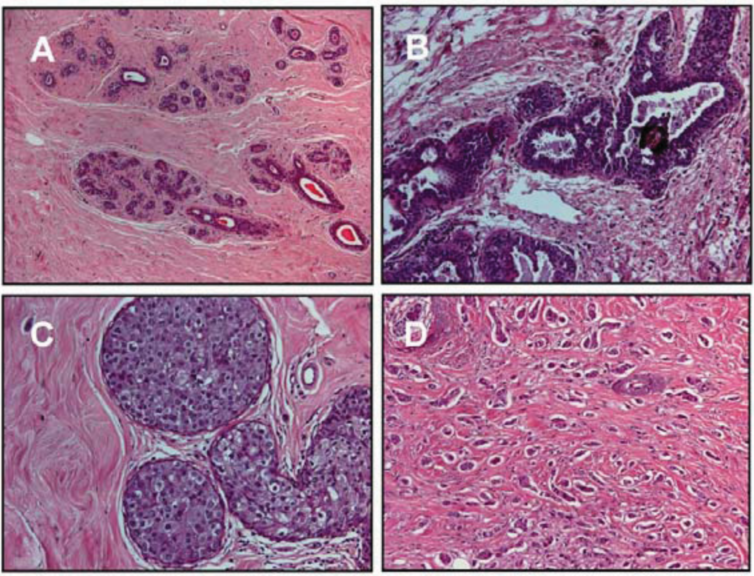

Figure 2. Breast histopathology. A) Normal breast tissue: ducts lined by a layer of epithelial cells are embedded into a stroma of connective tissue; B) Atypical Ductal Hyperplasia (ADH): ducts show an increased volume with lumen partially occupied by proliferating atypical cells; C) Ductal Carcinoma in situ (DCIS): the entire lumen of the ducts is occupied by markedly atypical cells, the malignant proliferation is wholly contained within the basal membrane; D) Invasive Ductal Carcinoma: the stroma is invaded by neoplastic cells, which organization resembles tubulo-acinar structures. (Pictures are a courtesy of Prof R Rossiello and Dr A. Apicella Department of Pathology "L. Armanni" II University of Naples, Italy).

1.3.1 Intraductal Proliferation of the Breast

There are three types of intraductal proliferation lesion of the breast: hyperplasia of the usual type (HUT), atypical ductal hyperplasia (ADH) and ductal carcinoma *in situ* (DCIS) (28). HUT is usually morphologically and phenotypically heterogeneous whereas ADH and DCIS are homogenous in cell type and marker expression (29). A follow-up study from Dupont and Page (30-32) indicated that HUT doubles the risk of developing invasive carcinomas. This view has been subsequently confirmed, but epidemiological and molecular studies cannot clearly demonstrate that malignant breast tumors originate from HUT directly or through ADH, because a shared cause could promote both type of lesions (33). ADH at the

present is seen as a risk factor and not as a direct precursor of DCIS or invasive lesions (34, 35). This is a rare condition detected in approximately 4% of benign breast biopsies and seen most commonly as incidental finding (35). The frequency of detection increases (31%) in presence of mammography microcalcification (33). ADH is often defined as ”a proliferative lesion that fulfill some but not all criteria for a diagnosis of low grade, non-comedo DCIS” (33, 36-39) (Figure 2B). It was estimated that a diagnosis of ADH increases four to five fold the risk of developing breast cancer in the general population (39). The risk is greater in premenopausal women (about six fold) (33, 40, 41) and in first-degree relatives of breast cancer patients (about ten fold) (42, 43). Although AH and ADH confer some kind of increased risk for breast cancer development, the only proliferative lesion that can be considered as a true precursor of invasive breast cancer is DCIS (42). Thus all invasive carcinomas originate as a carcinoma *in situ*, although this does not mean that all DCIS will progress to invasive tumor. DCIS is defined as a proliferation of neoplastic cells with no signs of invasion of the basal membrane (44, 45) (Figure 2C). It is a localized lesion involving only one region of the breast and in most of the cases only one quadrant (43, 46). Before the advent of mammography DCIS lesions where uncommon accounting for about 1 to 5% of breast cancer. After mammography screening was implemented pure DCIS accounts for 15 to 30% of all breast cancer cases (43, 47-49). Retrospective studies indicates that 30 to 50% of DCIS evolves toward invasive carcinomas within 6-10 years from the diagnosis. Invasive breast cancer occurs in the same region of the original lesion further suggesting that DCIS is a precursor. Progression to invasion is related to DCIS subtype with the comedo type that progress more frequently and rapidly (50).

1.3.2 Atypical Lobular Hyperplasia (ALH) and Lobular Carcinoma *in situ* (LCIS)

Atypical lobular hyperplasia and lobular carcinoma *in situ* are relatively infrequent lesions, representing incidental finding in breast biopsy performed for other indications. LCIS was first described in 1941 and shows morphological similarities with the cells of the lobular invasive carcinoma with the difference that foci of neoplastic cells are contained within the basal membrane (51). The term ALH was subsequently introduced to describe morphological similar but less well developed lesions (51-54). Since their first characterization ALH and LCIS become well-established histopathological entities in the classification of breast cancer. However, clinical diagnosis is not possible, because LCIS does not form palpable lump and is visible by mammography only in the rare case when a calcifying

subtype is present (51, 55). Since LCIS is clinically undetectable, its true incidence in the general population cannot be determined: the incidence of LCIS in otherwise benign biopsies has been estimated between 0.5% and 3.9% (56).

Initially LCIS was thought, as a precursor lesion of lobular invasive carcinoma, but following studies seems rather to suggest that they represent marker lesion rather than true invasive carcinoma precursors. In fact they are often multifocal and bilateral, and subsequent malignant tumors are more frequently of ductal rather than lobular type (51, 57). It has been estimated that women with a diagnosis of LCIS have a risk to develop invasive breast cancer 6.9 to 12 times higher than those without LCIS (58). A report combining results from nine studies revealed that 15% of the patients diagnosed with LCIS developed an ipsilater invasive carcinoma, and 9.3% a controlateral invasive tumor (33, 59, 60). Although ALH is often considered as the same that LCIS, patients with ALH show a lower risk of developing invasive carcinoma suggesting that the two lesions have to be considered separate entities (30)

1.3.3 Invasive Breast Cancer

About 80% of invasive breast tumors are ductal carcinomas, lobular carcinoma accounts for 8 to 14% of mammary cancer, whereas the remaining malignant tumors are classified as “special types” including tubular, medullar, cribriform, mucoid and papillary types. The clinical presentation of an invasive breast cancer is often palpable breast nodule. Mammography fails to detect 15% of the invasive lesions and in particular lobular carcinomas. In these cases, ultrasound and/or biopsy are needed to differentiate between benign and malignant breast lesions. Tumor stage is the main factor influencing treatment and prognosis of breast cancer patients (61). The most commonly used staging criteria is the one adopted by the American joint Committee (AJC) based on tumor size (T), lymph node status (N) and metastases (M) and subsequently incorporated in the Union International Contre le Cancer (UICC) staging system. The TNM system has been improved by the adding of a separate pathological classification that incorporates into the system tumor size and node status as assessed by pathologist (62). Data from several studies suggest that after 5 years from the diagnosis survival rate is 84% and 71% for women diagnosed with Stage I or Stage II disease respectively, but it decrease to 48% for Stage III and it is only 18% for Stage IV (63, 64). Lymph nodes status is the main prognostic factor, the rate of 5-years recurrence is approximately 20% in absence of lymph node metastases, whereas from 54-80% of the cases with more than 4 positive lymph nodes will recur (65). Expression of estrogen receptor (ER)

as determined by immunoistochemestry identifies a group of breast cancer cases with better prognosis. Two thirds of invasive breast tumors are ER positive, and 60% of them will respond to anti estrogenic therapy with tamoxifene (66). Her2-neu amplification can be demonstrated in a subset of breast cancers, and characterizes a subpopulation with general worst prognosis but potentially responsive to the treatment with anti-HER2 monoclonal antibody (67). Approximately one-fifth to two thirds of all patients with primary invasive breast cancer will eventually develop disseminated disease. Relapses occur at an approximately constant rate in the first 5-10 years than continue at a progressively slower rate indefinitely. Metastatic breast cancer cases have a survival rate of approximately 2 years. Breast cancer spreads through direct infiltration of the breast parenchyma, via lymphatic system, along mammary ducts and hematogenously to distant sites. Bone is the most common site of first distant relapse (70%) followed by lung (20%) and pleura (8%), more rare are metastasis to brain and liver (68-70).

2. PROMOTER HYPERMETHYLATION OF CANCER RELATED GENES IN BREAST CANCER

Cancer is the result from a multistep process characterized by the accumulation of genetic and epigenetic "hits" leading to uncontrolled growth and ultimately to the acquisition of metastatic potential (Figure 1B). Three types of genes are involved in carcinogenesis: oncogenes, tumor suppressor genes (TSGs) and stability (caretaker) genes. These cancer related genes are involved in a series of pathways that control the basic function of the cell: proliferation, communication with neighboring cells and with extra cellular matrix, senescence and programmed cell death (apoptosis) (71, 72). It is now becoming clear that there are many fewer pathways than genes, and they cross talk to one another forming a complex network of intracellular signals (72). Gene silencing by CpG promoter hypermethylation can modulate these pathways by acting directly on tumor suppressor genes and stability genes and indirectly on oncogenes through their regulators (Table 1). The analysis of methylation profiles in human cancer indicates that hypermethylation of some of the CpG islands are shared by multiple tumor types, whereas others are methylated in a tumor-type-specific manner (73, 74). In breast cancer the analysis of methylation distribution seems to indicate an association between methylation of the ESR1 promoter, and methylation at E-cadherin (CDH1), Thyroid hormone β1 (TRβ1),

Glutathione S-Transferase P1 (GSTP1), and Cyclin D2 (CCND2) (75, 76). Whereas an inverse correlation was found between hypermethylation at BRCA1 and Retinoic Acid Receptor β2 (RARβ2) loci (75, 77). This data further support the hypothesis that promoter hypermethylation does not occur randomly, and suggest the existence of specific selection process targeting key cancer related genes. Since the analysis of methylation profile of histologically distinct classes of breast cancer did not show significant differences (78), it is likely that the methylation subgroup identified represent separate biological entities with potential differences in sensitivity to therapy, occurrence of metastasis and overall prognosis.

Table 1. Genes hypermethylated in breast carcinogenesis.

Gene symbol	Gene name	Function
ADAM23	A disintegrin and metalloproteinase domain	Adhesion, proteolysis
AP2α	Activator-Protein-2α	Promotion of Apoptosis
APC	Adenomatous Polyposi Coli	Cell adhesion, signal transduction stabilization of the cytoskeleton, regulation of the cell cycle apoptosis.
ATM	Ataxia Teleangectasia Mutated	DNA repair
BAX	Bcl-2-X-associated protein	Apoptosis regulator
BCSG1	Breast Cancer Specyfic Gene 1; Synuclein γ	Increases motility and invasiveness
BRCA1	Breast Cancer type 1	DNA repair, cell cycle regulation
CDH1	E-cadherin	Adhesion, suppress invasion and metastasis
CDH13	H-cadherin	Adhesion
CLCA2	Calcium-activated-chloride-channel-2	Suppression of invasion and metastasis
CCND2	Cyclin D2	Cell cycle Regulator
DAPK	Death Associated Protein Kinase 1	Apoptosis
hDAB2IP	DOC-2/DAB2 interacting protein	Ras Regulator?
ESR1	Estrogen Receptor 1 (ERα)	Regulation of cell proliferation predictor of response to hormone therapy
ERβ	Estrogen Receptor 2 (ERβ)	Regulation of cell proliferation antagonizes effects of ERα
FHIT	Fragile Histidine Triad gene	Regulate apoptosis and cell proliferation
GPC3	Glypican-3	Apoptosis
GSTP1	Glutathione-S-Transferase P1	Carcinogen detoxification
HIC1	Hypermethylated in Cancer (HIC1)	Tumor suppressor?
HIN1	High in Normal 1	Putative cytokine inhibits cell growth
HOXA5	Homeo box A5	Upregulates p53, apoptosis
LN5	Laminin 5	Adhesion, suppression of invasiveness and metastasis
Maspin	Protease inhibitor-5	Inhibitor of angiogenesis

Table 1 (cont).

Gene symbol	Gene name	Function
NES1	Kallikrein 10	Inhibition of growth anchorage and tumor formation
Nm23H1	Non-metastatic-cells-23H1	Suppression of metastasis
NOEY2/ARH1	Ras homolog gene family member 1	Regulation of cyclin D1 and p21; inhibition of cell growth.
p16	Cyclin-dependent kinase inhibitor 2A	Cell cycle regulation involved in senescence
PAX5	Paired box gene 5	Inhibition of cell growth
Prostasin	Protease Serine 8	Suppression of invasion
PR	Progesteron receptors	Regulation of cell proliferation
PTEN	Phosphatase and tensin homolog deleted on chromosome 10	Apoptosis
RARβ	Retinoic acid Receptor β	Inhibition of proliferation, apoptosis senescence
RASSF1A	Ras Association domain family protein 1	Interaction with Ras?
RB1	Retinoblastoma 1	Cell cycle Regulation
RIZ1	Retinoblastoma protein interacting zinc finger	Downstream effector of estradiol?
SOCS1	Suppressor of cytokine signaling 1	Regulation of STAT activation
SYK	Spleen Tyrosine Kinase	Suppression of Invasion
THBS1	Thrombospondin I	Suppress angiogenesis and invasion
TIMP-3	Tissue inhibitor of metalloproteinase-3	Suppresses tumor growth invasion and metastasis
TGFBR2	TGFβ receptor Type II	Inhibition of cell growth
TMS1	Target of methylation Induced Silencing	Apoptosis
TP53	Transformation related Protein 53	Apoptosis, cell cycle regulation, inhibition of cell proliferation and invasion.
TRβ1	Thyroid Hormone receptor β1	Control of proliferation and differentiation
TRAIL	TNF-related-apoptosis inducing ligand	Apoptosis
TWIST	Twist related protein-1	$p14^{ARF}$ regulator
ZAC/LOT1	Pleomorphic adenoma gene-like	Apoptosis, Regulation of cell cycle
14-3-3σ	Stratifin	Cell cycle regulator

2.1 Self-sufficiency in Growth Signals

In a normal cell proliferation is positively regulated by growth stimulating signals. Endogenous and exogenous hormones as well as peptide growth factor play a pivotal role in the development of the mammary gland, where they are responsible for epithelial cell proliferation and morphogenesis (79, 80). Several experimental and epidemiological data

indicate a role for hormonal factors and in particular of estrogens in breast tumorigenesis. The incidence of sporadic breast cancer increases with age, reaches a peak between 45 and 50 years, and then declines slowly after menopause, a behavior reflecting the involvement of reproductive hormones (81). Moreover early age at menarche, late age at first birth, low parity, late menopause and hormone replacement therapies increase the risk of developing breast cancer, all these conditions reflecting hormonal patterns that lead to high levels of endogenous or exogenous estrogens (82). Interestingly several studies have reported complex crosstalk between estrogen modulated genes and peptide growth factors signaling cascades such as the Epidermal Growth Factor, (EGF), Insuline like Growth Factor (IGF) and Fibroblast Growth Factor (FGF) pathways (83-86).

2.1.1 Loss of Hormonal Control by Silencing of Nuclear Receptors

Many of the hormones implicated in maintaining mammary gland homeostasis act through members of the nuclear receptor superfamily, a large class of ligand-dependent transcription factors (87). Three classes of nuclear receptor have been identified: Type 1 or steroid receptor that include those for estrogens (ER), progesterone (PR); androgens (AR) glucorticoids (GR) and mineral corticoids (MR); Type II that includes those for all-*trans*-retinoic acid (RAR), thyroid hormone (TR) and vitamin D (VDR); Type III includes the so called orphan receptors for which an endogenous ligands have not been identified (64). Type I receptor, in absence of the ligand, are localized into the cytoplasm coupled with heath-shock protein. In presence of the agonist hormone they homodimerize and translocate to the nucleus were bind to palindromic response element. Type 2 receptors are localized in the nucleus and form heterodimer with the receptor for the 9-cis retinoic acid (RXR) binding constitutively to the response elements consisting of direct repeats where, in the absence of the ligand repress transcription (64). The activity of nuclear receptor is influenced by factors able to enhance (co-activators) or repress (co-repressor) the transcriptional activity (88).

Two Estrogen Receptors (ER) genes have been identified ESR1 encoding for ERα, and ESR2 encoding for ERβ (89-91). In the normal human breast ERα is expressed approximately by 10-30% of the luminal cells but not expressed by the myoepithelial cells (92, 93). In contrast, ERβ is expressed in both luminal and mioepithelial cells (94). The two estrogen receptors are highly conserved in their central DNA binding domain (C-terminus) but diverge in the amino-terminus (95), thus Erβ can activate the same genes regulated by Erα although in a less efficient manner. In cell that co expresses both receptors, ERβ act as an efficient inhibitor of the ERα transcriptional activity (63). Despite the clear role of estrogens in mammary cell

proliferation, the majority of proliferating cells in the adult breast do not express neither ERα nor ERβ (96). The prevailing concept is that estrogens regulate cell growth indirectly by inducing Erα positive cells to produce growth factor able to regulate proliferation in ERα negative cells (85, 97, 98). The ESR1 gene has a complex genomic organization, with at least eight promoters (Figure 3), whose utilization varies between different cell types (99). Therefore ERα expression results from the interplay between all the promoters, and their transcriptional regulators (76, 100-104). *In vitro* studies on breast cancer cell lines have demonstrated that the treatment with demethylating agents (i.e. 5-Azacytidine), can restore ERα expression. Hypermethylation of the first identified promoter, now named promoter A (Figure 3) (105), was investigated in several studies by methyl sensitive PCR or methylation specific restriction landmarks. Some levels of promoter hypermethylation were identified in 25% to 70% of the tumor analyzed (75, 106-108). Aberrant methylation of the promoter B, localized immediately upstream to promoter A, was also reported in breast cancer cell lines and primary tumors (109, 110), where correlated negatively with ERα expression (109). Recently hypermethylation of the distal F promoter, responsible for ERα transcription in bone tissues, was detected in ERα negative but not in ERα positive breast cancer cell lines (111). Transcription of the human ERβ gene also occurs from at least two different promoters 0K and 0N (Figure 3) generating two mRNA that differ at the 5'-untraslated regions. Zhao *et al*. (112) reported extensive methylation of the 0N promoter in breast cancer cell lines and primary tumors in contrast to promoter 0K that was unmethylated. The treatment of cell lines with demethylating agents was able to reactivate expression of the ERβ mRNA (112).

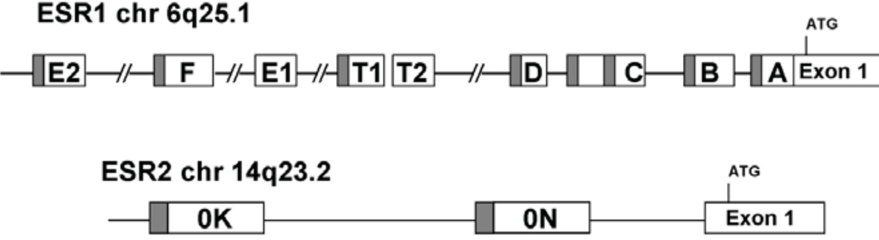

Figure 3. Genomic organization of 5'-untraslated regions (5'UTR) of human estrogen receptors ESR1 (ERα) and ESR2 (ERβ). Promoters are identified as gray boxes, and relative exons as white boxes. ESR1 has at least seven more promoters beside the first identified, and now named A. All mRNA isoforms have a common acceptor splice site in exon 1 at approximately 70 bp from the ATG, broken lines are observed splicing (105). ESR2 has at least two different promoters encoding mRNA isoforms that diverge in their 5'-UTR (112).

Conflicting data have been published regarding the correlation between ESR1 promoter methylation and receptor status determined by routine immunoistochemestry. In studies using MSP as well as those using methylation-sensitive restriction landmarks, aberrant methylation of ESR1 promoter A was found in only a proportion of tumors ER negative, as well as ESR1 promoter hypermethylation was detected in a significant number of ER positive breast cancers (75, 107-109, 113-115). A better correlation was found when ERα levels are determined by quantitative methods (107, 108). A recent study using microarray-based DNA methylation analysis, also indicate that methylation levels of ESR1 promoter A are not predictors of hormonal status. Surprising, in this study higher level of ESR1 methylation correlated with better prognosis in patients treated with tamoxifene (116). However, only one study has analyzed simultaneously two ESR1 promoters (A and B) finding complete ER negative status only when methylation was present in both (109).

The Progesterone Receptor (PR) gene encodes for two isoforms, PR-A and PR-B, both expressed in the luminal epithelial cells. PR-B is preferentially induced by ligand-bound Erα, and presence of progesterone receptor in cancer cells is indicative of a functional estrogen receptor (92, 104, 117, 118). Progesterone receptor isoforms are transcribed from two promoters and translated from two alternative ATG. They only differ for a stretch of about 165 aminoacids in amino terminus of PR-B, a region encoding a transactivating domain required for target genes that can be activated by PR-B but not by PR-A (118). In cell where PR-A is inactive, PR-B function as a strong transactivator of PR-dependent genes, whereas the binding of PR-A to the target gene can repress transcriptional activity of PR-B and other nuclear receptor including ERα (118, 119). Methylation status of the PR promoter was investigated by Lapidus *et al.* using methylation sensitive restriction analysis, they found three sites in the PR gene promoter that were unmethylated in normal breast and hypermethylated in 40% of breast tumors (107, 120).

Retinoids are dietary factors that possess antiproliferative, differentiative, immunomodulatory and apoptosis inducing properties, which act through Retinoic Acid Receptors (RARα, RARβ and RARγ) and Retinoids X Receptor (RXRα, RXRβ, and RXRγ) (121, 122). Both types of receptors are bound as RAR/RXR heterodimers or RXR homodimers to specific retinoic response elements (RAREs) located on the promoter region of target genes (123). In the absence of ligands, corepressor complexes interact with RAR-RXR heterodimes resulting in histone deacetylation, chromatine condensation and silencing of target genes. Agonist binding destabilizes the complex, and with the help of coactivators induces chromatin decondensation and receptor dependent transcription initiation (124). The

RARβ gene encodes for four different transcripts, among those the RARβ2 isoform transcribed from a promoter upstream to exon 3 is frequently lost in breast cancer, whereas RARα and RARγ as well as RXRβ are expressed in both normal breast and cancer cells (125, 126). The RARβ2 promoter region contains a RARE element, thus its expression can be induced by retinoic acid. (124). RARβ2 promoter methylation was found in 20 to 40% of primary breast cancer, and correlated with gene expression (75, 77, 127-129). In RARβ2 negative cell lines re-expression of the gene was demonstrated after treatment with demethylating agents and Histone DeACetylates (HDAC) inhibitors (127, 129-131). Interestingly, the frequency of RARβ2 hypermethylation was significantly higher in breast cancer metastasis to bone, brain and lung (~85%) (132). An interesting mechanism for RARβ epigenetic modulation was described in promielocytic leukemia. This neoplasm is characterized by the expression of the oncogenic fusion protein PML-RARα, which is able to induce hypermethylation of the RARE elements in the RARβ promoter, this effect is reversed by the treatment with retinoic acid (133).

Gene silencing by promoter hypermethylation was also detected in breast cancer for thyroid hormone receptors (TRs). TRs are type II nuclear receptors, thus in absence of the ligand they repress transcription, the binding with the ligand triodiothyronine (T3) destabilize the receptor and activates transcription of target genes. There are eight TRs isoforms encoded by two genes TRα and TRβ (134). In particular the isoform TRβ1 encoded by a gene located on chromosome 3p in a region frequently deleted in breast cancer, is hypermethylated in approximately 25% of the primary breast cancer (75, 135).

2.1.2 Self-activation of Peptide Growth Factors Signaling Pathways

In normal condition cell proliferation is finely regulated by secreted peptide with proliferative or growth inhibitory capability. Growth promoting factors interact with specific transmembrane receptors that initiate a cascade of signals leading ultimately to transcriptional activation of the cell cycle machinery. Self-activation of these transduction signals allows cancer cells to replicate in the absence of the proper mitogenic stimulus and regarding to the presence of inhibitory factors. The mitogen activated protein kinase (MAPK), and in particular, the ERK (extracellular-signal-regulated kinase) cascade is the most commonly involved in human cancers. The major activators of the ERK –pathway are peptide (i.e. EGF, IGF1, prolactin) that bind a tyrosine kinase transmembrane receptor leading to the activation of the G-protein Ras. Activated Ras interacts with a MAPK kinase kinase (Raf), that phosporylates a MAPK kinase (MEK), which finally activates

ERK (136, 137). Induction of the ERK pathway is usually associated with growth proliferation, but it can also determine inhibitory effects manifested by senescence, or apoptosis (137-139). In breast cancer the ERK-pathway is usually up regulated by over expression of the transmembrane epidermal growth factor receptor 2 (EGFR2 or HER2/neu or c-erB-2) as consequence of genomic amplification. Several data, however, indicates that promoter hypermethylation may also play a role in the deregulation of the ERK pathway in mammary tumors. The Ras Association domain Family 1A (RASSF1A) gene is methylated in 42-65% of breast cancer (136, 140-144). The function of this gene at the present is not known, but its homologue RASSF1C bind RAS in a GTP-dependent manner (145). It is possible that RASSF1A will interact with RAS in the same mode, mediating the inhibitory effects on the cell cycle. Loss of *RASSF1A* expression by methylation in human cancer may modify the balance of RAS activities toward a growth-promoting effect. Recently promoter hypermethylation was demonstrated for a novel member of the Ras GTPase activating family named DOC-2/DAB2 interacting protein (hDAB2IP) (146, 147). Aberrant methylation was detected in two CpG reach regions in approximately 50% of the cell lines and 40% of the primary tumors. For one of the two CpG rich regions examined a correlation with lymph node status was observed (148).

Cytokines are known to play an important role in breast cell functions, as trophic hormones and as mediators of host defense mechanisms (149). Like other cytokines IL-6 binds to a specific membrane receptor (IL6R) with activation of the Janus kinase (JAK) family leading ultimately to the phosphorylation of members of the STAT (Signal Transducer and Activators of Transcription) family of transcription factors, After phosphorylation STAT proteins can dimerize and translocate to the nucleus where they regulate transcription of several genes involved in cell growth and differentiation (150). The SOCS (Suppressor of Cytokine Signaling) family of proteins function as negative JAK/STAT regulator. Among the members of the SOCS family SOCS1 was found methylated in a subset of primary breast cancers. Aberrant methylation correlated with transcriptional silencing in breast cancer cell lines and treatment with 5-azacytidine restored expression (149).

2.2 Unresponsiveness to Growth Inhibitory Signals

Transforming Growth Factor (TGFβ) is a potent proliferation inhibitor of both normal mammary epithelial and breast cancer cells. The protein binds to a transmembrane receptor type II, which has a constitutively activated kinase domain (151). After interaction with TGFβ this receptor forms heterodimer with type I receptor that initiate the SMAD proteins signaling

cascade, which ultimately mediates the arrest of cell growth through inhibition of RB phosphorylation (151). ER positive breast cancer cell lines are refractory to the TGFβ effects, whereas ER negative cells usually maintain sensitivity (152). Some studies indicate that TGFβ resistance in ER positive breast cancer cell is related to loss of expression of the TGFβ receptor II (153, 154). Interestingly the treatment of ER positive cell lines with demethylating agents induces expression of TGFβ type II receptors mRNA and proteins (155, 156).

Other genes showing growth inhibition potential and down regulated by promoter hypermethylation in breast cancer are NOEY2, HIN1 and PAX5. NOEY2/ARH1 is a maternally imprinted gene sharing homology with RAS and RAP proteins able to suppress growth of breast and ovarian cancer cell lines through inhibition of the cyclin D1 and induction of $p21^{WAF1/CIP1}$ (157-159). The gene is down regulated in approximately 28-50% of breast cancer (157, 160); LOH is reported in 41% of mammary tumors and in most cases it involves the non-imprinted allele (157-159, 161). HIN1 (high in normal) is a putative cytokine expressed in normal breast tissues, but down regulated in the majority of breast cancer cell lines and primary tumors. Down regulation was associated with promoter hypermethylation in 94% of the cell lines and 74% of breast cancer (143, 162). Methylation was not found in BRCA1 familial breast cancers and sporadic tumors with a “BRCA1-like” histotype, suggesting that HIN1 methylation patterns may be associated with specific breast cancer subsets (163). HIN1 promoter methylation was significantly higher in distant metastases (~100%) as compared with primary tumors (132). PAX5 alpha and beta genes are transcription factors important in cell differentiation and development. They are located on chromosome 9p13 and transcribed from two different promoters with two alternative exon 1 (164, 165). Both promoter are methylated in approximately 65% of breast tumors, expression correlated with the density of methylation and in cell lines treatment with 5’Aza-2’-deoxycitidine restored expression (166). PAX5β encodes for a transcription factor that regulates expression of CD19 a protein able to negatively control cell growth (167). PAX5β loss of expression by promoter hypermethylation correlated with CD19 down regulation, thus PAX5β gene silencing might contribute to carcinogenesis by inhibiting the growth regulatory effects of CD19 (166).

2.3 Acquisition of Unlimited Replicative Potential

The cell cycle consists of an alternating DNA synthetic (S) and mitotic (M) phase, separated by gap phases (G1 and G2). From the time cells exit mitoses, they respond to extra cellular mitogens and antiproliferative agents until a restriction point in the late G1 phase is reached, after this, cell

division can be completed in the absence of extra cellular growth factor (168). The main cell cycle controllers are Cyclin-dependent kinases (cdks), which are characterized by a regulatory (cyclin) and a catalytic (cdk) subunit. In particular, cyclin D- and E- dependent kinases mediated the restriction point control. D-type cyclins interact with two catalytic partners (cdk4 and cdk6), which in response to mitogens phosphorylate the Rb protein with consequent release of E2Fs transcription factor and activation of target genes, which lead to S-phase entry. Cyclin E is activated by E2F and forms a complex with cdk2 reinforcing Rb phosphorylation. The shift in Rb phosphorylation from the mitogens responsive cyclin D cdk4/6 complex to mitogens independent cyclin E-cdk2 is in part responsible for the acquisition of mitogens independency at the restriction point (169). Loss of the Rb protein expression or alterations of the fine mechanism that control Rb phosphorylation are responsible for the uncontrolled cell growth seen in most if not all cancers. Rb mutation are not a frequent event in breast cancer as well as promoter hypermethylation is only reported in 9% of the mammary tumors (149). A putative Rb protein interactor is the RIZ1 gene (retinoblastoma protein-interacting zinc finger), inactivated by promoter hypermethylation in approximately 44% of breast cancers (170). This gene is a member of the nuclear/histone protein methyltransferase superfamily. Methylation of the promoter region correlated with lost or decreased expression and the treatment with demethylating agents was able to reactivate gene expression in RIZ1 negative cell lines (170). Some evidences indicate that RIZ1 is a downstream effector of the estrogen receptor, and it is involved in derepression of estradiol-induced genes (171, 172).

Cyclins play a pivotal role in the regulation of the cell cycle, up regulation of their expression has growth-promoting effects. CCND2 represent an exception, because it is up-regulated under growth arrest in normal cells, and its ectopic expression can effectively block cell cycle progression (173). These suggests that CCND2 is involved in the exit from cell cycle and in maintaining a non proliferative state (174). Down regulation of CCND2 expression was reported in both breast cancer cell lines and primary tumors and it was associated with promoter hypermethylation in a approximately half of the cases (75, 175, 176). The function of cyclin dependent kinases is finely regulated by two classes of inhibitors, the CIP/Kip family and the INK4 family. Initially the members of the Cip/kip family ($p21^{Cip1}$, $p27^{Kip1}$, $p57^{kip2}$) were just considered as cdk4/6 and cdk2 inhibitors. It is now known that they are also, required for the assembly of the active D-dependent holoenzymes, thereby facilitating cyclin E-cdk2 activation (169). The second class of cdk inhibitors represented by the INK4 family ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, $p19^{INK4d}$) act expressly on the cyclin D- dependent kinases. INK4 proteins sequester the cdk4/6 into

complexes releasing Cip/Kip proteins, and indirectly inhibiting cyclin E-cdk2, thereby determining cell cycle arrest (169). The ability to inhibit cell growth in G1 by INK4 proteins depends from the presence of a functional Rb protein. Down regulation of the genes of the INK4a family were described in breast cancer, with a prevalent loss of expression of the $p16^{INK4a}$ gene (177). Intragenic mutation and homozygous deletion were detected in both familial and sporadic cases (75, 178-182), and promoter hypermethylation is reported in approximately 15-20% of breast tumors (75, 77, 183-185). Inactivation of $p16^{INK4a}$ is mandatory for immortalization of human mammary epithelial cells (HMECs), and loss of expression during this process is regulated by promoter methylation (186). After isolation from healthy women, HMEC in colture exhibit an initial growth phase, followed by a plateau, from which emerges a subclone of proliferating cells characterized by $p16^{INK4a}$ methylation (variant HMEC, vHMEC) (187). These cells in continuous colture acquire telomeric dysfunction and accumulate chromosomal abnormalities similar to those seen in premalignant lesions (187). Interestingly, $p16^{INK4a}$ hypermethylation was found in focal patches of histological normal mammary tissue from healthy women (187, 188). On the basis of these data, it is surprising to detect $p16^{INK4a}$ promoter methylation in a small fraction of invasive breast cancer, and even more puzzling is to find up regulation rather than down modulation of $p16^{INK4a}$ expression in more advanced tumors (73, 75, 144, 189). One reason for these conflicting results can be that demethylation occurs later in breast carcinogenesis. Alternatively, $p16^{INK4a}$ inactivation by promoter hypermethylation could be only one of the possible mechanisms involved in the early stages of breast cancer development.

2.4 Evasion from Apoptosis

Programmed cell death (apoptosis) is a mechanism implicated in the control of cell number and in the elimination of dysfunctional cells. Extracellular death signals as well as intracellular stimuli like genotoxic damage can initiate the apoptotic program (190). A major checkpoint in the cell death-signaling cascade is mediated by the BCL-2 family of proteins, which is characterized by members with pro-apoptotic (BAX) or anti-apoptotic (BCL-2) function. Interestingly the Bax gene was recently found methylated in approximately 20% of primary breast cancers suggesting that this subset of tumors have a reduced susceptibility to apoptosis. Downstream to the BAX/BCL-2 checkpoint there are two major execution programs: the mitochondria intrinsic pathway and the caspase cascade. The caspase apoptotic pathway can be directly induced by ligand-bound activation of specific transmembrane receptors such as the members of the tumor necrosis

factor (TNF) family (72). The receptors for the TNF-related-apoptosis-inducing ligand (TRAIL) were recently found epigenetically modulated. TRAIL bind to two apoptotic (DR4 and DR5), and two potentially antiapoptotic receptors (DcR1 and DcR2) (191-193); methylation in breast cancer was reported for the DcR1 and the DcR2 receptor but not for DR4 and DR5 receptors. Promoter hypermethylation correlated with expression in primary tumors and cell lines, and treatment with demethylating agents restored expression (194). TRAIL receptors are also known to activate the NF-κB pathway that is also involved in the inhibition of apoptosis (195, 196). It is possible that changes in the expression of TRAIL receptors in cancer cells may prevalently activate the NF-κB as compared to the apoptotic pathway. The activation of the mitochondrial intrinsic pathway determines change in the mitochondrial membrane potential, production of ROS, opening of permeability transition pores and release of cytochrome c. Release of cytochrome c activates Apaf-1, which induces the caspases cascade leading ultimately to cell death. Caspases are activated by proteolytic cleavage after the association with adaptor proteins characterized by Caspase Recruitment Domains (CARD) (197). The TMS1 (Target of Methylation induced Silencing) gene encodes for a CARD protein which promotes apoptosis (198, 199). TMS1 promoter methylation was reported in 46% of breast cancer cell lines and 32% of primary tumors suggesting that it may play a role in mammary tumorigenesis (200-203).

An important component of the cellular response to stress is the p53 protein. After inappropriate stimuli activated p53 mediates cell cycle arrest and induces apoptosis, in addition the protein is a key constituent of the DNA-damage response machinery (72). In breast cancer p53 mutations are found only in a small proportion of the cases and promoter methylation is also infrequent (204, 205). A key regulator of p53 is the inhibitor MDM2, which bind the protein targeting p53 and itself for ubiquitination and subsequent degradation (206) . MDM2 expression is transcriptionally regulated by p53 creating a negative feedback that in a normal cell maintains p53 levels low. After inappropriate mitogenic signals, hypoxia, or in presence of DNA damage p53 protein is stabilized by inhibition of MDM2 and can translocate in the nucleus, leading ultimately to growth arrest and apoptosis (207). The main regulator of the p53/MDM2 interaction is $p14^{ARF}$ an alternative product of the INK4 locus (208-210). Although epigenetic modulation by methylation of $p14^{ARF}$ has been reported in cancer, it does not seem to play a direct role in breast tumorigenesis (73, 211). Among the protein that regulate $p14^{ARF}$ expression the Death Associated Protein kinase (DAPK) was found methylated in approximately 10% of breast cancers, whereas the Twist gene promoter was methylated in 28% of DCIS, and 42% of invasive tumors, increased frequency of gene promoter methylation was

also detected in distant metastasis (73, 132, 142, 143, 212). Recent data suggest that FHIT (Fragile Histidine Triad) may induce apoptosis and control cell growth through inactivation of MDM2 and p53 stabilization (213). FHIT is a diadenosine idrolase that spans the fragile site FRA3B at chromosome 3p14.2, a region frequently deleted in several tumor types including breast tumors. Promoter CpG methylation was detected in several tumor types and it seems to play a major role in FHIT inactivation in cancer (214). In breast cancer FHIT promoter hypermethylation was detected in 30-50% of the cases and methylation status correlated with loss or reduced gene expression (215, 216). The factors that regulate p53 synthesis have been less analyzed as compared to those controlling protein degradation. The Homeo box A5 HOXA5 is able to induce p53 transcription and its over expression induces p53-dependent apoptotic pathway (217, 218). Interestingly HOX5 is down regulated in 60% of breast cancers and both cell lines and primary tumor show coordinate loss of HOXA5 and p53 mRNA. The HOXA5 gene was methylated in 16 out of 20 p53-negative breast tumors but it was expressed in normal human mammary epithelial cells and immortalized HMEC (217). Among the proteins induced by p53, the 14-3-3σ protein (stratifin) is required for maintenance of G2 arrest (219). Promoter hypermethylation of the 14-3-3σ gene was found in 90% of invasive breast cancer, but its role in carcinogenesis is controversial because the gene is methylated with similar frequencies in normal breast and other non-cancerous tissues (219, 220). Umbricht *et al.* found 14-3-3σ methylation in 96% of invasive tumors, 83% of DCIS and 38% of atypical hyperplasia; no methylation was detected in HUT, and in normal tissues methylation was found in cancer patients but not in healthy individuals (221). Other gene found hypermethylated in breast cancer, which expression seems to be regulated by p53 is the transcriptional repressor HIC1 (222, 223). Although promoter hypermethylation is also found in normal breast tissues, its involvement in breast carcinogenesis cannot be excluded because in at least a subset of tumors with promoter hypermethylation was associated to LOH of the remaining allele (224-228). In animal models HIC1 homozygous disruption is lethal, whereas heterozygous disruption induces spontaneous malignant tumor including epithelial cancers. In these tumors the complete loss of function of the gene is associated to heavy methylation of the remaining allele (229).

Several other genes involved in the apoptotic pathways were found modulated by promoter hypermethylation. PTEN (Phosphates and tensin homolog deleted on chromosome 10) is a tumor suppressor gene implicated in the Cowden Syndrome and Bannayan-Zonana Syndrome both conditions characterized by predisposition to a variety of cancers and in particular to those of the breast (230-232). PTEN is a phosphatase that acts on proteins

and 3-phosphorylated phosphoinositides, including phosphatydilinositol (3,4,5) phosphate (PIP3), thus it can modulate signal transduction pathways involving lipid second messenger (233, 234). Through this mechanism PTEN can regulate the activity of the serine/threonine kinase AKT/PKB leading to suppression of apoptosis (235, 236). In sporadic breast cancers PTEN protein expression was reduced in 34% of the case with concomitant loss of expression as determined by immunohistochemestry in 60% of them (237). The expression of the ZAC/LOT1 gene was found silenced in breast cancer cell lines and primary tumors, and treatment with demethylating agents was able to restore expression in cell lines (238-240). Glypican 3 (GPC3) is a membrane bound heparan sulphate proteoglycan also down regulated in breast cancer cell lines (241). Some studies indicates that GPC3 reduced expression in breast tumors can be at least in part related to promoter hypermethylation (242, 243).

2.5 Inadequate Response to DNA Damage Induced by Endogenous and Exogenous Stimuli

A number of intricate networks have evolved in eukaryotic cells to respond to exogenous and endogenous genotoxic stimuli. Genes involved in these pathways play crucial roles in maintaining genomic integrity, and a defect in these processes may result in hypersensitivity to DNA damaging agents, genomic instability, and may lead ultimately to tumorigenesis. Two main forms of genetic instability are associated to tumors. One arises from the inactivation of DNA mismatch repair (MMR) genes, which leads to instability at nucleotide sequence level. This kind of instability is observed especially at short repeated sequences scattered through the genome named microsatellite, and it is defined as microsatellite instability (MSI) (244). The other form is Chromosomal Instability (CIN), characterized by gross chromosomal rearrangements. As MSI is related to the deregulation of the MMR system, it has been suggested that CIN in neoplastic cells is driven by an early inactivation of critical genes involved in the regulation of chromosomal stability (245). Since MSI and CIN are rarely found to coexist in tumors, one form of instability seems to be sufficient to drive tumor development or progression (244, 245).

Microsatellite instability is uncommon in breast cancer being detected from 0 to 20% of the breast cancer cases (246-249). According to these data, mutation and promoter hypermethylation of MMR genes are also a rare event in mammary carcinogenesis (73, 247). In contrast, karyotypic and fluorescence *in situ* hybridization (FISH) studies, have demonstrated variable states of chromosome structural variations in breast cancers, suggesting that CIN might be far more frequent than instability at nucleotide

level (250). A number of evidences indicate an involvement of the two major familial breast cancer susceptibility genes *BRCA1* and *BRCA2* in pathways induced by DNA damage. In particular both genes seem to play pivotal roles in the repair of double strands breaks (DSBs) (251, 252). BRCA1 and BRCA2 germ-line mutations predispose to early onset breast and ovarian cancer, and the majority of the mutations are non-sense producing a truncated protein. (253). In cancers from individual carrying BRCA1 mutation, the inactivation of the remaining allele is usually determined by LOH, but promoter hypermethylation can be an alternative mechanism for complete gene inactivation (77, 254). Although only a few somatic BRCA1 mutation were reported in sporadic breast cancer, LOH and reduced protein expression can be identified in 30-50% of the cases (255-266). However promoter hypermethylation does not seem to play a pivotal role in Brca1 down regulation in sporadic tumors, since it was reported in only 15% of invasive breast cancers (75, 267-271). BRCA2 promoter methylation was described in a few cases of sporadic ovarian cancers, but never detected in breast tumors (272, 273). Recently epigenetic silencing of the Ataxia Teleangectasia Mutated (ATM) gene, encoding for another key protein of the DSB pathway was reported in sporadic breast cancers (274). Epidemiology studies have previously indicated that ATM heterozygous mutation carriers (approximately 1% of the population) present a 4-5 fold increase of the risk to develop mammary cancer (275-278). Vo *et al*. found ATM proximal promoter methylation in 78% of stage II or greater breast cancer with highly significant correlation with mRNA expression (274).

Another class of genes involved in maintenance of genome stability prevents the damage induced by exogenous and endogenous chemicals. GSTP1 is a member of the glutathione-S-transferase superfamily implicated in detoxification of electrophilic compaund including several carcinogens. Down regulation of GSTP1 expression has been reported in numerous studies and it has been related to hypermethylation of the promoter CpG island (279-282). In breast cancer promoter hypermethylation was detected in approximately one third of the cases (73, 77, 280). An elegant hypothesis links estrogen metabolism to damage induced by GSTP1 inactivation, to carcinogenesis (283). In fact, estrogen metabolism generates reactive oxygen species (ROS) that are neutralized by GSTP1 (282). Thus, GSTP1 inactivation by promoter hypermethylation might lead to the accumulation of ROS with formation of DNA adduct and ultimately to mutation.

2.6 Acquisition of Tissue Invasiveness and Metastasizing Potential

Metastatic breast cancer is essential an incurable disease with less than 10% survival after 5 years (284). Until recently it has been thought that the acquisition of metastatic propensity during the multistep cancer progression required additional events (285). Recent data however seems to challenge this hypothesis suggesting that proclivity to metastasize is acquired early during tumorigenesis rather then near the end (286). Cell-cell and cell-extracellular matrix interactions are essential for the development and maintenance of an organism as well as proteolysis of the extracellular matrix is of vital importance for a series of tissue-remodeling processes, such as morphogenesis, wound healing, and inflammation. Loss of adhesion, tissue invasiveness and sustained angiogenesis are necessary for the acquisition of invasive properties during carcinogenesis, although they are not sufficient to drive the metastatic process (287).

Cadherins are a family of transmembrane glycoproteins that play pivotal role in the establishment and maintenance of normal tissue architecture (288, 289). The E-cadherin is mainly expressed in epithelial tissues and in normal breast prevalently in the luminal cells. In developmental animal models, E-cadherin expression is temporarily down regulated when budding lobules invade the stroma (290, 291). Reduced E-cadherin expression in mammary tumors correlates with loss of differentiation, invasiveness, increased tumor grade, metastases and overall worst prognosis (292-295). Complete loss of expression is found in 85% of the infiltrative lobular carcinomas, whereas variable levels of expression are found in invasive ductal carcinomas (290, 296-299). Loss of heterozigosity at E-cadherin gene locus (CDH1) is a frequent event in both IDC and ILC, but inactivation of the second allele by mutation was demonstrated only in ILC (300-303). In contrast, CDH1 promoter hypermethylation is found in approximately 40% of the breast cancer of both IDC and ILC types (157, 303, 304). Interestingly, the density of methylation at CpG sites seems to increase with progression from *in situ* carcinoma to metastatic tumors whereas no methylation was detected in pre-malignant lesions (75, 76, 305). Moreover in two separate studies CDH1 methylated status correlated with hypermethylation at the ESR1 promoter A (75, 76). Among the other cadherins the H-cadherin (CDH13) was found hypermethylated in approximately one third of primary breast tumors and cell lines. Treatment of CDH13 negative cell lines with 5-Aza-Cytidine was able to restore gene expression (76). Although the cell-to-cell adhesion properties of E-cadherin reside in the extracellular domain, its function depends from the interaction with a group of cytoplasmic proteins named catenins. In particular β-catenin is responsible for the anchorage of E-cad to

the actin cytoskeleton (288). This is intriguing because links E-cadherin to the Wnt (WiNgless Type) pathway involved in embryogenesis and organ development. Wnt proteins are a large family of cystein-rich secreted glycoproteins that bind to specific transmembrane receptors (Frizzled and LRP5/6) and activate β-catenin. In a normal cell β-catenin localize to the cytoplasm, and it is continuously degradated by phosphorylation and subsequent ubiquitination. Phosphorylation occurs in a multiprotein complex, which requires, among other proteins, the presence of the Adenomatous Poliposis Coli (APC) gene. In response to Wnt binding, β-catenin is stabilized, accumulates into the cytoplasm and translocates to the nucleus, where it regulates the expression of a set of genes involved in cell growth, morphology, motility and organ development (306, 307). Interestingly, E-cadherin was shown to inhibit β-catenin-mediated transactivation in an *in vitro* model, thus loss of E-cadherin expression may lead to oncogenic activation of the Wnt pathway (308). Abnormal accumulation of the β-catenin can also been determined by the absence of APC protein expression, and can affect signal transduction, stabilization of cytoskeleton, regulation of cell cycle and apoptosis (309). APC mutation or epigenetic inactivation play a key role in colorectal carcinogenesis and in particular in the early stages of disease progression (310). In breast cancer APC is mainly down regulated by epigenetic mechanisms; promoter hypermethylation is reported in 30-50% of the cases, and in breast cancer cell lines reduced protein expression correlate with methylated status (75, 303, 311). The activator protein-2α (AP2α) is a transcription factors involved in embryogenesis (312, 313). In the adult, AP2α is particularly important in epithelial cells where it has been involved in maintaining cell-to-cell adhesion through activation of E-cadherin (314), mediation of growth arrest through activation of $p21^{Cip1}$ (315) and promotion of apoptosis by interaction with the oncogene c-myc (316). Moreover AP2α can activate or repress several genes involved in breast tumorigenesis such as ER and Her-2-neu (315). Methylation of a small CpG region at the 3' end of exon 1 is methylated in breast cancer but not in normal tissues. Moreover methylation of this region correlates with gene silencing and in cell line the treatment with demethylating agents restores expression. Interestingly promoter methylation occurs in 75% of the invasive breast tumors but only in 16% of DCIS (317).

The interactions between epithelial cells and the extracellular matrix are fundamental for the structural integrity of breast tissue (318). Communication among epithelium and stroma occurs through extracellular proteins (laminins) and their transmembrane receptors (integrins). Attachment to the basement membrane requires the formation of structures called hemidesmosomes (HD). The major constituents of HD are integrin

$\alpha 6\beta 4$ and its ligand laminin 5 (LN5) (319). Laminin 5 specific for epithelium is a heterotrimeric protein and consist of three polypeptide chains $\alpha 3$, $\beta 3$ and $\gamma 2$ encoded by three genes LAMA3, LAMB3, and LANC2 (320). Alteration of hemidesmosomes and loss of expression of LN5 were described in breast cancer tumorigenesis (321, 322). The analysis of the CpG contained in the promoter region of the genes encoding for LN5 chains demonstrated promoter hypermethylation in 40% of breast cancer cell lines and 20% of the primary tumor. Very low levels of methylation were found in non-malignant breast tissues. Methylation of the LAMA3 and a mean chain methylation index were higher in high stage, larger size tumors as compared with low stage smaller size (323). Tissue Inhibitor of Metalloproteinases (TIMP) family members, inhibit the proteolytic activity of metalloproteinase, which may be necessary during tumor growth for angiogenesis, invasion and metastasis (324). In particular, TIMP-3 overexpression in tumor cells can induce apoptosis, and suppress growth and angiogenesis. TIMP-3 was found silenced by promoter hypermethylation in several human cancers including breast tumors (324). Genomic-bisulfite sequencing revealed that TIMP3 silencing correlated with the density of methylation and demethylating agents were able to restore expression in cell lines (325). Maspin is another protease inhibitor that belongs to the serpin family implicated in the inhibition of motility, invasion and metastasis (326, 327). Maspin is expressed in normal breast tissues, but down regulated in breast cancers (328). Promoter hypermethylation was reported in breast cancer cell lines and in a substantial fraction of DCIS (329). The A Disintegrin And Metalloproteinase domain (ADAM) family is implicated in both cell adhesion and regulation of proteolysis (330). Recently promoter hypermethylation of the ADAM23 protein was reported in approximately 60% of cell lines and primary breast tumors with higher degree of methylation in more advanced cases. Promoter hypermethylation was associated with reduced protein expression, and treatment of ADAM23 negative cell lines with demethylating agents restored gene expression. (331). The non-metastatic-23 gene family is characterized by *in vivo* metastasis suppressor activity (332). The nm-23H1 gene has two CpG in islands in the promoter region, and bisulfite sequencing demonstrated infrequent differential methylation in a panel of cell lines and 20 invasive ductal carcinomas. Treatment of the nm23-H1 cell lines with 5-aza-CdR, increased gene expression and reduced cell motility (333).

In animal models derived by human cancers, ion channels have been recently related to suppression of tissue invasiveness and metastatic potential. CLCA2 belong to the calcium activated chloride channel gene family, and is expressed in normal breast epithelial cells but not in breast cancer cell lines and primary tumors (334). When stably transfected in breast

cancer cell lines, CLCA2 is able to reduce tumorigenicity, invasiveness and the ability to colonize the lungs in nude mice (334). The treatment of CLCA2 negative breast cancer cell lines with demethylating agents restore expression, and bisulphate sequencing of the promoter CpG island of CLCA2 in primary tumors demonstrated hypermethylation (335).

Other genes implicated in the acquisition of metastatic propensity and regulated by promoter hypermethylation in primary breast tumors are the Spleen Tyrosine Kinase (SYK), Normal epithelial cell-specific-1 (NES1), and the Breast cancer specific gene 1 (BCSG1). SYK is a putative TSG implicated in the suppression of breast cancer invasion (336). In normal breast epithelium, only the full-length protein SYK(L) is expressed, whereas the shortened transcript SYK(S) is only detected in tumors (337). More than 30% of primary breast cancers show reduced SYK(L) expression which seems to be related to promoter hypermethylation. In SYK negative cell lines stable transfection of SYK(L) or treatment with 5-Aza-Cytidine were shown to inhibit invasiveness and motility (338, 339). NES1 is expressed normal breast but its expression is decreased in breast cancer cell lines and primary breast tumors (340). Although NES1 function is at present not known it seems to inhibit anchorage independent growth and tumor formation in nude mice (341). Hypermethylation of a CpG island spanning exon 3 was correlated to loss of gene expression in breast cancer cell lines and primary tumors. NES1-negative cell lines expressed the protein after treatment with demethylating agents (342). BCSG1 is a member of the neuronal protein family synuclein which overexpression increases cells motility *in vitro* and metastatic potential *in vivo* (343). Interestingly BCSG1 is not expressed in normal breast and highly expressed in advanced tumors. A study from Lu *et al*. suggests that BCSG1 aberrant expression in breast cancer might be related to epigenetic regulation (344). Finally, the angiogenesis inhibitor Thrombospondin 1 (THBS1) and the serine protease Prostasin were found hypermethylated in breast cell lines but so far no data are available on primary tumors (345, 346).

3. TIMING OF PROMOTER HYPERMETHYLATION DURING BREAST CANCER PROGRESSION

Although the past decades of research have shed new light into the epigenetic mechanisms involved in breast carcinogenesis, it is still difficult to establish a precise timing for promoter hypermethylation of the cancer related genes involved in mammary carcinogenesis. This is due to two

reasons. Data for only a few genes are available on pre-invasive lesions, as well as only a few studies have investigated gene promoter hypermethylation in distant metastases. Nevertheless, it seems clear that the number of methylated genes, as well as the frequency of hypermethylation for each individual gene increases during tumor progression. This was demonstrated for CCND2, ESR1, CDH1, AP2α, Twist and maspin going from DCIS to metastatic tumor (76, 135, 142, 144, 175, 317, 329). RASSF1A and 14-3-3σ were methylated in atypical hyperplasia, intraductal papillomas, and DCIS, but 14-3-3σ was also methylated in stroma and lymphocytes (142, 144, 221). In distant metastases from bone, brain and lung the frequency of methylation for CCND2, RASSF1A, Twist, RARβ and HIN1 was statistically significantly different as compared with the primary tumor (132). In another study the analysis of six cases of paired primary tumors and lymph node metastasis showed same methylation patterns for all but one case (347).

4. POTENTIAL CLINICAL APPLICATION OF CPG PROMOTER METHYLATION ANALYSIS

There is now a compelling body of evidence sustaining the importance of epigenetic mechanisms in the development and progression of breast cancer. Since the final goal would be to improve patient management the issue now is to develop new strategies based on the analysis of epigenetic events that may: 1) improve the ability of the pathologist to distinguish definitely malignant from indolent lesions; 2) help the clinician to differentiate between tumors with better or worst prognosis; 3) develop therapeutical strategies that may directly target epigenetic alterations specific of the cancer cells.

The detection of breast cancer in the early stages of development is the key for a successful treatment of the disease. Since promoter hypermethylation seems to occur early during tumor progression it represent a promising tool for the identification of tumor cells in clinical specimens. Methylated DNA can be detected with a very high degree of specificity even in the presence of an excess of unmethylated DNA (348). Fine Needle Aspiration (FNA) cytology is currently implemented into the diagnostic evaluation process of suspicious breast lesions. However this procedure has false negative rates ranging from 5 to 30%. In fact the accuracy of the analysis depends from the ability of the operator to collect the sample and to the proficiency of the cytopathologist in performing the morphological examination (349). Although aberrant promoter methylation was detected with high concordance between FNAs and primary tumors, not always the molecular analysis showed better sensitivity and specificity as compared to

cytological examination (350, 351). These may depend from the panel of genes tested, by analyzing RARβ, RASSF1A and CCND2 Pu *et al*. (350) found different methylation patterns between benign and malignant lesions. Whereas Jeronimo *et al*. (351) using a panel comprising CDH1, GSTP1, BRCA1 and RARβ did not find differences in methylation distribution. Thus as the number of methylated genes increase, more studies should be targeted to the identification of pattern of methylation that clearly distinguish between benign and malignant lesions. To improve early cancer detection other source of biological fluid accessible with minimal invasive techniques were investigated. Most breast cancers arise from the ductal epithelium, thus atypical and malignant cells can be found in ductal lavages or spontaneous produced ductal fluid (Nipple Aspirate). Cytomorphological analysis of these specimens is often unsatisfactory because of the small amount of cells recovered. The analysis of promoter methylation of CCND2, RARβ2 and Twist in ductal lavages allowed the identification of promoter hypermethylation in 17 of 20 fluids from women with a diagnosis of invasive carcinomas and in 2 of 7 fluids from DCIS, only 5 of 45 ductal lavages fluids from healthy women showed methylation at any of the gene tested. Pathologically confirmed breast cancer was subsequently diagnosed in two cases with abnormal cytology and methylated genes in the ductal lavages (212). Krassenstein *et al*. (352) analyzed nipple aspirates fluids from 15 invasive breast cancers and 7 carcinoma *in situ* for promoter hypermethylation of GSTP1, RARβ2, $p16^{INK4}$, $p14^{ARF}$, RASSF1A, and DAPK. Hypermethylation for at least one of the genes was detected in all tumors and identical gene hypermethylation was found in matched nipple aspirate fluid. Serum and plasma are more readily accessible bodily fluids, and collection of the sample does not require the presence of a specialist as it occurs for FNAs, nipple aspirates and ductal lavages Several reports have documented the presence of free DNA into serum or plasma from both healthy and cancer patients. However DNA concentration is ten times higher in cancer patients as compared with control subjects (353). These free DNA's may derive from cells that have invaded the circulatory system or alternatively may be released by the primary tumor from non-viable neoplastic cells. Dulaimi *et al*. (354) analyzed serum from 24 invasive breast cancers, 7 DCIS and 3 LCIS cases for hypermethylation of RASSF1A, DAPK and APC. Concomitant promoter hypermethylation for at least one gene was detected in 76% of the samples including DCIS, LCIS, stage I disease and invasive lobular carcinoma (354). It is long debated whether the presence of genetic and epigenetic alteration in serum/plasma of cancer patients has prognostic value. Two recent studies reported that detection of DNA methylation in serum of breast cancer patients with an high-throughput technique has indeed an independent prognostic value (355, 356).

An important characteristic of epigenetic events is that they are potentially reversible. Since complete gene silencing requires DNA methylation and histone deacetylation both demethylating agents and HDAC inhibitors are necessary to restore expression (357). The ability of 5-azacytidine to induce differentiation in cultured cells is known by decades, this compaund and its deoxy version 5-aza-2'deoxyctidine bind the DNA methyltransferase enzyme in a covalent complex with the DNA resulting in a loss of DNA methylation with each round of cell division (357). Several studies have demonstrated that re-expression of silenced genes with demethylating agents has a strong inhibitory effect on proliferation of cancer cells both *in vitro* and *in vivo*. However the clinical use of 5-azacytidine is limited by its mutagenic properties and especially by the toxicity. In recent years efforts have been made to identify new and less toxic demethylating drugs (358). Among these procainamide and the related drug procaine were effective in re-expressing ERα, RARβ2, and $p16^{INK4}$ in breast cancer cell lines (359, 360). On the side of HDAC Inhibitors the efficacy of phenyl butyric acid is currently tested in phase I and II clinical trials (359). The hope is that these new therapeutical approaches targeting the epigenetic modification present in the primary tumor, eventually in association with current chemotherapy and immunotherapy, will allow a better and more specific Treatment of breast cancer patients.

REFERENCES

1. Parkin D. M., Bray F. I. and Devesa S. S. Cancer burden in the year 2000. The global picture. Eur J Cancer, 2001;37 Suppl 8: S4-66.
2. Rudland P. S. "Mammary stem cells in normal development and cancer." In, *Stem cells*, C. S. Potten ed.: Academic Press, 1997.
3. Howard B. A. and Gusterson B. A. Human breast development. J Mammary Gland Biol Neoplasia, 2000;5: 119-137.
4. Osborne M. P. "Breast development and anatomy in Disease of the Breast". In, *Disease of the Breast*, Jay R. Harris, Lippman, Marc E., Morrow, Monica, Hellman, S. ed., Philadelphia: Lippincott-Raven Publ, 1996.
5. Potten C. S., Watson R. J., Williams G. T., Tickle S., Roberts S. A., Harris M. and Howell A. The effect of age and menstrual cycle upon proliferative activity of the normal human breast. Br J Cancer, 1988;58: 163-170.
6. Smalley M. and Ashworth A. Stem cells and breast cancer: A field in transit. Nat Rev Cancer, 2003;3: 832-44.
7. Kordon E. C. and Smith G. H. An entire functional mammary gland may comprise the progeny from a single cell. Development, 1998;125: 1921-1930.
8. Dontu G., El-Ashry D. and Wicha M. S. Breast cancer, stem/progenitor cells and the estrogen receptor. Trends Endocrinol Metab, 2004;15: 193-197.
9. Reya T., Morrison S. J., Clarke M. F. and Weissman I. L. Stem cells, cancer, and cancer stem cells. Nature, 2001;414: 105-111.

10. Pardal R., Clarke M. F. and Morrison S. J. Applying the principles of stem-cell biology to cancer. Nat Rev Cancer, 2003; 3: 895-902.
11. Dontu G., Al-Hajj M., Abdallah W. M., Clarke M. F. and Wicha M. S. Stem cells in normal breast development and breast cancer. Cell Prolif, 2003; 36 Suppl 1: 59-72.
12. Singh S. K., Clarke I. D., Terasaki M., Bonn V. E., Hawkins C., Squire J. and Dirks P. B. Identification of a cancer stem cell in human brain tumors. Cancer Res, 2003; 63: 5821-5828.
13. Ignatova T. N., Kukekov V. G., Laywell E. D., Suslov O. N., Vrionis F. D. and Steindler D. A. Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers in vitro. Glia, 2002; 39: 193-206.
14. Al-Hajj M., Wicha M. S., Benito-Hernandez A., Morrison S. J. and Clarke M. F. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A, 2003; 100: 3983-3988.
15. Naylor S., Smalley M. J., Robertson D., Gusterson B. A., Edwards P. A. and Dale T. C. Retroviral expression of Wnt-1 and Wnt-7b produces different effects in mouse mammary epithelium. J Cell Sci, 2000; 113 (Pt 12): 2129-2138.
16. Diallo R., Schaefer K. L., Poremba C., Shivazi N., Willmann V., Buerger H., Dockhorn-Dworniczak B. and Boecker W. Monoclonality in normal epithelium and in hyperplastic and neoplastic lesions of the breast. J Pathol, 2001; 193: 27-32.
17. Smith G. H. Experimental mammary epithelial morphogenesis in an in vivo model: evidence for distinct cellular progenitors of the ductal and lobular phenotype. Breast Cancer Res Treat, 1996; 39: 21-31.
18. Weissman I. L., Anderson D. J. and Gage F. Stem and progenitor cells: origins, phenotypes, lineage commitments, and transdifferentiations. Annu Rev Cell Dev Biol, 2001; 17: 387-403.
19. Smith C. A., Monaghan P. and Ellis J. Epithelial cells of the normal human breast. J Pathol, 1985; 146: 221-226.
20. Smith C. A., Monaghan P. and Neville A. M. Basal clear cells of the normal human breast. Virchows Arch A Pathol Anat Histopathol 1984;402: 319-329.
21. Ferguson D. J. An ultrastructural study of mitosis and cytokinesis in normal 'resting' human breast. Cell Tissue Res, 1988; 252: 581-587.
22. Stingl J., Eaves C. J., Kuusk U. and Emerman J. T. Phenotypic and functional characterization in vitro of a multipotent epithelial cell present in the normal adult human breast. Differentiation, 1998; 63: 201-213.
23. Stingl J., Eaves C. J., Zandieh I. and Emerman J. T. Characterization of bipotent mammary epithelial progenitor cells in normal adult human breast tissue. Breast Cancer Res Treat, 2001; 67: 93-109.
24. Gudjonsson T., Villadsen R., Nielsen H. L., Ronnov-Jessen L., Bissell M. J. and Petersen O. W. Isolation, immortalization, and characterization of a human breast epithelial cell line with stem cell properties. Genes Dev, 2002; 16: 693-706.
25. Pechoux C., Gudjonsson T., Ronnov-Jessen L., Bissell M. J. and Petersen O. W. Human mammary luminal epithelial cells contain progenitors to myoepithelial cells. Dev Biol, 1999;,206: 88-99.
26. Foote F. W. Lobular carcinoma *in situ*. A rare form of mammary cancer. Am J Pathol, 1941; 17: 491-496.
27. Reis-Filho J. S. and Lakhani S. R. The diagnosis and management of pre-invasive breast disease: genetic alterations in pre-invasive lesions. Breast Cancer Res, 2003; 5: 313-319.
28. Page D. L. and Dupont W. D. Anatomic indicators (histologic and cytologic) of increased breast cancer risk. Breast Cancer Res Treat, 1993; 28: 157-166.

29. Dupont W. D. and Page D. L. Risk factors for breast cancer in women with proliferative breast disease. N Engl J Med, 1985; 312: 146-151.
30. Fitzgibbons P. L., Henson D. E. and Hutter R. V. Benign breast changes and the risk for subsequent breast cancer: an update of the 1985 consensus statement. Cancer Committee of the College of American Pathologists. Arch Pathol Lab Med, 1998; 122: 1053-1055.
31. Boecker W., Buerger H., Schmitz K., Ellis I. A., van Diest P. J., Sinn H. P., Geradts J., Diallo R., Poremba C. and Herbst H. Ductal epithelial proliferations of the breast: a biological continuum? Comparative genomic hybridization and high-molecular-weight cytokeratin expression patterns. J Pathol ,2001; 195: 415-421.
32. Boecker W., Moll R., Dervan P., Buerger H., Poremba C., Diallo R. I., Herbst H., Schmidt A., Lerch M. M. and Buchwalow I. B. Usual ductal hyperplasia of the breast is a committed stem (progenitor) cell lesion distinct from atypical ductal hyperplasia and ductal carcinoma in situ. J Pathol, 2002; 198: 458-467.
33. Page D. L., Dupont W. D., Rogers L. W. and Rados M. S. Atypical hyperplastic lesions of the female breast. A long-term follow-up study. Cancer ,1985; 55: 2698-2708.
34. Bartow S. A., Pathak D. R., Black W. C., Key C. R. and Teaf S. R. Prevalence of benign, atypical, and malignant breast lesions in populations at different risk for breast cancer. A forensic autopsy study. Cancer, 1987; 60: 2751-2760.
35. Stomper P. C., Cholewinski S. P., Penetrante R. B., Harlos J. P. and Tsangaris T. N. Atypical hyperplasia: frequency and mammographic and pathologic relationships in excisional biopsies guided with mammography and clinical examination. Radiology, 1993; 189: 667-671.
36. Page D. L. and Jensen R. A. Evaluation and management of high risk and premalignant lesions of the breast. World J Surg, 1994; 18: 32-38.
37. Dupont W. D., Parl F. F., Hartmann W. H., Brinton L. A., Winfield A. C., Worrell J. A., Schuyler P. A. and Plummer W. D. Breast cancer risk associated with proliferative breast disease and atypical hyperplasia. Cancer, 1993; 71: 1258-1265.
38. Ma L. and Boyd N. F. Atypical hyperplasia and breast cancer risk: a critique. Cancer Causes Control, 1992; 3: 517-525.
39. London S. J., Connolly J. L., Schnitt S. J. and Colditz G. A. A prospective study of benign breast disease and the risk of breast cancer. Jama, 1992; 267: 941-944.
40. Tavassoli F. A. and Norris H. J. A comparison of the results of long-term follow-up for atypical intraductal hyperplasia and intraductal hyperplasia of the breast. Cancer, 1990; 65: 518-529.
41. Page D. L. and Dupont W. D. Indicators of increased breast cancer risk in humans. J Cell Biochem Suppl, 1992; 16G: 175-182.
42. van Dongen J. A., Fentiman I. S., Harris J. R., Holland R., Peterse J. L., Salvadori B. and Stewart H. J. In-situ breast cancer: the EORTC consensus meeting. Lancet, 1989;,2: 25-27.
43. Page D. L., Dupont W. D., Rogers L. W. and Landenberger M. Intraductal carcinoma of the breast: follow-up after biopsy only. Cancer, 1982; 49: 751-758.
44. Faverly D. R., Burgers L., Bult P. and Holland R. Three dimensional imaging of mammary ductal carcinoma in situ: clinical implications. Semin Diagn Pathol, 1994; 11: 193-198.
45. Holland R., Hendriks J. H., Vebeek A. L., Mravunac M. and Schuurmans Stekhoven J. H. Extent, distribution, and mammographic/histological correlations of breast ductal carcinoma in situ. Lancet, 1990; 335: 519-522.
46. Lagios M. D. Duct carcinoma in situ. Pathology and treatment. Surg Clin North Am, 1990; 70: 853-871.

47. Page D. L., Dupont W. D., Rogers L. W., Jensen R. A. and Schuyler P. A. Continued local recurrence of carcinoma 15-25 years after a diagnosis of low grade ductal carcinoma in situ of the breast treated only by biopsy. Cancer, 1995; 76: 1197-1200.
48. Betsill W. L., Jr., Rosen P. P., Lieberman P. H. and Robbins G. F. Intraductal carcinoma. Long-term follow-up after treatment by biopsy alone. Jama, 1978; 239: 1863-1867.
49. Ketcham A. S. and Moffat F. L. Vexed surgeons, perplexed patients, and breast cancers which may not be cancer. Cancer, 1990; 65: 387-393.
50. Couch F. J., Weber, B.L. "Breast Cancer". In, *The Genetic Basis of Human Cancer*, Bert Vogelstein, Kinzler, Kenneth W. ed., New York: McGraw-Hill, 1998.
51. Haagensen C. D., Lane N., Lattes R. and Bodian C. Lobular neoplasia (so-called lobular carcinoma in situ) of the breast. Cancer, 1978; 42: 737-769.
52. Wheeler J. E., Enterline H. T., Roseman J. M., Tomasulo J. P., McIlvaine C. H., Fitts W. T., Jr. and Kirshenbaum J. Lobular carcinoma in situ of the breast. Long-term followup. Cancer, 1974; 34: 554-563.
53. Sapino A., Frigerio A., Peterse J. L., Arisio R., Coluccia C. and Bussolati G. Mammographically detected in situ lobular carcinomas of the breast. Virchows Arch, 2000; 436: 421-430.
54. Georgian-Smith D. and Lawton T. J. Calcifications of lobular carcinoma in situ of the breast: radiologic-pathologic correlation. AJR Am J Roentgenol, 2001; 176: 1255-1259.
55. Page D. L., Kidd T. E., Jr., Dupont W. D., Simpson J. F. and Rogers L. W. Lobular neoplasia of the breast: higher risk for subsequent invasive cancer predicted by more extensive disease. Hum Pathol, 1991; 22: 1232-1239.
56. Kinne D. W. "Clinical management of lobular carcinoma in situ". In, *Breast Diseases*, JR Harris, Hellman, S., Henderson, I.C., Kinne, D.W. ed., Philadelphia: Lippincott, 1991.
57. Andersen J. A. Lobular carcinoma in situ. A long-term follow-up in 52 cases. Acta Pathol Microbiol Scand [A], 1974; 82: 519-533.
58. Andersen J. A. Lobular carcinoma in situ of the breast. An approach to rational treatment. Cancer, 1977; 39: 2597-2602.
59. Page D. L., Schuyler P. A., Dupont W. D., Jensen R. A., Plummer W. D., Jr. and Simpson J. F. Atypical lobular hyperplasia as a unilateral predictor of breast cancer risk: a retrospective cohort study. Lancet, 2003; 361: 125-129.
60. Marshall L. M., Hunter D. J., Connolly J. L., Schnitt S. J., Byrne C., London S. J. and Colditz G. A. Risk of breast cancer associated with atypical hyperplasia of lobular and ductal types. Cancer Epidemiol Biomarkers Prev, 1997; 6: 297-301.
61. Singletary S. E. and Greene F. L. Revision of breast cancer staging: the 6th edition of the TNM Classification. Semin Surg Oncol, 2003; 21: 53-59.
62. Sainsbury J. R., Anderson T. J. and Morgan D. A. ABC of breast diseases: breast cancer. Bmj, 2000; 321: 745-750.
63. McDonnell D. P. and Norris J. D. Connections and regulation of the human estrogen receptor. Science, 2002; 296: 1642-1644.
64. Tsai M. J. and O'Malley B. W. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem, 1994; 63: 451-486.
65. Valagussa P., Bonadonna G. and Veronesi U. Patterns of relapse and survival following radical mastectomy. Analysis of 716 consecutive patients. Cancer, 1978; 41: 1170-1178.
66. Tamoxifen for early breast cancer: an overview of the randomised trials. Early Breast Cancer Trialists' Collaborative Group. Lancet, 1998; 351: 1451-1467.

67. Menard S., Pupa S. M., Campiglio M. and Tagliabue E. Biologic and therapeutic role of HER2 in cancer. Oncogene, 2003; 22: 6570-6578.
68. Fornier M., Munster P. and Seidman A. D. Update on the management of advanced breast cancer. Oncology (Huntingt), 1999; 13: 647-658; discussion 660, 663-664.
69. Clark G. M., Sledge G. W., Jr., Osborne C. K. and McGuire W. L. Survival from first recurrence: relative importance of prognostic factors in 1,015 breast cancer patients. J Clin Oncol, 1987; 5: 55-61.
70. Rutqvist L. E. and Wallgren A. Long-term survival of 458 young breast cancer patients. Cancer, 1985; 55: 658-665.
71. Fearon E. R. and Vogelstein B. A genetic model for colorectal tumorigenesis. Cell, 1990; 61: 759-767.
72. Vogelstein B. and Kinzler K. W. Cancer genes and the pathways they control. Nat Med, 2004; 10: 789-799.
73. Esteller M., Corn P. G., Baylin S. B. and Herman J. G. A gene hypermethylation profile of human cancer. Cancer Res, 2001; 61: 3225-3229.
74. Costello J. F., Fruhwald M. C., Smiraglia D. J., Rush L. J., Robertson G. P., Gao X., Wright F. A., Feramisco J. D., Peltomaki P., Lang J. C., Schuller D. E., Yu L., Bloomfield C. D., Caligiuri M. A., Yates A., Nishikawa R., Su Huang H., Petrelli N. J., Zhang X., O'Dorisio M. S., Held W. A., Cavenee W. K. and Plass C. Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. Nat Genet, 2000; 24: 132-138.
75. Parrella P., Poeta M. L., Gallo A. P., Prencipe M., Scintu M., Apicella A., Rossiello R., Liguoro G., Seripa D., Gravina C., Rabitti C., Rinaldi M., Nicol T., Tommasi S., Paradiso A., Schittulli F., Altomare V. and Fazio V. M. Nonrandom distribution of aberrant promoter methylation of cancer-related genes in sporadic breast tumors. Clin Cancer Res, 2004; 10: 5349-5354.
76. Nass S. J., Herman J. G., Gabrielson E., Iversen P. W., Parl F. F., Davidson N. E. and Graff J. R. Aberrant methylation of the estrogen receptor and E-cadherin 5' CpG islands increases with malignant progression in human breast cancer. Cancer Res, 2000; 60: 4346-4348.
77. Esteller M., Fraga M. F., Guo M., Garcia-Foncillas J., Hedenfalk I., Godwin A. K., Trojan J., Vaurs-Barriere C., Bignon Y. J., Ramus S., Benitez J., Caldes T., Akiyama Y., Yuasa Y., Launonen V., Canal M. J., Rodriguez R., Capella G., Peinado M. A., Borg A., Aaltonen L. A., Ponder B. A., Baylin S. B. and Herman J. G. DNA methylation patterns in hereditary human cancers mimic sporadic tumorigenesis. Hum Mol Genet, 2001; 10: 3001-3007.
78. Bae Y. K., Brown A., Garrett E., Bornman D., Fackler M. J., Sukumar S., Herman J. G. and Gabrielson E. Hypermethylation in histologically distinct classes of breast cancer. Clin Cancer Res, 2004; 10: 5998-6005.
79. Nandi S., Guzman R. C. and Yang J. Hormones and mammary carcinogenesis in mice, rats, and humans: a unifying hypothesis. Proc Natl Acad Sci U S A, 1995; 92: 3650-7.
80. Kato S., Kitamoto T., Masuhiro Y. and Yanagisawa J. Molecular mechanism of a cross-talk between estrogen and growth-factor signaling pathways. Oncology, 1998; 55 Suppl 1: 5-10.
81. Pike M. C., Spicer D. V., Dahmoush L. and Press M. F. Estrogens, progestogens, normal breast cell proliferation, and breast cancer risk. Epidemiol Rev, 1993; 15: 17-35.
82. Group E. H. a. B. C. C. Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. J Natl Cancer Inst, 2002; 94: 606-616.
83. Henderson B. E. and Feigelson H. S. Hormonal carcinogenesis. Carcinogenesis, 2000; 21: 427-433.

84. Kato S. Estrogen receptor-mediated cross-talk with growth factor signaling pathways. Breast Cancer, 2001; 8: 3-9.
85. Yee D. and Lee A. V. Crosstalk between the insulin-like growth factors and estrogens in breast cancer. J Mammary Gland Biol Neoplasia, 2000; 5: 107-115.
86. Hall J. M., Couse J. F. and Korach K. S. The multifaceted mechanisms of estradiol and estrogen receptor signaling. J Biol Chem, 2001; 276: 36869-36872.
87. Mangelsdorf D. J., Thummel C., Beato M., Herrlich P., Schutz G., Umesono K., Blumberg B., Kastner P., Mark M. and Chambon P. The nuclear receptor superfamily: the second decade. Cell, 1995; 83: 835-839.
88. McKenna N. J., Lanz R. B. and O'Malley B. W. Nuclear receptor coregulators: cellular and molecular biology. Endocr Rev, 1999; 20: 321-44.
89. Mosselman S., Polman J. and Dijkema R. ER beta: identification and characterization of a novel human estrogen receptor. FEBS Lett, 1996; 392: 49-53.
90. Enmark E., Pelto-Huikko M., Grandien K., Lagercrantz S., Lagercrantz J., Fried G., Nordenskjold M. and Gustafsson J. A. Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. J Clin Endocrinol Metab, 1997; 82: 4258-4265.
91. Green S., Walter P., Greene G., Krust A., Goffin C., Jensen E., Scrace G., Waterfield M. and Chambon P. Cloning of the human oestrogen receptor cDNA. J Steroid Biochem, 1986; 24: 77-83.
92. Russo J., Ao X., Grill C. and Russo I. H. Pattern of distribution of cells positive for estrogen receptor alpha and progesterone receptor in relation to proliferating cells in the mammary gland. Breast Cancer Res Treat, 1999; 53: 217-227.
93. Petersen O. W., Hoyer P. E. and van Deurs B. Frequency and distribution of estrogen receptor-positive cells in normal, nonlactating human breast tissue. Cancer Res, 1987; 47: 5748-5751.
94. Speirs V., Skliris G. P., Burdall S. E. and Carder P. J. Distinct expression patterns of ER alpha and ER beta in normal human mammary gland. J Clin Pathol, 2002; 55: 371-374.
95. Kuiper G. G., Carlsson B., Grandien K., Enmark E., Haggblad J., Nilsson S. and Gustafsson J. A. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology, 1997; 138: 863-870.
96. Clarke R. B., Howell A., Potten C. S. and Anderson E. Dissociation between steroid receptor expression and cell proliferation in the human breast. Cancer Res, 1997; 57: 4987-4991.
97. Ethier S. P. Growth factor synthesis and human breast cancer progression. J Natl Cancer Inst, 1995; 87: 964-973.
98. Lippman M. E. and Dickson R. B. Growth control of normal and malignant breast epithelium. Prog Clin Biol Res, 1990; 354A: 147-178.
99. Ponglikitmongkol M., Green S. and Chambon P. Genomic organization of the human oestrogen receptor gene. Embo J, 1988; 7: 3385-3388.
100. Keaveney M., Klug J., Dawson M. T., Nestor P. V., Neilan J. G., Forde R. C. and Gannon F. Evidence for a previously unidentified upstream exon in the human oestrogen receptor gene. J Mol Endocrinol, 1991; 6: 111-115.
101. Grandien K. Determination of transcription start sites in the human estrogen receptor gene and identification of a novel, tissue-specific, estrogen receptor-mRNA isoform. Mol Cell Endocrinol, 1996; 116: 207-212.
102. Thompson D. A., McPherson L. A., Carmeci C., deConinck E. C. and Weigel R. J. Identification of two estrogen receptor transcripts with novel 5' exons isolated from a MCF7 cDNA library. J Steroid Biochem Mol Biol, 1997; 62: 143-153.

103. Flouriot G., Griffin C., Kenealy M., Sonntag-Buck V. and Gannon F. Differentially expressed messenger RNA isoforms of the human estrogen receptor-alpha gene are generated by alternative splicing and promoter usage. Mol Endocrinol, 1998; 12: 1939-1954.
104. Piva R., Gambari R., Zorzato F., Kumar L. and del Senno L. Analysis of upstream sequences of the human estrogen receptor gene. Biochem Biophys Res Commun, 1992; 183: 996-1002.
105. Kos M., Reid G., Denger S. and Gannon F. Minireview: genomic organization of the human ERalpha gene promoter region. Mol Endocrinol, 2001; 15: 2057-2063.
106. Ottaviano Y. L., Issa J. P., Parl F. F., Smith H. S., Baylin S. B. and Davidson N. E. Methylation of the estrogen receptor gene CpG island marks loss of estrogen receptor expression in human breast cancer cells. Cancer Res, 1994; 54: 2552-2555.
107. Lapidus R. G., Ferguson A. T., Ottaviano Y. L., Parl F. F., Smith H. S., Weitzman S. A., Baylin S. B., Issa J. P. and Davidson N. E. Methylation of estrogen and progesterone receptor gene 5' CpG islands correlates with lack of estrogen and progesterone receptor gene expression in breast tumors. Clin Cancer Res, 1996; 2: 805-810.
108. Lapidus R. G., Nass S. J., Butash K. A., Parl F. F., Weitzman S. A., Graff J. G., Herman J. G. and Davidson N. E. Mapping of ER gene CpG island methylation-specific polymerase chain reaction. Cancer Res, 1998; 58: 2515-2519.
109. Iwase H., Omoto Y., Iwata H., Toyama T., Hara Y., Ando Y., Ito Y., Fujii Y. and Kobayashi S. DNA methylation analysis at distal and proximal promoter regions of the oestrogen receptor gene in breast cancers. Br J Cancer, 1999; 80: 1982-1986.
110. Yoshida T., Eguchi H., Nakachi K., Tanimoto K., Higashi Y., Suemasu K., Iino Y., Morishita Y. and Hayashi S. Distinct mechanisms of loss of estrogen receptor alpha gene expression in human breast cancer: methylation of the gene and alteration of trans-acting factors. Carcinogenesis, 2000; 21: 2193-2201.
111. Penolazzi L., Lambertini E., Giordano S., Sollazzo V., Traina G., del Senno L. and Piva R. Methylation analysis of the promoter F of estrogen receptor alpha gene: effects on the level of transcription on human osteoblastic cells. J Steroid Biochem Mol Biol, 2004; 91: 1-9.
112. Zhao C., Lam E. W., Sunters A., Enmark E., De Bella M. T., Coombes R. C., Gustafsson J. A. and Dahlman-Wright K. Expression of estrogen receptor beta isoforms in normal breast epithelial cells and breast cancer: regulation by methylation. Oncogene, 2003; 22: 7600-7606.
113. Falette N. S., Fuqua S. A., Chamness G. C., Cheah M. S., Greene G. L. and McGuire W. L. Estrogen receptor gene methylation in human breast tumors. Cancer Res, 1990; 50: 3974-3978.
114. Hori M., Iwasaki M., Yoshimi F., Asato Y. and Itabashi M. Hypermethylation of the Estrogen Receptor Alpha Gene Is Not Related to Lack of Receptor Protein in Human Breast Cancer. Breast Cancer, 1999; 6: 79-86.
115. Kay P. H., Hahnel R., Gunn H., Harmon D. and Song J. Hypermethylation of HpaII recognition sequences within the 5' coding region of the estrogen receptor gene is not associated with estrogen receptor negativity in primary breast tumours. Anticancer Res, 1998; 18: 1709-1712.
116. Widschwendter M., Siegmund K. D., Muller H. M., Fiegl H., Marth C., Muller-Holzner E., Jones P. A. and Laird P. W. Association of breast cancer DNA methylation profiles with hormone receptor status and response to tamoxifen. Cancer Res, 2004; 64: 3807-3813.

117. Kastner P., Krust A., Turcotte B., Stropp U., Tora L., Gronemeyer H. and Chambon P. Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. Embo J, 1990; 9: 1603-1614.
118. Sartorius C. A., Melville M. Y., Hovland A. R., Tung L., Takimoto G. S. and Horwitz K. B. A third transactivation function (AF3) of human progesterone receptors located in the unique N-terminal segment of the B-isoform. Mol Endocrinol,1994; 8: 1347-1360.
119. McDonnell D. P., Shahbaz M. M., Vegeto E. and Goldman M. E. The human progesterone receptor A-form functions as a transcriptional modulator of mineralocorticoid receptor transcriptional activity. J Steroid Biochem Mol Biol, 1994; 48: 425-432.
120. Ferguson A. T., Lapidus R. G. and Davidson N. E. Demethylation of the progesterone receptor CpG island is not required for progesterone receptor gene expression. Oncogene, 1998; 17: 577-583.
121. Ki Hong W., Lippman S. M., Hittelman W. N. and Lotan R. Retinoid chemoprevention of aerodigestive cancer: from basic research to the clinic. Clin Cancer Res, 1995; 1: 677-686.
122. Pastorino U., Infante M., Maioli M., Chiesa G., Buyse M., Firket P., Rosmentz N., Clerici M., Soresi E. and Valente M. Adjuvant treatment of stage I lung cancer with high-dose vitamin A. J Clin Oncol, 1993; 11: 1216-1222.
123. Minucci S. and Pelicci P. G. Retinoid receptors in health and disease: co-regulators and the chromatin connection. Semin Cell Dev Biol, 1999; 10: 215-225.
124. Altucci L. and Gronemeyer H. The promise of retinoids to fight against cancer. Nat Rev Cancer, 2001; 1: 181-193.
125. de The H., Marchio A., Tiollais P. and Dejean A. Differential expression and ligand regulation of the retinoic acid receptor alpha and beta genes. Embo J 1989;8: 429-433.
126. Hoffmann B., Lehmann J. M., Zhang X. K., Hermann T., Husmann M., Graupner G. and Pfahl M. A retinoic acid receptor-specific element controls the retinoic acid receptor-beta promoter. Mol Endocrinol, 1990; 4: 1727-1736.
127. Widschwendter M., Berger J., Hermann M., Muller H. M., Amberger A., Zeschnigk M., Widschwendter A., Abendstein B., Zeimet A. G., Daxenbichler G. and Marth C. Methylation and silencing of the retinoic acid receptor-beta2 gene in breast cancer. J Natl Cancer Inst, 2000; 92: 826-832.
128. Widschwendter M., Berger J., Muller H. M., Zeimet A. G. and Marth C. Epigenetic downregulation of the retinoic acid receptor-beta2 gene in breast cancer. J Mammary Gland Biol Neoplasia, 2001; 6: 193-201.
129. Sirchia S. M., Ferguson A. T., Sironi E., Subramanyan S., Orlandi R., Sukumar S. and Sacchi N. Evidence of epigenetic changes affecting the chromatin state of the retinoic acid receptor beta2 promoter in breast cancer cells. Oncogene, 2000; 19: 1556-1563.
130. Arapshian A., Kuppumbatti Y. S. and Mira-y-Lopez R. Methylation of conserved CpG sites neighboring the beta retinoic acid response element may mediate retinoic acid receptor beta gene silencing in MCF-7 breast cancer cells. Oncogene, 2000; 19: 4066-4070.
131. Bovenzi V., Le N. L., Cote S., Sinnett D., Momparler L. F. and Momparler R. L. DNA methylation of retinoic acid receptor beta in breast cancer and possible therapeutic role of 5-aza-2'-deoxycytidine. Anticancer Drugs, 1999; 10: 471-476.
132. Mehrotra J., Vali M., McVeigh M., Kominsky S. L., Fackler M. J., Lahti-Domenici J., Polyak K., Sacchi N., Garrett-Mayer E., Argani P. and Sukumar S. Very high frequency of hypermethylated genes in breast cancer metastasis to the bone, brain, and lung. Clin Cancer Res, 2004; 10: 3104-3109.

133. Di Croce L., Raker V. A., Corsaro M., Fazi F., Fanelli M., Faretta M., Fuks F., Lo Coco F., Kouzarides T., Nervi C., Minucci S. and Pelicci P. G. Methyltransferase recruitment and DNA hypermethylation of target promoters by an oncogenic transcription factor. Science, 2002; 295: 1079-1082.
134. O'Shea P. J. and Williams G. R. Insight into the physiological actions of thyroid hormone receptors from genetically modified mice. J Endocrinol, 2002; 175: 553-570.
135. Li Z., Meng Z. H., Chandrasekaran R., Kuo W. L., Collins C. C., Gray J. W. and Dairkee S. H. Biallelic inactivation of the thyroid hormone receptor beta1 gene in early stage breast cancer. Cancer Res, 2002; 62: 1939-1943.
136. Yan P. S., Shi H., Rahmatpanah F., Hsiau T. H., Hsiau A. H., Leu Y. W., Liu J. C. and Huang T. H. Differential distribution of DNA methylation within the RASSF1A CpG island in breast cancer. Cancer Res, 2003; 63: 6178-6186.
137. McCormick F. Signal transduction. How receptors turn Ras on. Nature, 1993; 363: 15-16.
138. Serrano M., Lin A. W., McCurrach M. E., Beach D. and Lowe S. W. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell, 1997; 88: 593-602.
139. Downward J. Ras signalling and apoptosis. Curr Opin Genet Dev, 1998; 8: 49-54.
140. Burbee D. G., Forgacs E., Zochbauer-Muller S., Shivakumar L., Fong K., Gao B., Randle D., Kondo M., Virmani A., Bader S., Sekido Y., Latif F., Milchgrub S., Toyooka S., Gazdar A. F., Lerman M. I., Zabarovsky E., White M. and Minna J. D. Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. J Natl Cancer Inst, 2001; 93: 691-699.
141. Dammann R., Yang G. and Pfeifer G. P. Hypermethylation of the cpG island of Ras association domain family 1A (RASSF1A), a putative tumor suppressor gene from the 3p21.3 locus, occurs in a large percentage of human breast cancers. Cancer Res, 2001; 61: 3105-3109.
142. Fackler M. J., McVeigh M., Evron E., Garrett E., Mehrotra J., Polyak K., Sukumar S. and Argani P. DNA methylation of RASSF1A, HIN-1, RAR-beta, Cyclin D2 and Twist in in situ and invasive lobular breast carcinoma. Int J Cancer, 2003; 107: 970-975.
143. Fackler M. J., McVeigh M., Mehrotra J., Blum M. A., Lange J., Lapides A., Garrett E., Argani P. and Sukumar S. Quantitative multiplex methylation-specific PCR assay for the detection of promoter hypermethylation in multiple genes in breast cancer. Cancer Res 2004;64: 4442-4452.
144. Lehmann U., Langer F., Feist H., Glockner S., Hasemeier B. and Kreipe H. Quantitative assessment of promoter hypermethylation during breast cancer development. Am J Pathol, 2002; 160: 605-612.
145. Vos M. D., Ellis C. A., Bell A., Birrer M. J. and Clark G. J. Ras uses the novel tumor suppressor RASSF1 as an effector to mediate apoptosis. J Biol Chem, 2000; 275: 35669-33672.
146. Chen H., Pong R. C., Wang Z. and Hsieh J. T. Differential regulation of the human gene DAB2IP in normal and malignant prostatic epithelia: cloning and characterization. Genomics, 2002; 79: 573-581.
147. Wang Z., Tseng C. P., Pong R. C., Chen H., McConnell J. D., Navone N. and Hsieh J. T. The mechanism of growth-inhibitory effect of DOC-2/DAB2 in prostate cancer. Characterization of a novel GTPase-activating protein associated with N-terminal domain of DOC-2/DAB2. J Biol Chem, 2002; 277: 12622-12631.
148. Dote H., Toyooka S., Tsukuda K., Yano M., Ouchida M., Doihara H., Suzuki M., Chen H., Hsieh J. T., Gazdar A. F. and Shimizu N. Aberrant promoter methylation in human

DAB2 interactive protein (hDAB2IP) gene in breast cancer. Clin Cancer Res, 2004; 10: 2082-2089.

149. Sutherland K. D., Lindeman G. J., Choong D. Y., Wittlin S., Brentzell L., Phillips W., Campbell I. G. and Visvader J. E. Differential hypermethylation of SOCS genes in ovarian and breast carcinomas. Oncogene, 2004; 23: 7726-7733.
150. Hirano T., Ishihara K. and Hibi M. Roles of STAT3 in mediating the cell growth, differentiation and survival signals relayed through the IL-6 family of cytokine receptors. Oncogene, 2000; 19: 2548-2556.
151. Benson J. R. Role of transforming growth factor beta in breast carcinogenesis. Lancet Oncol, 2004; 5: 229-239.
152. Arteaga C. L., Tandon A. K., Von Hoff D. D. and Osborne C. K. Transforming growth factor beta: potential autocrine growth inhibitor of estrogen receptor-negative human breast cancer cells. Cancer Res, 1988; 48: 3898-3904.
153. Kalkhoven E., Roelen B. A., de Winter J. P., Mummery C. L., van den Eijnden-van Raaij A. J., van der Saag P. T. and van der Burg B. Resistance to transforming growth factor beta and activin due to reduced receptor expression in human breast tumor cell lines. Cell Growth Differ, 1995; 6: 1151-1161.
154. Sun L., Wu G., Willson J. K., Zborowska E., Yang J., Rajkarunanayake I., Wang J., Gentry L. E., Wang X. F. and Brattain M. G. Expression of transforming growth factor beta type II receptor leads to reduced malignancy in human breast cancer MCF-7 cells. J Biol Chem, 1994; 269: 26449-2655.
155. Ammanamanchi S., Kim S. J., Sun L. Z. and Brattain M. G. Induction of transforming growth factor-beta receptor type II expression in estrogen receptor-positive breast cancer cells through SP1 activation by 5-aza-2'-deoxycytidine. J Biol Chem, 1998; 273: 16527-16534.
156. Ammanamanchi S. and Brattain M. G. Restoration of transforming growth factor-beta signaling through receptor RI induction by histone deacetylase activity inhibition in breast cancer cells. J Biol Chem, 2004; 279: 32620-3265.
157. Yu Y., Xu F., Peng H., Fang X., Zhao S., Li Y., Cuevas B., Kuo W. L., Gray J. W., Siciliano M., Mills G. B. and Bast R. C., Jr. NOEY2 (ARHI), an imprinted putative tumor suppressor gene in ovarian and breast carcinomas. Proc Natl Acad Sci U S A, 1999; 96: 214-219.
158. Yu Y., Fujii S., Yuan J., Luo R. Z., Wang L., Bao J., Kadota M., Oshimura M., Dent S. R., Issa J. P. and Bast R. C., Jr. Epigenetic regulation of ARHI in breast and ovarian cancer cells. Ann N Y Acad Sci, 2003; 983: 268-277.
159. Yuan J., Luo R. Z., Fujii S., Wang L., Hu W., Andreeff M., Pan Y., Kadota M., Oshimura M., Sahin A. A., Issa J. P., Bast R. C., Jr. and Yu Y. Aberrant methylation and silencing of ARHI, an imprinted tumor suppressor gene in which the function is lost in breast cancers. Cancer Res, 2003; 63: 4174-4180.
160. Wang L., Hoque A., Luo R. Z., Yuan J., Lu Z., Nishimoto A., Liu J., Sahin A. A., Lippman S. M., Bast R. C., Jr. and Yu Y. Loss of the expression of the tumor suppressor gene ARHI is associated with progression of breast cancer. Clin Cancer Res, 2003; 9: 3660-3666.
161. Hisatomi H., Nagao K., Wakita K. and Kohno N. ARHI/NOEY2 inactivation may be important in breast tumor pathogenesis. Oncology, 2002; 62: 136-140.
162. Krop I. E., Sgroi D., Porter D. A., Lunetta K. L., LeVangie R., Seth P., Kaelin C. M., Rhei E., Bosenberg M., Schnitt S., Marks J. R., Pagon Z., Belina D., Razumovic J. and Polyak K. HIN-1, a putative cytokine highly expressed in normal but not cancerous mammary epithelial cells. Proc Natl Acad Sci U S A, 2001; 98: 9796-9801.

163. Krop I., Maguire P., Lahti-Domenici J., Lodeiro G., Richardson A., Johannsdottir H. K., Nevanlinna H., Borg A., Gelman R., Barkardottir R. B., Lindblom A. and Polyak K. Lack of HIN-1 methylation in BRCA1-linked and "BRCA1-like" breast tumors. Cancer Res, 2003; 63: 2024-2027.
164. Busslinger M., Klix N., Pfeffer P., Graninger P. G. and Kozmik Z. Deregulation of PAX-5 by translocation of the Emu enhancer of the IgH locus adjacent to two alternative PAX-5 promoters in a diffuse large-cell lymphoma. Proc Natl Acad Sci U S A, 1996; 93: 6129-6134.
165. Stapleton P., Weith A., Urbanek P., Kozmik Z. and Busslinger M. Chromosomal localization of seven PAX genes and cloning of a novel family member, PAX-9. Nat Genet, 1993; 3: 292-298.
166. Palmisano W. A., Crume K. P., Grimes M. J., Winters S. A., Toyota M., Esteller M., Joste N., Baylin S. B. and Belinsky S. A. Aberrant promoter methylation of the transcription factor genes PAX5 alpha and beta in human cancers. Cancer Res, 2003; 63: 4620-4625.
167. Ledbetter J. A., Rabinovitch P. S., June C. H., Song C. W., Clark E. A. and Uckun F. M. Antigen-independent regulation of cytoplasmic calcium in B cells with a 12-kDa B-cell growth factor and anti-CD19. Proc Natl Acad Sci U S A, 1988; 85: 1897-1901.
168. Pardee A. B. G1 events and regulation of cell proliferation. Science, 1989; 246: 603-608.
169. Sherr C. J. The Pezcoller lecture: cancer cell cycles revisited. Cancer Res, 2000; 60: 3689-3695.
170. Du Y., Carling T., Fang W., Piao Z., Sheu J. C. and Huang S. Hypermethylation in human cancers of the RIZ1 tumor suppressor gene, a member of a histone/protein methyltransferase superfamily. Cancer Res, 2001; 61: 8094-8099.
171. Abbondanza C., Medici N., Nigro V., Rossi V., Gallo L., Piluso G., Belsito A., Roscigno A., Bontempo P., Puca A. A., Molinari A. M., Moncharmont B. and Puca G. A. The retinoblastoma-interacting zinc-finger protein RIZ is a downstream effector of estrogen action. Proc Natl Acad Sci U S A, 2000; 97: 3130-3135.
172. Carling T., Kim K. C., Yang X. H., Gu J., Zhang X. K. and Huang S. A histone methyltransferase is required for maximal response to female sex hormones. Mol Cell Biol, 2004; 24: 7032-7042.
173. Meyyappan M., Atadja P. W. and Riabowol K. T. Regulation of gene expression and transcription factor binding activity during cellular aging. Biol Signals 1996;5: 130-138.
174. Meyyappan M., Wong H., Hull C. and Riabowol K. T. Increased expression of cyclin D2 during multiple states of growth arrest in primary and established cells. Mol Cell Biol, 1998;,18: 3163-3172.
175. Evron E., Umbricht C. B., Korz D., Raman V., Loeb D. M., Niranjan B., Buluwela L., Weitzman S. A., Marks J. and Sukumar S. Loss of cyclin D2 expression in the majority of breast cancers is associated with promoter hypermethylation. Cancer Res, 2001; 61: 2782-2787.
176. Virmani A., Rathi A., Heda S., Sugio K., Lewis C., Tonk V., Takahashi T., Roth J. A., Minna J. D., Euhus D. M. and Gazdar A. F. Aberrant methylation of the cyclin D2 promoter in primary small cell, nonsmall cell lung and breast cancers. Int J Cancer, 2003; 107: 341-345.
177. Brenner A. J., Paladugu A., Wang H., Olopade O. I., Dreyling M. H. and Aldaz C. M. Preferential loss of expression of p16(INK4a) rather than p19(ARF) in breast cancer. Clin Cancer Res, 1996; 2: 1993-1998.

178. Berns E. M., Klijn J. G., Smid M., van Staveren I. L., Gruis N. A. and Foekens J. A. Infrequent CDKN2 (MTS1/p16) gene alterations in human primary breast cancer. Br J Cancer, 1995; 72: 964-967.
179. Calvano J. E., Rush E. B., Tan L. K., Rosen P. P., Borgen P. I. and Van Zee K. J. Absence of p16 gene (CDKN2) deletions in microdissected primary breast carcinoma specimens. Ann Surg Oncol, 1997;,4: 416-420.
180. Quesnel B., Fenaux P., Philippe N., Fournier J., Bonneterre J., Preudhomme C. and Peyrat J. P. Analysis of p16 gene deletion and point mutation in breast carcinoma. Br J Cancer, 1995; 72: 351-353.
181. Gorgoulis V. G., Koutroumbi E. N., Kotsinas A., Zacharatos P., Markopoulos C., Giannikos L., Kyriakou V., Voulgaris Z., Gogas I. and Kittas C. Alterations of p16-pRb pathway and chromosome locus 9p21-22 in sporadic invasive breast carcinomas. Mol Med, 1998; 4: 807-822.
182. Borg A., Sandberg T., Nilsson K., Johannsson O., Klinker M., Masback A., Westerdahl J., Olsson H. and Ingvar C. High frequency of multiple melanomas and breast and pancreas carcinomas in CDKN2A mutation-positive melanoma families. J Natl Cancer Inst, 2000; 92: 1260-1266.
183. Herman J. G., Merlo A., Mao L., Lapidus R. G., Issa J. P., Davidson N. E., Sidransky D. and Baylin S. B. Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. Cancer Res, 1995; 55: 4525-4530.
184. Woodcock D. M., Linsenmeyer M. E., Doherty J. P. and Warren W. D. DNA methylation in the promoter region of the p16 (CDKN2/MTS-1/INK4A) gene in human breast tumours. Br J Cancer, 1999;7 9: 251-256.
185. Silva J., Silva J. M., Dominguez G., Garcia J. M., Cantos B., Rodriguez R., Larrondo F. J., Provencio M., Espana P. and Bonilla F. Concomitant expression of p16INK4a and p14ARF in primary breast cancer and analysis of inactivation mechanisms. J Pathol, 2003; 199: 289-297.
186. Foster S. A., Wong D. J., Barrett M. T. and Galloway D. A. Inactivation of p16 in human mammary epithelial cells by CpG island methylation. Mol Cell Biol, 1998; 18: 1793-1801.
187. Crawford Y. G., Gauthier M. L., Joubel A., Mantei K., Kozakiewicz K., Afshari C. A. and Tlsty T. D. Histologically normal human mammary epithelia with silenced p16(INK4a) overexpress COX-2, promoting a premalignant program. Cancer Cell, 2004; 5: 263-273.
188. Holst C. R., Nuovo G. J., Esteller M., Chew K., Baylin S. B., Herman J. G. and Tlsty T. D. Methylation of p16(INK4a) promoters occurs in vivo in histologically normal human mammary epithelia. Cancer Res, 2003; 63: 1596-1601.
189. Hui R., Macmillan R. D., Kenny F. S., Musgrove E. A., Blamey R. W., Nicholson R. I., Robertson J. F. and Sutherland R. L. INK4a gene expression and methylation in primary breast cancer: overexpression of p16INK4a messenger RNA is a marker of poor prognosis. Clin Cancer Res, 2000; 6: 2777-2787.
190. Hajra K. M. and Liu J. R. Apoptosome dysfunction in human cancer. Apoptosis, 2004; 9: 691-704.
191. Pan G., O'Rourke K., Chinnaiyan A. M., Gentz R., Ebner R., Ni J. and Dixit V. M. The receptor for the cytotoxic ligand TRAIL. Science, 1997; 276: 111-113.
192. Ashkenazi A. Targeting death and decoy receptors of the tumour-necrosis factor superfamily. Nat Rev Cancer, 2002; 2: 420-430.
193. Ashkenazi A. and Dixit V. M. Apoptosis control by death and decoy receptors. Curr Opin Cell Biol, 1999; 11: 255-260.

194. Shivapurkar N., Toyooka S., Toyooka K. O., Reddy J., Miyajima K., Suzuki M., Shigematsu H., Takahashi T., Parikh G., Pass H. I., Chaudhary P. M. and Gazdar A. F. Aberrant methylation of trail decoy receptor genes is frequent in multiple tumor types. Int J Cancer, 2004; 109: 786-792.
195. Chaudhary P. M., Eby M., Jasmin A., Bookwalter A., Murray J. and Hood L. Death receptor 5, a new member of the TNFR family, and DR4 induce FADD-dependent apoptosis and activate the NF-kappaB pathway. Immunity, 1997; 7: 821-830.
196. Schneider P., Thome M., Burns K., Bodmer J. L., Hofmann K., Kataoka T., Holler N. and Tschopp J. TRAIL receptors 1 (DR4) and 2 (DR5) signal FADD-dependent apoptosis and activate NF-kappaB. Immunity, 1997; 7: 831-836.
197. Cohen G. M. Caspases: the executioners of apoptosis. Biochem J, 1997; 326 (Pt 1): 1-16.
198. Masumoto J., Taniguchi S., Ayukawa K., Sarvotham H., Kishino T., Niikawa N., Hidaka E., Katsuyama T., Higuchi T. and Sagara J. ASC, a novel 22-kDa protein, aggregates during apoptosis of human promyelocytic leukemia HL-60 cells. J Biol Chem, 1999; 274: 33835-33838.
199. Conway K. E., McConnell B. B., Bowring C. E., Donald C. D., Warren S. T. and Vertino P. M. TMS1, a novel proapoptotic caspase recruitment domain protein, is a target of methylation-induced gene silencing in human breast cancers. Cancer Res, 2000; 60: 6236-6242.
200. Virmani A., Rathi A., Sugio K., Sathyanarayana U. G., Toyooka S., Kischel F. C., Tonk V., Padar A., Takahashi T., Roth J. A., Euhus D. M., Minna J. D. and Gazdar A. F. Aberrant methylation of TMS1 in small cell, non small cell lung cancer and breast cancer. Int J Cancer, 2003; 106: 198-204.
201. Levine J. J., Stimson-Crider K. M. and Vertino P. M. Effects of methylation on expression of TMS1/ASC in human breast cancer cells. Oncogene, 2003; 22: 3475-3488.
202. Yokoyama T., Sagara J., Guan X., Masumoto J., Takeoka M., Komiyama Y., Miyata K., Higuchi K. and Taniguchi S. Methylation of ASC/TMS1, a proapoptotic gene responsible for activating procaspase-1, in human colorectal cancer. Cancer Lett, 2003; 202: 101-108.
203. Guan X., Sagara J., Yokoyama T., Koganehira Y., Oguchi M., Saida T. and Taniguchi S. ASC/TMS1, a caspase-1 activating adaptor, is downregulated by aberrant methylation in human melanoma. Int J Cancer, 2003; 107: 202-208.
204. Pharoah P. D., Day N. E. and Caldas C. Somatic mutations in the p53 gene and prognosis in breast cancer: a meta-analysis. Br J Cancer, 1999; 80: 1968-1973.
205. Kang J. H., Kim S. J., Noh D. Y., Park I. A., Choe K. J., Yoo O. J. and Kang H. S. Methylation in the p53 promoter is a supplementary route to breast carcinogenesis: correlation between CpG methylation in the p53 promoter and the mutation of the p53 gene in the progression from ductal carcinoma in situ to invasive ductal carcinoma. Lab Invest, 2001; 81: 573-579.
206. Chene P. Inhibition of the p53-MDM2 interaction: targeting a protein-protein interface. Mol Cancer Res, 2004 ;2: 20-28.
207. Zhang H. G., Wang J., Yang X., Hsu H. C. and Mountz J. D. Regulation of apoptosis proteins in cancer cells by ubiquitin. Oncogene, 2004; 23: 2009-2015.
208. Quelle D. E., Zindy F., Ashmun R. A. and Sherr C. J. Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. Cell, 1995; 83: 993-1000.

209. Kamijo T., Zindy F., Roussel M. F., Quelle D. E., Downing J. R., Ashmun R. A., Grosveld G. and Sherr C. J. Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. Cell, 1997; 91: 649-659.
210. Mao L., Merlo A., Bedi G., Shapiro G. I., Edwards C. D., Rollins B. J. and Sidransky D. A novel p16INK4A transcript. Cancer Res, 1995; 55: 2995-2997.
211. Esteller M., Cordon-Cardo C., Corn P. G., Meltzer S. J., Pohar K. S., Watkins D. N., Capella G., Peinado M. A., Matias-Guiu X., Prat J., Baylin S. B. and Herman J. G. p14ARF silencing by promoter hypermethylation mediates abnormal intracellular localization of MDM2. Cancer Res, 2001; 61: 2816-2821.
212. Evron E., Dooley W. C., Umbricht C. B., Rosenthal D., Sacchi N., Gabrielson E., Soito A. B., Hung D. T., Ljung B., Davidson N. E. and Sukumar S. Detection of breast cancer cells in ductal lavage fluid by methylation-specific PCR. Lancet, 2001; 357: 1335-1336.
213. Nishizaki M., Sasaki J., Fang B., Atkinson E. N., Minna J. D., Roth J. A. and Ji L. Synergistic tumor suppression by coexpression of FHIT and p53 coincides with FHIT-mediated MDM2 inactivation and p53 stabilization in human non-small cell lung cancer cells. Cancer Res, 2004; 64: 5745-5752.
214. Ingvarsson S. FHIT alterations in breast cancer. Semin Cancer Biol, 2001; 11: 361-366.
215. Zochbauer-Muller S., Fong K. M., Maitra A., Lam S., Geradts J., Ashfaq R., Virmani A. K., Milchgrub S., Gazdar A. F. and Minna J. D. 5' CpG island methylation of the FHIT gene is correlated with loss of gene expression in lung and breast cancer. Cancer Res, 2001; 61: 3581-3585.
216. Yang Q., Nakamura M., Nakamura Y., Yoshimura G., Suzuma T., Umemura T., Shimizu Y., Mori I., Sakurai T. and Kakudo K. Two-hit inactivation of FHIT by loss of heterozygosity and hypermethylation in breast cancer. Clin Cancer Res, 2002; 8: 2890-2893.
217. Raman V., Martensen S. A., Reisman D., Evron E., Odenwald W. F., Jaffee E., Marks J. and Sukumar S. Compromised HOXA5 function can limit p53 expression in human breast tumours. Nature, 2000; 405: 974-978.
218. Chen H., Chung S. and Sukumar S. HOXA5-induced apoptosis in breast cancer cells is mediated by caspases 2 and 8. Mol Cell Biol, 2004; 24: 924-935.
219. Chan T. A., Hermeking H., Lengauer C., Kinzler K. W. and Vogelstein B. 14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage. Nature, 1999; 401: 616-620.
220. Ferguson A. T., Evron E., Umbricht C. B., Pandita T. K., Chan T. A., Hermeking H., Marks J. R., Lambers A. R., Futreal P. A., Stampfer M. R. and Sukumar S. High frequency of hypermethylation at the 14-3-3 sigma locus leads to gene silencing in breast cancer. Proc Natl Acad Sci U S A, 2000; 97: 6049-6054.
221. Umbricht C. B., Evron E., Gabrielson E., Ferguson A., Marks J. and Sukumar S. Hypermethylation of 14-3-3 sigma (stratifin) is an early event in breast cancer. Oncogene, 2001; 20: 3348-3353.
222. Guerardel C., Deltour S., Pinte S., Monte D., Begue A., Godwin A. K. and Leprince D. Identification in the human candidate tumor suppressor gene HIC-1 of a new major alternative TATA-less promoter positively regulated by p53. J Biol Chem, 2001; 276: 3078-3089.
223. Wales M. M., Biel M. A., el Deiry W., Nelkin B. D., Issa J. P., Cavenee W. K., Kuerbitz S. J. and Baylin S. B. p53 activates expression of HIC-1, a new candidate tumour suppressor gene on 17p13.3. Nat Med, 1995; 1: 570-577.
224. Rathi A., Virmani A. K., Harada K., Timmons C. F., Miyajima K., Hay R. J., Mastrangelo D., Maitra A., Tomlinson G. E. and Gazdar A. F. Aberrant methylation of

the HIC1 promoter is a frequent event in specific pediatric neoplasms. Clin Cancer Res, 2003; 9: 3674-3678.
225. Rathi A., Virmani A. K., Schorge J. O., Elias K. J., Maruyama R., Minna J. D., Mok S. C., Girard L., Fishman D. A. and Gazdar A. F. Methylation profiles of sporadic ovarian tumors and nonmalignant ovaries from high-risk women. Clin Cancer Res, 2002; 8: 3324-33231.
226. Rood B. R., Zhang H., Weitman D. M. and Cogen P. H. Hypermethylation of HIC-1 and 17p allelic loss in medulloblastoma. Cancer Res, 2002; 62: 3794-3797.
227. Fujii H., Biel M. A., Zhou W., Weitzman S. A., Baylin S. B. and Gabrielson E. Methylation of the HIC-1 candidate tumor suppressor gene in human breast cancer. Oncogene, 1998; 16: 2159-2164.
228. Parrella P., Scintu M., Prencipe M., Poeta M. L., Gallo A. P., Rabitti C., Rinaldi M., Tommasi S., Paradiso A., Schittulli F., Valori V. M., Toma S., Altomare V. and Fazio V. M. HIC1 promoter methylation and 17p13.3 allelic loss in invasive ductal carcinoma of the breast. Cancer Lett, 2004.
229. Chen W. Y., Zeng X., Carter M. G., Morrell C. N., Chiu Yen R. W., Esteller M., Watkins D. N., Herman J. G., Mankowski J. L. and Baylin S. B. Heterozygous disruption of Hic1 predisposes mice to a gender-dependent spectrum of malignant tumors. Nat Genet, 2003; 33: 197-202.
230. Waite K. A. and Eng C. Protean PTEN: form and function. Am J Hum Genet, 2002; 70: 829-844.
231. Marsh D. J., Dahia P. L., Zheng Z., Liaw D., Parsons R., Gorlin R. J. and Eng C. Germline mutations in PTEN are present in Bannayan-Zonana syndrome. Nat Genet, 1997; 16: 333-334.
232. Liaw D., Marsh D. J., Li J., Dahia P. L., Wang S. I., Zheng Z., Bose S., Call K. M., Tsou H. C., Peacocke M., Eng C. and Parsons R. Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. Nat Genet, 1997; 16: 64-67.
233. Maehama T. and Dixon J. E. PTEN: a tumour suppressor that functions as a phospholipid phosphatase. Trends Cell Biol, 1999; 9: 125-128.
234. Maehama T. and Dixon J. E. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem, 1998; 273: 13375-13378.
235. Lu Y., Lin Y. Z., LaPushin R., Cuevas B., Fang X., Yu S. X., Davies M. A., Khan H., Furui T., Mao M., Zinner R., Hung M. C., Steck P., Siminovitch K. and Mills G. B. The PTEN/MMAC1/TEP tumor suppressor gene decreases cell growth and induces apoptosis and anoikis in breast cancer cells. Oncogene, 1999; 18: 7034-45.
236. Stambolic V., Suzuki A., de la Pompa J. L., Brothers G. M., Mirtsos C., Sasaki T., Ruland J., Penninger J. M., Siderovski D. P. and Mak T. W. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. Cell ,1998; 95: 29-39.
237. Khan S., Kumagai T., Vora J., Bose N., Sehgal I., Koeffler P. H. and Bose S. PTEN promoter is methylated in a proportion of invasive breast cancers. Int J Cancer, 2004; 112: 407.
238. Varrault A., Bilanges B., Mackay D. J., Basyuk E., Ahr B., Fernandez C., Robinson D. O., Bockaert J. and Journot L. Characterization of the methylation-sensitive promoter of the imprinted ZAC gene supports its role in transient neonatal diabetes mellitus. J Biol Chem, 2001; 276: 18653-18656.
239. Bilanges B., Varrault A., Basyuk E., Rodriguez C., Mazumdar A., Pantaloni C., Bockaert J., Theillet C., Spengler D. and Journot L. Loss of expression of the candidate

tumor suppressor gene ZAC in breast cancer cell lines and primary tumors. Oncogene, 1999; 18: 3979-3988.

240. Abdollahi A., Pisarcik D., Roberts D., Weinstein J., Cairns P. and Hamilton T. C. LOT1 (PLAGL1/ZAC1), the candidate tumor suppressor gene at chromosome 6q24-25, is epigenetically regulated in cancer. J Biol Chem, 2003; 278: 6041-609.
241. Gonzalez A. D., Kaya M., Shi W., Song H., Testa J. R., Penn L. Z. and Filmus J. OCI-5/GPC3, a glypican encoded by a gene that is mutated in the Simpson-Golabi-Behmel overgrowth syndrome, induces apoptosis in a cell line-specific manner. J Cell Biol, 1998; 141: 1407-1414.
242. Xiang Y. Y., Ladeda V. and Filmus J. Glypican-3 expression is silenced in human breast cancer. Oncogene, 2001; 20: 7408-7412.
243. Chen C. M., Chen H. L., Hsiau T. H., Hsiau A. H., Shi H., Brock G. J., Wei S. H., Caldwell C. W., Yan P. S. and Huang T. H. Methylation target array for rapid analysis of CpG island hypermethylation in multiple tissue genomes. Am J Pathol, 2003; 163: 37-45.
244. Boland C. R., Thibodeau S. N., Hamilton S. R., Sidransky D., Eshleman J. R., Burt R. W., Meltzer S. J., Rodriguez-Bigas M. A., Fodde R., Ranzani G. N. and Srivastava S. A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res, 1998; 58: 5248-5257.
245. Duesberg P., Rausch C., Rasnick D. and Hehlmann R. Genetic instability of cancer cells is proportional to their degree of aneuploidy. Proc Natl Acad Sci U S A, 1998; 95: 13692-13697.
246. Anbazhagan R., Fujii H. and Gabrielson E. Microsatellite instability is uncommon in breast cancer. Clin Cancer Res, 1999; 5: 839-844.
247. Adem C., Soderberg C. L., Cunningham J. M., Reynolds C., Sebo T. J., Thibodeau S. N., Hartmann L. C. and Jenkins R. B. Microsatellite instability in hereditary and sporadic breast cancers. Int J Cancer, 2003; 107: 580-582.
248. Muller A., Edmonston T. B., Corao D. A., Rose D. G., Palazzo J. P., Becker H., Fry R. D., Rueschoff J. and Fishel R. Exclusion of breast cancer as an integral tumor of hereditary nonpolyposis colorectal cancer. Cancer Res, 2002; 62: 1014-1019.
249. Risinger J. I., Barrett J. C., Watson P., Lynch H. T. and Boyd J. Molecular genetic evidence of the occurrence of breast cancer as an integral tumor in patients with the hereditary nonpolyposis colorectal carcinoma syndrome. Cancer, 1996; 77: 1836-18343.
250. Yoon D. S., Wersto R. P., Zhou W., Chrest F. J., Garrett E. S., Kwon T. K. and Gabrielson E. Variable levels of chromosomal instability and mitotic spindle checkpoint defects in breast cancer. Am J Pathol, 2002; 161: 391-397.
251. Scully R., Chen J., Plug A., Xiao Y., Weaver D., Feunteun J., Ashley T. and Livingston D. M. Association of BRCA1 with Rad51 in mitotic and meiotic cells. Cell, 1997; 88: 265-275.
252. Sharan S. K., Morimatsu M., Albrecht U., Lim D. S., Regel E., Dinh C., Sands A., Eichele G., Hasty P. and Bradley A. Embryonic lethality and radiation hypersensitivity mediated by Rad51 in mice lacking Brca2. Nature, 1997; 386: 804-810.
253. Miki Y., Swensen J., Shattuck-Eidens D., Futreal P. A., Harshman K., Tavtigian S., Liu Q., Cochran C., Bennett L. M. and Ding W. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science, 1994; 266: 66-71.
254. Neuhausen S. L. and Marshall C. J. Loss of heterozygosity in familial tumors from three BRCA1-linked kindreds. Cancer Res, 1994; 54: 6069-6072.

255. Beckmann M. W., Picard F., An H. X., van Roeyen C. R., Dominik S. I., Mosny D. S., Schnurch H. G., Bender H. G. and Niederacher D. Clinical impact of detection of loss of heterozygosity of BRCA1 and BRCA2 markers in sporadic breast cancer. Br J Cancer, 1996; 73: 1220-126.
256. Rio P. G., Pernin D., Bay J. O., Albuisson E., Kwiatkowski F., De Latour M., Bernard-Gallon D. J. and Bignon Y. J. Loss of heterozygosity of BRCA1, BRCA2 and ATM genes in sporadic invasive ductal breast carcinoma. Int J Oncol, 1998; 13: 849-853.
257. Sorlie T., Andersen T. I., Bukholm I. and Borresen-Dale A. L. Mutation screening of BRCA1 using PTT and LOH analysis at 17q21 in breast carcinomas from familial and non-familial cases. Breast Cancer Res Treat, 1998; 48: 259-264.
258. Gonzalez R., Silva J. M., Dominguez G., Garcia J. M., Martinez G., Vargas J., Provencio M., Espana P. and Bonilla F. Detection of loss of heterozygosity at RAD51, RAD52, RAD54 and BRCA1 and BRCA2 loci in breast cancer: pathological correlations. Br J Cancer, 1999; 81: 503-509.
259. Katsama A., Sourvinos G., Zachos G. and Spandidos D. A. Allelic loss at the BRCA1, BRCA2 and TP53 loci in human sporadic breast carcinoma. Cancer Lett, 2000; 150: 165-170.
260. Futreal P. A., Liu Q., Shattuck-Eidens D., Cochran C., Harshman K., Tavtigian S., Bennett L. M., Haugen-Strano A., Swensen J., Miki Y. and et al. BRCA1 mutations in primary breast and ovarian carcinomas. Science, 1994; 266: 120-122.
261. Papa S., Seripa D., Merla G., Gravina C., Giai M., Sismondi P., Rinaldi M., Serra A., Saglio G. and Fazio V. M. Identification of a possible somatic BRCA1 mutation affecting translation efficiency in an early-onset sporadic breast cancer patient. J Natl Cancer Inst, 1998; 90: 1011-1012.
262. Thompson M. E., Jensen R. A., Obermiller P. S., Page D. L. and Holt J. T. Decreased expression of BRCA1 accelerates growth and is often present during sporadic breast cancer progression. Nat Genet, 1995; 9: 444-450.
263. Yoshikawa K., Ogawa T., Baer R., Hemmi H., Honda K., Yamauchi A., Inamoto T., Ko K., Yazumi S., Motoda H., Kodama H., Noguchi S., Gazdar A. F., Yamaoka Y. and Takahashi R. Abnormal expression of BRCA1 and BRCA1-interactive DNA-repair proteins in breast carcinomas. Int J Cancer 2000;88: 28-36.
264. Magdinier F., Ribieras S., Lenoir G. M., Frappart L. and Dante R. Down-regulation of BRCA1 in human sporadic breast cancer; analysis of DNA methylation patterns of the putative promoter region. Oncogene, 1998; 17: 3169-3176.
265. Sourvinos G. and Spandidos D. A. Decreased BRCA1 expression levels may arrest the cell cycle through activation of p53 checkpoint in human sporadic breast tumors. Biochem Biophys Res Commun, 1998; 245: 75-80.
266. Wilson C. A., Ramos L., Villasenor M. R., Anders K. H., Press M. F., Clarke K., Karlan B., Chen J. J., Scully R., Livingston D., Zuch R. H., Kanter M. H., Cohen S., Calzone F. J. and Slamon D. J. Localization of human BRCA1 and its loss in high-grade, non-inherited breast carcinomas. Nat Genet, 1999; 21: 236-240.
267. Khanna K. K. and Jackson S. P. DNA double-strand breaks: signaling, repair and the cancer connection. Nat Genet, 2001; 27: 247-254.
268. Rice J. C., Massey-Brown K. S. and Futscher B. W. Aberrant methylation of the BRCA1 CpG island promoter is associated with decreased BRCA1 mRNA in sporadic breast cancer cells. Oncogene, 1998; 17: 1807-1812.
269. Catteau A., Harris W. H., Xu C. F. and Solomon E. Methylation of the BRCA1 promoter region in sporadic breast and ovarian cancer: correlation with disease characteristics. Oncogene, 1999; 18: 1957-1965.

270. Esteller M., Hamilton S. R., Burger P. C., Baylin S. B. and Herman J. G. Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. Cancer Res, 1999; 59: 793-797.
271. Rice J. C., Ozcelik H., Maxeiner P., Andrulis I. and Futscher B. W. Methylation of the BRCA1 promoter is associated with decreased BRCA1 mRNA levels in clinical breast cancer specimens. Carcinogenesis, 2000; 21: 1761-1765.
272. Hilton J. L., Geisler J. P., Rathe J. A., Hattermann-Zogg M. A., DeYoung B. and Buller R. E. Inactivation of BRCA1 and BRCA2 in ovarian cancer. J Natl Cancer Inst, 2002; 94: 1396-1406.
273. Collins N., Wooster R. and Stratton M. R. Absence of methylation of CpG dinucleotides within the promoter of the breast cancer susceptibility gene BRCA2 in normal tissues and in breast and ovarian cancers. Br J Cancer ,1997; 76: 1150-1156.
274. Vo Q. N., Kim W. J., Cvitanovic L., Boudreau D. A., Ginzinger D. G. and Brown K. D. The ATM gene is a target for epigenetic silencing in locally advanced breast cancer. Oncogene, 2004; 58:9432-9437.
275. Swift M., Morrell D., Massey R. B. and Chase C. L. Incidence of cancer in 161 families affected by ataxia-telangiectasia. N Engl J Med, 1991; 325: 1831-1836.
276. Inskip H. M., Kinlen L. J., Taylor A. M., Woods C. G. and Arlett C. F. Risk of breast cancer and other cancers in heterozygotes for ataxia-telangiectasia. Br J Cancer, 1999; 79: 1304-1307.
277. Olsen J. H., Hahnemann J. M., Borresen-Dale A. L., Brondum-Nielsen K., Hammarstrom L., Kleinerman R., Kaariainen H., Lonnqvist T., Sankila R., Seersholm N., Tretli S., Yuen J., Boice J. D., Jr. and Tucker M. Cancer in patients with ataxia-telangiectasia and in their relatives in the nordic countries. J Natl Cancer Inst, 2001; 93: 121-127.
278. Chenevix-Trench G., Spurdle A. B., Gatei M., Kelly H., Marsh A., Chen X., Donn K., Cummings M., Nyholt D., Jenkins M. A., Scott C., Pupo G. M., Dork T., Bendix R., Kirk J., Tucker K., McCredie M. R., Hopper J. L., Sambrook J., Mann G. J. and Khanna K. K. Dominant negative ATM mutations in breast cancer families. J Natl Cancer Inst 2002;94: 205-215.
279. Harden S. V., Sanderson H., Goodman S. N., Partin A. A., Walsh P. C., Epstein J. I. and Sidransky D. Quantitative GSTP1 methylation and the detection of prostate adenocarcinoma in sextant biopsies. J Natl Cancer Inst, 2003; 95: 1634-1637.
280. Esteller M., Corn P. G., Urena J. M., Gabrielson E., Baylin S. B. and Herman J. G. Inactivation of glutathione S-transferase P1 gene by promoter hypermethylation in human neoplasia. Cancer Res, 1998; 58: 4515-4518.
281. Lee W. H., Morton R. A., Epstein J. I., Brooks J. D., Campbell P. A., Bova G. S., Hsieh W. S., Isaacs W. B. and Nelson W. G. Cytidine methylation of regulatory sequences near the pi-class glutathione S-transferase gene accompanies human prostatic carcinogenesis. Proc Natl Acad Sci U S A, 1994; 91: 11733-11737.
282. Tchou J. C., Lin X., Freije D., Isaacs W. B., Brooks J. D., Rashid A., De Marzo A. M., Kanai Y., Hirohashi S. and Nelson W. G. GSTP1 CpG island DNA hypermethylation in hepatocellular carcinomas. Int J Oncol, 2000; 16: 663-676.
283. Esteller M. Epigenetic lesions causing genetic lesions in human cancer: promoter hypermethylation of DNA repair genes. Eur J Cancer, 2000; 36: 2294-300.
284. Landis S. H., Murray T., Bolden S. and Wingo P. A. Cancer statistics, 1998. CA Cancer J Clin, 1998; 48: 6-29.
285. Boyd D. Invasion and metastasis. Cancer Metastasis Rev, 1996; 15: 77-89.
286. Bernards R. Cancer: cues for migration. Nature, 2003; 425: 247-248.

287. Welch D. R., Steeg P. S. and Rinker-Schaeffer C. W. Molecular biology of breast cancer metastasis. Genetic regulation of human breast carcinoma metastasis. Breast Cancer Res, 2000; 2: 408-416.
288. Behrens J. Cadherins and catenins: role in signal transduction and tumor progression. Cancer Metastasis Rev, 1999; 18: 15-30.
289. Meiners S., Brinkmann V., Naundorf H. and Birchmeier W. Role of morphogenetic factors in metastasis of mammary carcinoma cells. Oncogene, 1998; 16: 9-20.
290. Rasbridge S. A., Gillett C. E., Sampson S. A., Walsh F. S. and Millis R. R. Epithelial (E-) and placental (P-) cadherin cell adhesion molecule expression in breast carcinoma. J Pathol, 1993; 169: 245-250.
291. Daniel C. W., Strickland P. and Friedmann Y. Expression and functional role of E- and P-cadherins in mouse mammary ductal morphogenesis and growth. Dev Biol, 1995; 169: 511-519.
292. Heimann R., Lan F., McBride R. and Hellman S. Separating favorable from unfavorable prognostic markers in breast cancer: the role of E-cadherin. Cancer Res, 2000; 60: 298-304.
293. Hunt N. C., Douglas-Jones A. G., Jasani B., Morgan J. M. and Pignatelli M. Loss of E-cadherin expression associated with lymph node metastases in small breast carcinomas. Virchows Arch, 1997; 430: 285-289.
294. Oka H., Shiozaki H., Kobayashi K., Inoue M., Tahara H., Kobayashi T., Takatsuka Y., Matsuyoshi N., Hirano S., Takeichi M. and et al. Expression of E-cadherin cell adhesion molecules in human breast cancer tissues and its relationship to metastasis. Cancer Res, 1993; 53: 1696-1701.
295. Siitonen S. M., Kononen J. T., Helin H. J., Rantala I. S., Holli K. A. and Isola J. J. Reduced E-cadherin expression is associated with invasiveness and unfavorable prognosis in breast cancer. Am J Clin Pathol, 1996; 105: 394-402.
296. Gamallo C., Palacios J., Suarez A., Pizarro A., Navarro P., Quintanilla M. and Cano A. Correlation of E-cadherin expression with differentiation grade and histological type in breast carcinoma. Am J Pathol, 1993; 142: 987-993.
297. Moll R., Mitze M., Frixen U. H. and Birchmeier W. Differential loss of E-cadherin expression in infiltrating ductal and lobular breast carcinomas. Am J Pathol 1993;143: 1731-1742.
298. Vos C. B., Cleton-Jansen A. M., Berx G., de Leeuw W. J., ter Haar N. T., van Roy F., Cornelisse C. J., Peterse J. L. and van de Vijver M. J. E-cadherin inactivation in lobular carcinoma in situ of the breast: an early event in tumorigenesis. Br J Cancer, 1997; 76: 1131-1133.
299. Huiping C., Sigurgeirsdottir J. R., Jonasson J. G., Eiriksdottir G., Johannsdottir J. T., Egilsson V. and Ingvarsson S. Chromosome alterations and E-cadherin gene mutations in human lobular breast cancer. Br J Cancer, 1999; 81: 1103-1110.
300. Berx G., Cleton-Jansen A. M., Strumane K., de Leeuw W. J., Nollet F., van Roy F. and Cornelisse C. E-cadherin is inactivated in a majority of invasive human lobular breast cancers by truncation mutations throughout its extracellular domain. Oncogene, 1996; 13: 1919-1925.
301. Berx G., Cleton-Jansen A. M., Nollet F., de Leeuw W. J., van de Vijver M., Cornelisse C. and van Roy F. E-cadherin is a tumour/invasion suppressor gene mutated in human lobular breast cancers. Embo J, 1995; 14: 6107-6115.
302. Cleton-Jansen A. M. E-cadherin and loss of heterozygosity at chromosome 16 in breast carcinogenesis: different genetic pathways in ductal and lobular breast cancer? Breast Cancer Res, 2002; 4: 5-8.

303. Sarrio D., Moreno-Bueno G., Hardisson D., Sanchez-Estevez C., Guo M., Herman J. G., Gamallo C., Esteller M. and Palacios J. Epigenetic and genetic alterations of APC and CDH1 genes in lobular breast cancer: relationships with abnormal E-cadherin and catenin expression and microsatellite instability. Int J Cancer, 2003; 106: 208-215.
304. Graff J. R., Herman J. G., Lapidus R. G., Chopra H., Xu R., Jarrard D. F., Isaacs W. B., Pitha P. M., Davidson N. E. and Baylin S. B. E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. Cancer Res, 1995; 55: 5195-5199.
305. Graff J. R., Gabrielson E., Fujii H., Baylin S. B. and Herman J. G. Methylation patterns of the E-cadherin 5' CpG island are unstable and reflect the dynamic, heterogeneous loss of E-cadherin expression during metastatic progression. J Biol Chem, 2000; 275: 2727-2732.
306. Wijnhoven B. P., Dinjens W. N. and Pignatelli M. E-cadherin-catenin cell-cell adhesion complex and human cancer. Br J Surg, 2000; 87: 992-1005.
307. Conacci-Sorrell M., Zhurinsky J. and Ben-Ze'ev A. The cadherin-catenin adhesion system in signaling and cancer. J Clin Invest, 2002; 109: 987-991.
308. Yang S. Z., Kohno N., Yokoyama A., Kondo K., Hamada H. and Hiwada K. Decreased E-cadherin augments beta-catenin nuclear localization: studies in breast cancer cell lines. Int J Oncol, 2001; 18: 541-548.
309. Fearnhead N. S., Britton M. P. and Bodmer W. F. The ABC of APC. Hum Mol Genet, 2001; 10: 721-733.
310. Fearnhead N. S., Wilding J. L. and Bodmer W. F. Genetics of colorectal cancer: hereditary aspects and overview of colorectal tumorigenesis. Br Med Bull, 2002; 64: 27-43.
311. Virmani A. K., Rathi A., Sathyanarayana U. G., Padar A., Huang C. X., Cunnigham H. T., Farinas A. J., Milchgrub S., Euhus D. M., Gilcrease M., Herman J., Minna J. D. and Gazdar A. F. Aberrant methylation of the adenomatous polyposis coli (APC) gene promoter 1A in breast and lung carcinomas. Clin Cancer Res, 2001; 7: 1998-2004.
312. Mitchell P. J., Timmons P. M., Hebert J. M., Rigby P. W. and Tjian R. Transcription factor AP-2 is expressed in neural crest cell lineages during mouse embryogenesis. Genes Dev, 1991; 5: 105-119.
313. Schorle H., Meier P., Buchert M., Jaenisch R. and Mitchell P. J. Transcription factor AP-2 essential for cranial closure and craniofacial development. Nature, 1996; 381: 235-238.
314. Batsche E., Muchardt C., Behrens J., Hurst H. C. and Cremisi C. RB and c-Myc activate expression of the E-cadherin gene in epithelial cells through interaction with transcription factor AP-2. Mol Cell Biol, 1998; 18: 3647-3658.
315. Hilger-Eversheim K., Moser M., Schorle H. and Buettner R. Regulatory roles of AP-2 transcription factors in vertebrate development, apoptosis and cell-cycle control. Gene, 2000; 260: 1-12.
316. Zeng Y. X., Somasundaram K. and el-Deiry W. S. AP2 inhibits cancer cell growth and activates p21WAF1/CIP1 expression. Nat Genet, 1997; 15: 78-82.
317. Douglas D. B., Akiyama Y., Carraway H., Belinsky S. A., Esteller M., Gabrielson E., Weitzman S., Williams T., Herman J. G. and Baylin S. B. Hypermethylation of a small CpGuanine-rich region correlates with loss of activator protein-2alpha expression during progression of breast cancer. Cancer Res, 2004; 64: 1611-1620.
318. Adams J. C. and Watt F. M. Regulation of development and differentiation by the extracellular matrix. Development, 1993; 117: 1183-1198.

319. Bergstraesser L. M., Srinivasan G., Jones J. C., Stahl S. and Weitzman S. A. Expression of hemidesmosomes and component proteins is lost by invasive breast cancer cells. Am J Pathol, 1995; 147: 1823-1839.
320. Stahl S., Weitzman S. and Jones J. C. The role of laminin-5 and its receptors in mammary epithelial cell branching morphogenesis. J Cell Sci, 1997; 110 (Pt 1): 55-63.
321. Martin K. J., Kwan C. P., Nagasaki K., Zhang X., O'Hare M. J., Kaelin C. M., Burgeson R. E., Pardee A. B. and Sager R. Down-regulation of laminin-5 in breast carcinoma cells. Mol Med, 1998; 4: 602-613.
322. Giannelli G. and Antonaci S. Biological and clinical relevance of Laminin-5 in cancer. Clin Exp Metastasis, 2000; 18: 439-443.
323. Sathyanarayana U. G., Padar A., Huang C. X., Suzuki M., Shigematsu H., Bekele B. N. and Gazdar A. F. Aberrant promoter methylation and silencing of laminin-5-encoding genes in breast carcinoma. Clin Cancer Res, 2003; 9: 6389-94.
324. Gomez D. E., Alonso D. F., Yoshiji H. and Thorgeirsson U. P. Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. Eur J Cell Biol, 1997; 74: 111-22.
325. Bachman K. E., Herman J. G., Corn P. G., Merlo A., Costello J. F., Cavenee W. K., Baylin S. B. and Graff J. R. Methylation-associated silencing of the tissue inhibitor of metalloproteinase-3 gene suggest a suppressor role in kidney, brain, and other human cancers. Cancer Res, 1999; 59: 798-802.
326. Zou Z., Anisowicz A., Hendrix M. J., Thor A., Neveu M., Sheng S., Rafidi K., Seftor E. and Sager R. Maspin, a serpin with tumor-suppressing activity in human mammary epithelial cells. Science, 1994; 263: 526-529.
327. Maass N., Nagasaki K., Ziebart M., Mundhenke C. and Jonat W. Expression and regulation of tumor suppressor gene maspin in breast cancer. Clin Breast Cancer, 2002; 3: 281-287.
328. Domann F. E., Rice J. C., Hendrix M. J. and Futscher B. W. Epigenetic silencing of maspin gene expression in human breast cancers. Int J Cancer, 2000; 85: 805-810.
329. Futscher B. W., O'Meara M. M., Kim C. J., Rennels M. A., Lu D., Gruman L. M., Seftor R. E., Hendrix M. J. and Domann F. E. Aberrant methylation of the maspin promoter is an early event in human breast cancer. Neoplasia, 2004; 6: 380-389.
330. Cal S., Freije J. M., Lopez J. M., Takada Y. and Lopez-Otin C. ADAM 23/MDC3, a human disintegrin that promotes cell adhesion via interaction with the alphavbeta3 integrin through an RGD-independent mechanism. Mol Biol Cell, 2000; 11: 1457-1469.
331. Costa F. F., Verbisck N. V., Salim A. C., Ierardi D. F., Pires L. C., Sasahara R. M., Sogayar M. C., Zanata S. M., Mackay A., O'Hare M., Soares F., Simpson A. J. and Camargo A. A. Epigenetic silencing of the adhesion molecule ADAM23 is highly frequent in breast tumors. Oncogene, 2004; 23: 1481-1488.
332. Heimann R., Ferguson D. J. and Hellman S. The relationship between nm23, angiogenesis, and the metastatic proclivity of node-negative breast cancer. Cancer Res, 1998; 58: 2766-2771.
333. Hartsough M. T., Clare S. E., Mair M., Elkahloun A. G., Sgroi D., Osborne C. K., Clark G. and Steeg P. S. Elevation of breast carcinoma Nm23-H1 metastasis suppressor gene expression and reduced motility by DNA methylation inhibition. Cancer Res, 2001; 61: 2320-2327.
334. Gruber A. D. and Pauli B. U. Tumorigenicity of human breast cancer is associated with loss of the Ca2+-activated chloride channel CLCA2. Cancer Res, 1999; 59: 5488-5491.
335. Li X., Cowell J. K. and Sossey-Alaoui K. CLCA2 tumour suppressor gene in 1p31 is epigenetically regulated in breast cancer. Oncogene, 2004; 23: 1474-1480.

336. Coopman P. J., Do M. T., Barth M., Bowden E. T., Hayes A. J., Basyuk E., Blancato J. K., Vezza P. R., McLeskey S. W., Mangeat P. H. and Mueller S. C. The Syk tyrosine kinase suppresses malignant growth of human breast cancer cells. Nature, 2000; 406: 742-747.
337. Wang L., Duke L., Zhang P. S., Arlinghaus R. B., Symmans W. F., Sahin A., Mendez R. and Dai J. L. Alternative splicing disrupts a nuclear localization signal in spleen tyrosine kinase that is required for invasion suppression in breast cancer. Cancer Res, 2003; 63: 4724-4730.
338. Yuan Y., Mendez R., Sahin A. and Dai J. L. Hypermethylation leads to silencing of the SYK gene in human breast cancer. Cancer Res, 2001; 61: 5558-55561.
339. Yuan Y., Liu H., Sahin A. and Dai J. L. Reactivation of SYK expression by inhibition of DNA methylation suppresses breast cancer cell invasiveness. Int J Cancer, 2004; 113:654-659.
340. Dhar S., Bhargava R., Yunes M., Li B., Goyal J., Naber S. P., Wazer D. E. and Band V. Analysis of normal epithelial cell specific-1 (NES1)/kallikrein 10 mRNA expression by in situ hybridization, a novel marker for breast cancer. Clin Cancer Res, 2001; 7: 3393-8.
341. Goyal J., Smith K. M., Cowan J. M., Wazer D. E., Lee S. W. and Band V. The role for NES1 serine protease as a novel tumor suppressor. Cancer Res, 1998; 58: 4782-4786.
342. Li B., Goyal J., Dhar S., Dimri G., Evron E., Sukumar S., Wazer D. E. and Band V. CpG methylation as a basis for breast tumor-specific loss of NES1/kallikrein 10 expression. Cancer Res, 2001; 61: 8014-8021.
343. Jia T., Liu Y. E., Liu J. and Shi Y. E. Stimulation of breast cancer invasion and metastasis by synuclein gamma. Cancer Res, 1999; 59: 742-727.
344. Lu A., Gupta A., Li C., Ahlborn T. E., Ma Y., Shi E. Y. and Liu J. Molecular mechanisms for aberrant expression of the human breast cancer specific gene 1 in breast cancer cells: control of transcription by DNA methylation and intronic sequences. Oncogene, 2001; 20: 5173-5185.
345. Li Q., Ahuja N., Burger P. C. and Issa J. P. Methylation and silencing of the Thrombospondin-1 promoter in human cancer. Oncogene, 1999; 18: 3284-3289.
346. Chen L. M. and Chai K. X. Prostasin serine protease inhibits breast cancer invasiveness and is transcriptionally regulated by promoter DNA methylation. Int J Cancer, 2002; 97: 323-329.
347. Cavalli L. R., Urban C. A., Dai D., de Assis S., Tavares D. C., Rone J. D., Bleggi-Torres L. F., Lima R. S., Cavalli I. J., Issa J. P. and Haddad B. R. Genetic and epigenetic alterations in sentinel lymph nodes metastatic lesions compared to their corresponding primary breast tumors. Cancer Genet Cytogenet, 2003; 146: 33-40.
348. Widschwendter M. and Jones P. A. DNA methylation and breast carcinogenesis. Oncogene, 2002; 21: 5462-5482.
349. Barrows G. H., Anderson T. J., Lamb J. L. and Dixon J. M. Fine-needle aspiration of breast cancer. Relationship of clinical factors to cytology results in 689 primary malignancies. Cancer, 1986; 58: 1493-1498.
350. Pu R. T., Laitala L. E., Alli P. M., Fackler M. J., Sukumar S. and Clark D. P. Methylation profiling of benign and malignant breast lesions and its application to cytopathology. Mod Pathol, 2003; 16: 1095-1101.
351. Jeronimo C., Costa I., Martins M. C., Monteiro P., Lisboa S., Palmeira C., Henrique R., Teixeira M. R. and Lopes C. Detection of gene promoter hypermethylation in fine needle washings from breast lesions. Clin Cancer Res, 2003; 9: 3413-3417.

352. Krassenstein R., Sauter E., Dulaimi E., Battagli C., Ehya H., Klein-Szanto A. and Cairns P. Detection of breast cancer in nipple aspirate fluid by CpG island hypermethylation. Clin Cancer Res, 2004; 10: 28-32.
353. Wong I. H. Methylation profiling of human cancers in blood: molecular monitoring and prognostication (review). Int J Oncol, 2001; 19: 1319-1324.
354. Dulaimi E., Hillinck J., de C., II, Al-Saleem T. and Cairns P. Tumor suppressor gene promoter hypermethylation in serum of breast cancer patients. Clin Cancer Res, 2004; 10: 6189-6193.
355. Muller H. M., Widschwendter A., Fiegl H., Ivarsson L., Goebel G., Perkmann E., Marth C. and Widschwendter M. DNA methylation in serum of breast cancer patients: an independent prognostic marker. Cancer Res, 2003; 63: 7641-7645.
356. Muller H. M., Fiegl H., Widschwendter A. and Widschwendter M. Prognostic DNA methylation marker in serum of cancer patients. Ann N Y Acad Sci, 2004; 1022: 44-49.
357. Egger G., Liang G., Aparicio A. and Jones P. A. Epigenetics in human disease and prospects for epigenetic therapy. Nature, 2004; 429: 457-463.
358. Bender C. M., Pao M. M. and Jones P. A. Inhibition of DNA methylation by 5-aza-2'-deoxycytidine suppresses the growth of human tumor cell lines. Cancer Res, 1998; 58: 95-101.
359. Segura-Pacheco B., Trejo-Becerril C., Perez-Cardenas E., Taja-Chayeb L., Mariscal I., Chavez A., Acuna C., Salazar A. M., Lizano M. and Duenas-Gonzalez A. Reactivation of tumor suppressor genes by the cardiovascular drugs hydralazine and procainamide and their potential use in cancer therapy. Clin Cancer Res, 2003; 9: 1596-1603.
360. Villar-Garea A., Fraga M. F., Espada J. and Esteller M. Procaine is a DNA-demethylating agent with growth-inhibitory effects in human cancer cells. Cancer Res, 2003; 63: 4984-4989.

Chapter 6

EPIGENETIC DYSREGULATION OF MASPIN (SERPINB5) IN CANCER INVASION AND METASTASIS

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Abstract: The goal of this chapter is to promote the value of studying maspin regulation as a paradigm for loss of transcriptional control during cancer progression and to highlight the importance of this endeavor in developing a comprehensive picture of the epigenetics of the malignant phenotype. We will attempt to do this through a discussion of the structure and functions of the serpin superfamily of proteins, with an emphasis on maspin, its discovery as a tumor suppressor, and its functional role in cancer. The control of maspin expression in normal tissue by epigenetic mechanisms will be described and how this underlying mechanism is compromised in cancer leading to the inappropriate silencing of maspin in cancers derived from maspin-positive cell types, as well as the activation of maspin in cancers derived from normally maspin-negative cell types. Finally, we will close with speculation that maspin may represent an inaugural member of a class of cell-type restricted genes involved in cancer cause and progression that are controlled by epigenetic mechanisms. During transformation, epigenetic instability and mischief results in a loss of control in the expression of these genes. We propose that these genes, through metastable epigenetic switching mechanisms, can be turned off and on in response to environmental stresses and cues in the cancer cell, thereby allowing tumor cells a phenotypic plasticity that appears necessary for the challenges a tumor cell and its progeny must undertake to migrate from primary tumor site to distant metastatic site. It is proposed that this epigenetic switch can be targeted by therapeutics designed to transcriptional reprogram tumor cells and flip the switch back to non-malignant behavior.

Key words: maspin, methylation, histone, chromatin, tissue-specific, expression, p53

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1. SERPINS AND MASPIN

Serpins (**Ser**ine **P**roteinase **In**hibitors) are a super family of proteins with hundreds of members distributed through the Animal and Plant kingdoms (1) Examples of serpins are also found in viruses, but homologs have not been identified in prokaryotes or fungi. Additionally, serpins are found in some model organisms (*D. Melanogaster. C. Elegans, and A. Thalian*a), but not others (*S. Pombe, Cerevisiae*). Serpins are important to human health and disease because of their roles in disparate biochemical pathways, which include blood homeostasis, hormone transport, and neuronal function (2). As such, disruption of normal serpin function through mutation or loss of expression contributes to a wide range of human diseases that include cirrhosis, emphysema, dementia, and cancer (3).

Serpins are variable glycosylated proteins that are monomers in their active state. The typical serpin is 350 to 500 amino acids and folds into 3 beta sheets, 9 alpha helices, and a reactive site loop (RSL) necessary for cognate proteinase recognition and subsequent suicide substrate-like inhibition (2). Most serpins inhibit serine proteinases of the chymotrypsinogen family; however, some serpins are able to inhibit cysteine proteinases, such as cathepsins. Not all serpins are protease inhibitors, however, with one example being cortisol binding globulin. While still not fully resolved, maspin appears to be another example of a non-inhibitory serpin, or at least a serine protease inhibitor with additional functions (4-7).

Analysis of 219 serpin sequences from animals, plants and viruses produced a phylogenetic tree that divides the serpins into 16 branches or clades (families) (1). These 16 clades are categorized A through P and are named based on their prototypical member. Maspin is a member of clade B and is named the ovalbumin, intracellular family of serpins (ov-serpins). The ov-serpins can be found in gene clusters on chromosomes 6 and 18 (8). Maspin, whose RefSeq name is SerpinB5 (clade B, member 5), is found on chromosome 18.q21.33 along with many other serpins of the ov-serpin class that are from centromere to telomere serpin B5 (Maspin), B12, B13 (Hurpin), B4 (SCCA2), B3 (SCCA1), B11, B7 (Megsin), B2 (PAI2), B10 (Bomapin), and B8 – the names in parentheses refer to the gene’s common name.

2. MASPIN DISCOVERY

Maspin or mammary serpin was one of the first tumor suppressor genes cloned using expression genetics, which followed the idea that investigation of heredity at the level of RNA and gene expression would reveal many new

genes and pathways involved in oncogenesis (9). Using the techniques of subtractive hybridization and differential display, Ruth Sager and her laboratory used this pioneering concept to discover scores of genes down-regulated at the level of gene transcription that was not due to gene mutation or deletion (10). Maspin is a prime example of the genes found by the Sager laboratory as a result of using this approach. Since its discovery as a tumor suppressor in breast cancer in 1994 to the present, 2004, over 175 articles have have been published that involve maspin biology, with aberrant expression of maspin being reported in more than 10 different types of human cancer.

The original publication reported on the results of a functional genomics screen that was used to identify genes that were down-regulated in breast cancer when compared to normal breast cells grown under similar conditions (11). More than 30 such genes were identified; one of the genes isolated being maspin. The functional studies described in the original report decidedly showed that maspin has tumor suppressor function in breast cancer and are discussed briefly below. Breast cancer cells transfected with a full length maspin cDNA showed a reduction in the number and size of tumors that formed in the mouse mammary fat pad xenograft model. Importantly, the reintroduction of maspin completely also blocked the malignant potential of the tumors that did form in the mouse xenografts, as no metastases were found in the lymph nodes or lungs of maspin transfected breast cancer cells, in contrast to vector-only transfected cells where metastatic tumors were found. In contrast to the *in vivo* effects of maspin transfection, in cell culture maspin transfectants showed no difference in growth rates compared to control and vector-only transfected cells, which suggest that the inhibitory effect on tumor formation and dissemination was not a result of a simple maspin-induced growth inhibition. Indeed, functional analysis of this phenotype *in vitro* revealed that the maspin transfectants have a diminished invasive potential compared to control or vector only-transfected cells. Immunohistochemical analysis of human breast cancers for maspin protein expression indicated that maspin was lost in a high percentage of lymph node metastases and pleural effusions. Taken together, the results presented in this study implicate strongly a critical role for the loss of maspin expression in the evolution of human breast cancer.

An important aspect of the Sager laboratory paradigm was that most genes down-regulated in cancer are not mutated, but rather the genes are inappropriately silenced, and that expression genetics could reveal these culprits in cancer (12). As such, these inappropriately silenced genes that participate in cancer initiation and/or progression represent potential targets of gene reactivation or transcriptional reprogramming, and could represent

potential therapeutic opportunities in the treatment of cancer. Maspin represents an excellent example of this paradigm.

As revealed in the initial report, Southern blot analysis showed the presence of the maspin gene in breast cancer cells where maspin was no longer expressed. Additionally, the maspin gene was not grossly rearranged and sequence analysis revealed that the maspin coding sequence was not mutated. Furthermore, treatment of cells with the tumor promoter TPA was able to re-induce maspin gene expression. It is tempting to speculate that many of the other genes that the Sager laboratory isolated using this paradigm may very well be inappropriately silenced by the same mechanisms responsible for silencing maspin gene expression

In summary, maspin is a prime example of a tumor suppressor gene cloned by gene expression screening. A host of subsequent studies have confirmed the initial observations regarding maspin and extended them to clearly indicate that maspin plays an important role in normal breast development and its loss contributes to the development of metastatic breast cancer. While these early studies clearly defined a role for maspin as a tumor suppressor the detailed molecular mechanism by which this occurs has remained elusive and incomplete, although recent work suggests that these days are limited. Many questions regarding maspin emerged from these studies and can be largely grouped under 2 general headings:

1) What specific molecular mechanisms does maspin use to exert its tumor suppressor properties, and

2) What mechanisms control maspin gene expression and how are they compromised in carcinogenesis.

The remainder of this review will briefly discuss the biologic role of the maspin protein, in an effort to emphasize the potential multifaceted roles and therefore importance of maspin in human cancer, especially breast cancer. The majority of discussion will be reserved for the control of maspin gene expression in normal tissue and the multiple mechanisms by which maspin may be inappropriately silenced (or activated) in normal and cancerous human tissue.

3. MOLECULAR MECHANISMS OF MASPIN PROTEIN FUNCTION

The importance of maspin as a tumor suppressor has led to a large number of studies designed to determine the molecular function of maspin. Excellent reagents for the analysis of the 42kd maspin protein have been generated, and include recombinant and purified maspin protein, specific, sensitive and commercially available antibodies, retro- and adenoviruses that

express maspin, maspin transgenic mice, and the crystal structure of the protein. The early work from the Sager and Hendrix laboratories' clearly indicated that maspin inhibits cell motility and invasion, and that maspin exerted these effects at the cell surface (11, 13, 14). These and other investigators provided experimental evidence that maspin exerted its effect at the cell surface and increased adherence to components of the basement membrane, such as fibronectin and laminin (14-17). Yeast 2-hybrid studies indicated that maspin protein binding partners included collagen types I and III (18). Recent studies by the Hendrix laboratory suggests that the signaling pathway through which maspin exerts its inhibitory effects on cell motility is through the RHO GTPase pathway effecting Rac activity (19). In addition to maspin's effects on cell adherence and motility, elegant studies by the Zhang laboratory demonstrated, using both *in vitro* and *in vivo* models that maspin could inhibit angiogenesis by directly inhibiting endothelial cell migration towards basic fibroblast growth factor and vascular endothelial growth factor (20). Recently, structural biologists solved the three dimensional structure of maspin to 3.1 angstrom resolution (6). In general, the solved structure supports the basic aspects of maspin's function described above. Maspin's RSL, which is sufficient for its tumor suppressor properties, is accessible and assumes a fixed conformation (17, 21). The structural results obtained in this study also support earlier speculation that the bulky amino acid residues that populate maspin's hinge region make it highly unlikely to act as an inhibitory serpin using the standard serpin loop-insertion mechanism (5, 7).

Not surprisingly, like many other tumor suppressor genes and oncogenes, maspin plays a critical role in normal organismal development. The same holds true for maspin, convincingly shown by work from the Zhang laboratory (16, 22-24). Mice in which both copies of the maspin gene were "knocked out" were embryonic lethal at the peri-implantation stage. Deletion of the maspin gene disrupted the formation of the endodermal layer and epiblast morphogenesis. Further studies indicate that deletion of maspin disrupts visceral endoderm function by reducing cell adhesion and proliferation (16). In maspin gene addition experiments, the Zhang laboratory selectively drove high levels of maspin expression in the mammary gland of the mouse using the whey acidic protein (WAP) promoter (23). Studies in this mouse model showed that maspin was important for normal mammary development and its overexpression inhibited lobular-alveolar structures in pregnancy and disrupted normal mammary gland differentiation. When these maspin transgenic mice were crossed with the Whey Acidic Protein T-antigen (WAP-Tag) mouse model of mammary tumor progression, it was found that the overexpression of maspin inhibited formation of tumor metastases, but not the tumor incidence. The reduced rate of metastases, *in vivo*, was associated with an increased

apoptotic fraction in the tumors, and decrease in tumor cell migratory activity, and blood microvessel density (24).

In summary, the maspin protein is likely a multifunctional protein although it does not appear to be an inhibitory serpin. The RSL of maspin appears to be sufficient for its antitumor properties and reintroduction of this protein into tumor model systems can reduce tumor burden, dissemination, and metastases. Strategies that reintroduce maspin genetically or through delivery of the protein have been attempted to decrease the rate of tumor progression in models of human cancer, and are discussed in Section 7.

4. MASPIN GENETICS AND EPIGENETICS

The maspin gene, located at chromosome 18.q21.33 consists of seven exons spread over a little less than 30kb, with the first exon being non-coding. Three mRNA species have been detected to arise from this gene. The main transcript that produces the mature protein is approximately 3kb by northern blot analysis, with the two other transcripts of unknown function (and are not always detectable), also arising from the maspin gene. One transcript is approximately 1.2 kb and shares extensive homology with the 5' end of the maspin gene, and the other transcript being approximately 4.6 kb that appears to be an incomplete splice variant of maspin (http://genome.ucsc.edu/). While expression of the gene is frequently down-regulated in cancer, the gene appears not to be deleted, rearranged or mutated (11).

In normal cells, expression of maspin is limited largely to epithelial cell types, such as breast, prostate, skin, oral, and bronchial epithelial cells, although exceptions exist, such as corneal stromal cells (17). In contrast maspin is not expressed in most non-epithelial cell types, for example, peripheral blood mononuclear cells, cardiac muscle, fibroblasts, chondrocytes. The expression of maspin is regulated at the level of transcription, and therefore detailed analysis of the maspin promoter should provide insights into the mechanisms of transcriptional control. In addition, expression of maspin is frequently lost or down-regulated in cancer, but the gene appears not to be deleted, rearranged or mutated. Taken together these data suggest that examination of the promoter region would provide meaningful insights into the mechanism of gene control and aberrant silencing.

The earliest studies on the maspin promoter emanated, not surprisingly, from the Sager laboratory. Studies in breast and prostate cancer cells, using deletion and mutation constructs of the maspin promoter in chloramphenicol acetyltransferase reporter assays and electromobility shift assays, provided

the first data regarding transcription factors important to basal maspin expression (25, 26). These studies clearly demonstrated the importance of the AP-1 and ets transcription factors in the positive regulation of maspin in breast and prostate tissue, and set the stage for all subsequent work regarding the control of maspin transcription. Exciting recent work identified p53 binding sites in the maspin promoter region (27). These investigators demonstrated that introduction of wild-type p53 could activate maspin expression in prostate cacner cells as well as the p53 wild-type breast cancer cell line, MCF7. Our laboratories' followed up these observations with microarray-based studies in which we examined p53 target genes (28). The analysis of the resultant gene expression using multidimensional scaling supported the hypothesis that maspin is a p53-inducible gene. In this study we also demonstrated direct binding of p53 to the maspin promoter using *in vivo* chromatin immunoprecipitation assays. Finally, a pair of studies published in the past few months provides the intriguing observation, that the p53 homolog p63, which can bind the same recognition sequence as p53, can substitute for p53 and induce maspin gene expression when expressed in cancer cells (29, 30). The important role of p63 in development and differentiation of epithelial cell types including epidermis, mammary, prostate – all maspin positive cell types – suggests the intriguing possibility that cell type specific maspin expression is established at least in part through p63 dependent mechanism during development.

We used the UCSC genome bioinformatics portal (http://genome.ucsc.edu/) to perform a Mulitz alignment (phastCONS) of the maspin promoter. Using a phylogenetic hidden Markov model, the Multiz alignment provides a measure of evolutionary conservation of genome regions between human, chimp, mouse, rat, dog, as well as other organisms. This alignment has revealed a variety of interesting features about the maspin promoter. First, this alignment shows that the recognition sequences of the primary transcription factors discussed above show identity (e.g., AP-1 and ets) or high homology (e.g., p53) among the human, chimp, mouse, rat, and dog maspin promoter regions. Second, based on the genomic data produced and analyzed for the human genome, it appears that the transcription start site is actually 123 nucleotides further 3' than originally reported (25). Figure 1 shows a schematic of the maspin promoter with the relevant transcription factor response elements along with the updated numbering system.

Also shown in Figure 1 is the location of CpG dinucleotides located in the maspin promoter. Analysis of this region containing the 22 CpG and using the original stringency described by Frommer and Gardiner-Garden (31), this maspin promoter area fits the criteria of a CpG island**.** In addition, other features, such as the fact that this region overlaps the promoter and

transcriptional start site supports the CpG "islandness" of the region. In our estimation this is where the story gets a little more interesting.

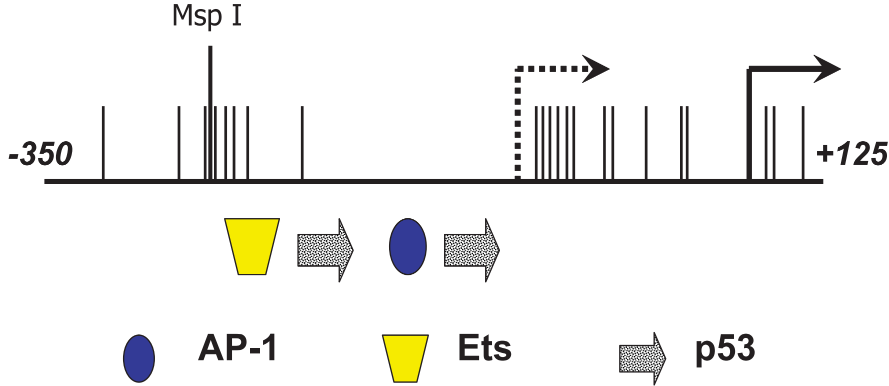

Figure 1. The Maspin Gene Has a CpG-rich Promoter. Diagram of the maspin promoter. The location of the relevant transcription factor response elements is shown. The bent dotted line arrow shows the originally reported transcription start site, and the solid bent arrow shows the transcription start site obtained through the USCS genome bioinformatics web portal. The vertical tick marks show the locations of the 22 CpG dinucleotides. The Msp I site, used in assays of chromatin accessibility, is also shown. The numbering system shown refers to nucleotide position relative to transcription start.

Analysis of the patterns of CpG methylation in the maspin promoter of normal cell types derived from a variety of different tissues revealed striking differences in CpG methylation that were tightly linked to the expression state of maspin and the overall chromatin structure of the region (32). Normal maspin-positive cell types (e.g., mammary, prostate, skin epithelium) have a uniformly unmethylated maspin promoter, the associated histones H3 and H4 are hyperacetylated, the K9 position of histone H3 is unmethylated, and the chromatin is in an accessible conformation. In contrast, maspin-negative tissue (e.g., lymphocytes, fibroblasts, chondrocytes) have a completely methylated maspin promoter, which is linked to hypoacetylated histones H3 and H4, methylation of K9 in histone H3, and an inaccessible chromatin structure (32) and unpublished results). Furthermore treatment of immortalized maspin-negative cells (such as the fibroblast strain VA-13) with the inhibitor of DNA methylation, 5-aza-2'deoxy-cytidine, led to a dose-dependent activation in maspin expression. These results provide evidence for a definitive role for CpG methylation in the establishment and maintenance of cell type specific gene expression, and provide support to the hypothesis proposed more than 25 years ago, that

CpG methylation participates in the cell-type specific control of gene expression (33, 34).

Can this observation be modeled in mouse? It appears the answer may be no, unfortunately. The multiz alignment offers additional interesting insights, in this regard. There are 22 CpG sites in the region around the human maspin transcriptional start. In chimpanzee, 20 of the 22 CpG sites seen in the human promoter are aligned and conserved (the two CpG sites not conserved are CpT sites in chimp). In stark contrast, over this same region, the mouse maspin promoter is decidedly not CpG-rich as there are only 9 CpG sites in the same corresponding region and the alignment of the mouse CpG sites are not conserved with the human or chimp promoter. A similar CpG-poor maspin promoter is found in rat and dog. Nonetheless, pilot studies were performed in our laboratories' to assess CpG methylation of the mouse maspin promoter in maspin-positive and maspin-negative cells of mouse origin. Results from this very small pilot study are shown in Figure 2). In both maspin-positive and maspin-negative cells, high levels of methylation of the CpG sites in this CG-poor region are seen, not only in the 9 CpG sites that are found in the region covered by the human CpG island, but also 2 more CpG sites further upstream (-408 and -402).

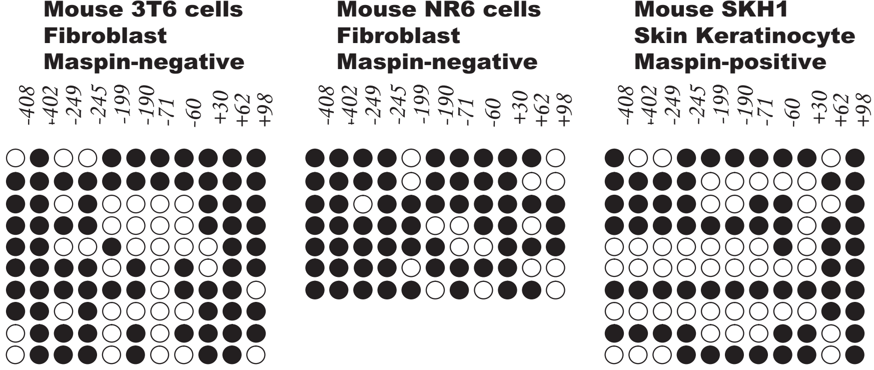

Figure 2. Methylation analysis in the mouse maspin promoter by sodium bisulfite sequencing. Cytosine methylation profile obtained for 11 CpG sites in the mouse Maspin promoter region from 3 different mouse cell lines. Each row of circles represents the cytosine methylation pattern obtained from individual clones of the Maspin promoter, as well as the position of each CpG site relative to transcription start. Open circles indicate unmethylated CpG sites; filled circles indicate methylated CpG sites.

In summary, whether the maspin promoter is a CpG island is somewhat irrelevant; what is relevant is that CpG methylation and other epigenetic mechanisms participate in the control of the expression of cell type-specific genes, of which maspin is the inaugural member. Like most mammalian

genes, maspin expression is clearly regulated at many levels. Solid experimental evidence exists for a role for multiple transcription factors participating and cooperating in the transcriptional control of the maspin gene. In addition to this chromatin landscape, the maspin promoter is also decorated with epigenetic modifications on histones and CpG cytosines. Cell-type specific control is tightly linked to the degree of CpG methylation, histone H3 and H4 acetylation and H3 K9 methylation. As such, the example of maspin clearly supports the hypothesis that DNA methylation participates in the control of cell type specific expression and by extension cellular differentiation, a theory posited three decades ago (33, 34).

5. MASPIN EPIGENETICS AND BREAST CANCER

The original report from the Sager lab intrigued us because we knew that aberrant methylation was a hallmark of cancer and inappropriate gene silencing (35-37). With respect to gene expression in breast cancer cells, we reproduced and extended their results in showing that maspin expression is lost in a high percentage of breast cancer cells (38). In an attempt to decipher the mechanism of the inappropriate silencing, we followed the Sager paradigm that the silencing was not genetic, but epigenetic in nature. In our original report, we used bisulfite sequencing to analyze DNA methylation patterns and levels in breast cancer cells, and we discovered that silencing of the maspin gene was frequently associated with aberrant methylation of the maspin promoter. In addition, using nuclease sensitivity assays, we showed that this aberrant CpG methylation was associated with an inaccessible chromatin structure. Relief of this constrained state should allow for re-expression of the gene by allowing the transcription factors present to access their cognate binding sites within the maspin promoter. Treatment of a different breast cancer cell lines with the epigenetic modifier 5-aza-2'-deoxycytidine was capable of reactivating maspin expression, strongly suggesting that at least one facet of the defect in maspin expression is epigenetic in nature. This initial *in vitro* work has been confirmed and extended by a variety of other groups, with more recent data showing that histones associated with the maspin promoter become hypoacetylated in breast cancer (28, 39), as well as enriched for K9 methylation of histone H3 (unpublished observations). Table 1 contains our analysis of the maspin promoter in a variety of different human breast cancer cell lines.

Maspin was originally isolated due to its down-regulation in advanced breast cancer. A number of correlative clinical studies have corroborated these findings (40-44). Studies on maspin expression in breast cancer by Maass and colleagues have moved its loss to an earlier point in the temporal

events in breast carcinogenesis by finding loss of maspin can occur in ductal carcinoma *in situ* (DCIS), prior to frank carcinoma and metastatic growth (45).

Table 1. Maspin in Human Breast Cancer Cell Lines.

Cell line	Maspin Expression	Promoter Methylation	Histone Acetylation H3	Histone Acetylation H4	Chromatin State
HMEC	++++	-	+	+	OPEN
MCF10	++++	-	+	+	OPEN
UACC 245	++	-			
ZR-71	+	-			
MCF7	-	+	-	-	CLOSED
BT549	-	+	-	-	
HS578T	-	+			
MDA-MB-435	-	+	-	-	CLOSED
MDA-MB-231	-	+	-	-	CLOSED
MDA-MB-157	-	+			
MDA-MB-453	-	+			
UACC 1179	-	+	-	-	CLOSED
UACC 893	-	+			
UACC 3133	-	+			
UACC 2087	-	-			
MDA-MB-468	-	-			

Maspin expression was determined by quantitative real time PCR using TaqMan chemistry. Maspin promoter methylation state was determined by sodium bisulfite sequencing. The histone acetylation state of the Maspin promoter was determined by chromatin immunoprecipitation assays using real time PCR with TaqMan chemistry. Chromatin structure was determined using a nuclease sensitivity assay coupled to linker-mediated PCR.

We wished to determine if aberrant methylation of the maspin promoter occurred in breast cancer *in vivo*. The cell type specific patterns of maspin promoter methylation make the analysis of methylation changes in tumor tissue non-trivial; only purified cell populations will lead to a definitive answer. In a small study of 30 DCIS specimens and two healthy controls we used immunohistochemical analysis, laser capture microdissection, and bisulfite sequencing to assess epigenetic silencing of maspin in human cancer (46). Results also showed that 57% of the DCIS lost maspin expression; and for those for which sufficient amounts of genomic DNA could be isolated by laser capture microdissection, we found loss of maspin immunoreactivity was frequently, but not always, accompanied by aberrant methylation of the maspin promoter. We found that the neoplasms

themselves often displayed significant heterogeneity in maspin staining within the tumor population, while normal tissue stained uniformly throughout the duct with intense staining in myoepithelial cells, consistent with earlier studies (47, 48). Taken together with the earlier work of Maass, these results indicate that maspin silencing can be an early event in breast carcinogenesis, preceding metastatic growth. Additionally, these studies also suggest that other mechanisms, in addition to CpG methylation, participate and perhaps cooperate with aberrant methylation to inappropriately silence maspin expression early in breast carcinogenesis.

6. EPIGENETIC DYSREGULATION IN OTHER CANCERS

Since its original identification in breast cancer, changes in maspin expression in cancer have been widely reported – many of these reports provide data that supports the association of maspin gene expression changes with epigenetic changes, with both silencing due to aberrant hypermethylation and activation due to aberrant hypomethylation. Table 2 shows the cancers in which differences in maspin expression have been found between the tumor and the corresponding normal differentiated cell, and if the differences in expression have been linked directly to methylation state.

The loss of maspin expression seen in some tumors is confounded by the activation of maspin expression seen in other tumors. These seemingly conflicting results have caused some confusion and concern regarding the tumor suppressor role of maspin. A few points; however, should be made regarding the observations of maspin activation in cancer. The seemingly paradoxical activation of maspin in some tumors was described in correlative clinical studies where a role for maspin in the disease process has not been ascertained. One possible explanation for these contradictory results is that the maspin protein has different functions in different cell types depending on the spectrum of other maspin interacting proteins that are expressed within a given cell type and environment. Secondly, complex epigenetic changes occur to the genomes of cancer cells, and we have shown that the cell type specific expression of maspin is controlled by the cytosine methylation state of its promoter. As epigenetic modifications by their nature are metastable, it could be envisioned that as the tumor evolves, a variety of epigenetic states emerge, that which is most favorable to the current microenvironment conditions will likely be selected for and survive. Most epigenetic changes, however, will likely be neutral or unfavorable to tumor growth and dissemination. As such the activation of maspin in the

tumorigenic counterparts of normal cells that do not express maspin (e.g., pancreas) may be a result of complex epigenomic changes in cytosine methylation of which demethylation of the maspin promoter was a part. Whether this inappropriate activation of maspin expression imparts any significant phenotypic changes on the tumor at particular stages of cancer progression or whether its activation is an epiphenomenon that reflects loss of methylation homeostasis remains to be determined. Preliminary *in vitro* studies carried out in our laboratory does show that adenoviral delivery of maspin to non-expressing pancreatic cancer cells actually inhibits their ability to invade a matrigel membrane, suggesting that maspin retains this functional attribute seen in prostate and breast epithelial cells. Considering that pancreatic cancer is distinctly different than breast and prostate cancers, this anti-invasive effect of maspin may not be sufficient to disrupt pancreatic malignancy.

Table 2. Human Cancer, Maspin Expression, and the Epigenetic State.

Tumor Type	Maspin Expression	Maspin Promoter Methylation	References
Breast	Silenced	Hypermethylation	(28, 38, 39, 46, 49, 50)
Prostate	Decreased	No Change	(13, 15, 50-52)
Pancreas	Activated	Hypomethylation	(53-57)
Thyroid	Activated	Hypomethylation	(58, 59)
Ovarian	Activated	Hypomethylation	(60) and unpublished results
Lung	Activated	Hypomethyaltion	(29, 61-63)
Gastric	Conflicting	Hypomethylation	(64-68)
Melanoma	Activated	Hypomethylation	(69)
Oral	Decreased	Unknown	(70, 71)
Salivary	Variable	Unknown	(72)
Bladder	Silenced	Unknown	(73, 74)
Colon	Variable	Unknown	(75, 76)

Maspin expression and promoter methylation status for each tumor type were taken from the references provided in the last column. Expression, in this case, refers to either mRNA or protein expression. DNA methylation state was provided if the referenced studies assayed CpG methylation status directly.

From these apparently conflicting results, it is clear additional studies will be required to unravel the complex question of how maspin expression may modify the malignant phenotype in some cell types and potentially not others. Nevertheless, it is clear the CpG methylation state plays an important role in in controlling maspin expression in normal tissue, and that this epigenetic control is compromised during human carcinogenesis.

7. MASPIN AS A MOLECULAR TARGET

Maspin as a molecular target in cancer has some appeal; however, in this approach the therapeutic agent(s) must reactivate or stimulate maspin, which today is more difficult than inhibiting an overexpressed mutant gene product, for example. Nonetheless, a number of features about maspin make it appealing as a target of opportunity. First, maspin is present and important to the function and development of normal tissues, indicative of its likely significance as an important node in cell physiology. Second, the loss of maspin transcends disease boundaries and is seen in a number of epithelial tumors. Third, the loss of maspin is clearly important for the persistence of the tumor as well as its metastatic potential. Indeed, confidence in maspin as a target is bolstered by experimental gene therapy studies where systemic maspin gene delivery inhibited breast tumor progression (77).

Two primary approaches to a maspin-directed therapy have been studied in the laboratory. In the first approach, only maspin activity is targeted for reintroduction to the tumor. Early reports focused on the delivery of recombinant mapsin proteins to the tumor, and *in vitro* studies demonstrated impressive anti-tumor properties (13, 14, 19, 21). Although delivery of large proteins as therapeutic agents currently has pragmatic limitations, maspin investigators have also shown that the maspin RSL is sufficient for the antitumor activity of maspin (17). This data opens new promise to small peptide or molecule mimetics of the maspin RSL as potential therapeutic agents. Also, as stated above, liposomal delivery of a maspin transgene in a syngeneic mouse model of breast cancer inhibited both the primary as well as metastatic tumor growth through increased apoptosis of the tumor cells (77). Taken together, these specifically-targeted approaches towards maspin-based cancer therapeutics offer an excellent hope in the future.

A second approach, not specific as the approaches above, are instead selective in nature, and can be considered targeted towards the transcriptional reprogramming of the cancer cell. Because of the epigenetic nature of maspin silencing in many cancers, therapeutics that target CpG methylation or histone modification state may actually prune the epigenetic landscape back to its ordered state, and inhibit the cancer phenotype. The approach should be considered selective because it does not only target maspin. In fact, the concept of transcriptional reprogramming should not only target maspin, but also other epigenetically silenced genes that are involved in cancer initiation and progression. Perhaps the scores of other genes originally isolated by the Sager laboratory through the expression genetics approach may be such targets.

Essentially, the goal of this selective, but non-specific, approach is to repair the tumor cells' epigenotype, which should result in a benign

differentiated phenotype or an apoptotic response. A variety of studies have shown in multiple systems where maspin is epigenetically silenced, inhibitors of DNA methylation and histone deacetylase can reactivate maspin expression (28, 38, 39, 56, 57, 73, 78-81). Other studies have provided solid rationale and scientific data that the addition of epigenetic modifiers, such as 5-aza-2'-deoxycytidine, with standard breast cancer therapeutics, such as taxotere, may lead to more efficacious breast cancer treatment regimens (78).

Our laboratories' have attempted to develop therapeutic approaches using maspin as a model of epigenetic and genetic regulation. The idea being that repair of both epigenetic and genetic lesions may be necessary to most effectively reprogram the transcriptional profile of tumors cells (28). To this end, we combined the epigenetic modifier 5-aza-2'-deoxycytidine with adenoviral delivery of wild type p53 to repair a genetic defect. Treatment with either agent alone led to a reactivation of maspin, but only 1 to 10% of normal basal levels of maspin; however, when these agents were given sequentially maspin expression reached near normal levels. Further supporting the selective rather than specific nature of this pharmacologic approach, similar synergistic reactivation of gene expression was found for another gene, desmocollin 3, in our 5-aza-2'-deoxycytidine plus wild type p53 study. Desmocollin 3 is relevant because it appears to display tumor suppressor function. Like maspin; Desmocollin 3 functions normally to inhibit motility, is a p53 target gene, and is also aberrantly methylated in breast cancer (28, 82-86).

Unfortunately, viral gene therapy of wild-type p53 has not been very successful to date. Although there are a host of reasons why wild-type p53 gene therapy has not been successful, one reason may be that p53 was not capable of fully reactivating the critical bank of p53 target genes necessary to elicit an anti-tumor response, due perhaps to an epigenetic component of gene silencing. As viral gene delivery systems have technical hurdles to overcome, it is perhaps possible that in the future small molecule mimetics of p53 may prove useful in this approach.

In summary, significant activity targeting maspin in cancer therapy is underway. Maspin specific approaches using the maspin gene or protein may ultimately prove useful for cancer therapy. Alternatively, a more selective or generalized approach that is pharmacologically directed at the entire bank of epigenetically silenced genes that participate in human cancer may prove useful. It appears that the challenges and promises of therapies designed to reactivate or repair tumor suppressor genes and their products is accurately reflected in the model of maspin biology.

8. MASPIN AS A PARADIGM OF EPIGENTIC CONTROL OF CELL TYPE SPECIFIC EXPRESSION

The serpins are a large and functionally diverse gene superfamily that participate in a variety of human pathologies when dysregulated (3). Dysregulation of the serpin, maspin, is most closely linked to cancer progression. Maspin does not appear to be a serine protease inhibitor, but instead exerts its effects through other means, such as inhibition of cell motility, invasiveness, and angiogenic potential.

Maspin is a clear example of an autosomal gene controlled by epigenetic mechanisms in normal tissue. Normal epithelial tissues that are maspin-positive uniformly have an unmethylated maspin promoter, which is associated with acetylated histones H3 and H4. These epigenetic modifications are closely linked to a chromatin structure that renders the promoter available for DNA-protein interaction. In contrast, normal cell types derived from maspin-negative mesenchymal tissues uniformly have a methylated maspin promoter, which is associated with underacetylated histones H3 and H4 and a chromatin structure that is not compatible with DNA-protein interaction.

In breast cancer, maspin gene expression is lost, but not by mutation or deletion. Instead, maspin is lost through epigenetic silencing. The epigenetic state of the maspin gene related to its silencing in breast cancer cells closely resembles the epigenetic state of normal mesenchymal tissues that maintain maspin in a silent and inaccessible state; breast cancer cells acquire a repressive chromatin structure through aberrant methylation of the promoter, deacetylation of the associated histones H3 and H4, as well as methylation of K9 residue of histone H3. Conversely, it is also noted that tumors derived from maspin-negative cell types often activate maspin gene expression, which is accompanied by demethylation of the maspin promoter and hyperacetylation of histones H3 and H4,

The aberrant methylation events associated with epigenetic silencing in cancer does not seem to effect all loci that are lost during human carcinogenesis. Instead, epigenetic silencing seems to selectively inactivate a distinct subset of loci, which in turn is frequently tumor type-specific (87, 88). As such, genes that are normally controlled by epigenetic mechanisms may very well be common and important targets for this type of metastable silencing. Additionally, genes silenced by epigenetic mechanisms are unique in that epigenetic silencing is potentially reversible.

In cancer cells that undergo metastatic dispersal, we suggest that critical, but potential transient, epigenetically-driven changes in gene expression patterns occur. A gene that may promote survival in one physiological

context (e.g., primary tumor site) may be deleterious in another context (e.g., invasive disseminating tumor), and vice versa. We propose that epigenetic regulation through CpG methylation and histone modification provides a mechanism by which cell type-specific genes can be turned on and off without direct mutation to the gene itself. For this reason, alterations in gene expression patterns at "the flip of an epigenetic switch" would allow for adaptation of (tumor) cells to their environment is an appealing and viable possibility. In other words, epigenetic control of genes is unique because it is reversible, and thereby can confer a high degree of plasticity to the malignant phenotype.

If this prediction is correct, then other cell-type specific genes controlled by epigenetic mechanisms should also be present. As such, candidate gene approaches can be used for the discovery of such genes, as well as non-biased global scanning approaches. A candidate gene approach has already identified MCJ, and others such as HoxA5 and 14-3-3 sigma have been speculated (89, 90). Indeed, we have recently found that the 14-3-3 sigma CpG island is virtually identical to the epigenetic landscape we found for maspin in epithelial and mesenchymal cell types (manuscript submitted).

In conclusion the serpin, maspin, displays a number of important and unique characteristics, both in the control of its gene expression and the function of the protein. Great strides in understanding maspin biology have been made over the past decade, since its discovery, and some of these advances have extended beyond maspin to provide a greater understanding of mammalian biology as a whole. Of course, because maspin plays an important part in human cancer, future discoveries will be necessary to translate our understanding of maspin biology to the improvement in the detection, prognostication, and treatment of human cancer.

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REFERENCES

1. Silverman G. A., Bird P. I., Carrell R. W., Church F. C., Coughlin P. B., Gettins P. G., Irving J. A., Lomas D. A., Luke C. J., Moyer R. W., Pemberton P. A., Remold-O'Donnell E., Salvesen G. S., Travis J. and Whisstock J. C. The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Evolution, mechanism of inhibition, novel functions, and a revised nomenclature. J Biol Chem, 2001; 276: 33293-33296.
2. Irving J. A., Pike R. N., Lesk A. M. and Whisstock J. C. Phylogeny of the serpin superfamily: implications of patterns of amino acid conservation for structure and function. Genome Res, 2000; 10: 1845-1864.
3. Lomas D. A. and Carrell R. W. Serpinopathies and the conformational dementias. Nat Rev Genet, 2002; 3: 759-768.
4. Fitzpatrick P. A., Wong D. T., Barr P. J. and Pemberton P. A. Functional implications of the modeled structure of maspin. Protein Eng, 1996; 9: 585-589.
5. Hopkins P. C. and Whisstock J. Function of maspin. Science, 1994; 265: 1893-1894.
6. Al-Ayyoubi M., Gettins P. G. and Volz K. Crystal structure of human maspin, a serpin with anti-tumor properties: Maspin's reactive center loop is exposed but constrained. J Biol Chem, 2004.
7. Pemberton P. A., Wong D. T., Gibson, H. L., Kiefer M. C., Fitzpatrick P. A., Sager R. and Barr P. J. The tumor suppressor maspin does not undergo the stressed to relaxed transition or inhibit trypsin-like serine proteases. Evidence that maspin is not a protease inhibitory serpin. J Biol Chem, 1995; 270: 15832-15837.
8. Silverman G. A., Whisstock J. C., Askew D. J., Pak S. C., Luke C. J., Cataltepe S., Irving J. A. and Bird P. I. Human clade B serpins (ov-serpins) belong to a cohort of evolutionarily dispersed intracellular proteinase inhibitor clades that protect cells from promiscuous proteolysis. Cell Mol Life Sci, 2004; 61: 301-325.
9. Pardee A. B. Ruth Sager, Geneticist. In: M. J. C. Hendrix (ed.), 2002; Maspin, pp. 1-7. Georgetown: Landes Biosciences.
10. Zhang M. S., S: Pardee A. B. Maspin in the Sager Laboratory. In: M. J. C. Hendrix (ed.), 2002; Maspin. Georgetown: Landes Biosciences.
11. Zou Z., Anisowicz A., Hendrix M. J., Thor A., Neveu M., Sheng S., Rafidi K., Seftor E. and Sager R. Maspin, a serpin with tumor-suppressing activity in human mammary epithelial cells. Science, 1994; 263: 526-529.
12. Sager R., Sheng S., Anisowicz A., Sotiropoulou G., Zou Z., Stenman G., Swisshelm K., Chen Z., Hendrix M. J., Pemberton P, and et al. RNA genetics of breast cancer: maspin as paradigm. Cold Spring Harb Symp Quant Biol, 1994; 59: 537-546.
13. Sheng S., Carey J., Seftor E. A., Dia, L., Hendrix M. J. and Sager R. Maspin acts at the cell membrane to inhibit invasion and motility of mammary and prostatic cancer cells. Proc Natl Acad Sci U S A, 1996; 93: 11669-11674.
14. Seftor R. E., Seftor E. A., Sheng S., Pemberton P. A., Sager R. and Hendrix M. J. maspin suppresses the invasive phenotype of human breast carcinoma. Cancer Res, 1998; 58: 5681-5685.
15. Abraham S., Zhang W., Greenberg N. and Zhang M. Maspin functions as tumor suppressor by increasing cell adhesion to extracellular matrix in prostate tumor cells. J Urol, 2003; 169: 1157-1161.
16. Gao F., Shi H. Y., Daughty C., Cella N. and Zhang M. Maspin plays an essential role in early embryonic development. Development, 2004; 131: 1479-1489.

17. Ngamkitidechakul C., Warejcka D. J., Burke J. M., O'Brien W. J. and Twining S. S. Sufficiency of the reactive site loop of maspin for induction of cell-matrix adhesion and inhibition of cell invasion. Conversion of ovalbumin to a maspin-like molecule. J Biol Chem, 2003; 278: 31796-31806.
18. Blacque O. E. and Worrall D. M. Evidence for a direct interaction between the tumor suppressor serpin, maspin, and types I and III collagen. J Biol Chem, 2002; 277: 10783-10788.
19. Odero-Marah V. A., Khalkhali-Ellis Z., Chunthapong J., Amir S., Sefto, R. E., Seftor E. A. and Hendrix M. J. Maspin regulates different signaling pathways for motility and adhesion in aggressive breast cancer cells. Cancer Biol Ther, 2003; 2: 398-403.
20. Zhang M., Volpert O., Shi Y. H. and Bouck N. Maspin is an angiogenesis inhibitor. Nat Med, 2000; 6: 196-199.
21. Sheng S., Pemberton P. A. and Sager R. Production, purification, and characterization of recombinant maspin proteins. J Biol Chem, 1994; 269: 30988-30993.
22. Shi H. Y., Lydon J. P. and Zhang M. Hormonal defect in maspin heterozygous mice reveals a role of progesterone in pubertal ductal development. Mol Endocrinol, 2004; 18: 2196-2207.
23. Zhang M., Magit D., Botteri F., Shi H. Y., He K., Li M., Furth P. and Sager R. Maspin plays an important role in mammary gland development. Dev Biol, 1999; 215: 278-287.
24. Zhang M., Shi Y., Magit D., Furth P. A. and Sager R. Reduced mammary tumor progression in WAP-TAg/WAP-maspin bitransgenic mice. Oncogene, 2000; 19: 6053-6058.
25. Zhang M., Maass N., Magit D. and Sager R. Transactivation through Ets and Ap1 transcription sites determines the expression of the tumor-suppressing gene maspin. Cell Growth Differ 1997; 8: 179-186.
26. Zhang M., Magit D. and Sager R. Expression of maspin in prostate cells is regulated by a positive ets element and a negative hormonal responsive element site recognized by androgen receptor. Proc Natl Acad Sci U S A, 1997; 94: 5673-5678.
27. Zou Z., Gao C., Nagaich A. K., Connell T., Saito S., Moul J. W., Seth P., Appella E. and Srivastava S. p53 regulates the expression of the tumor suppressor gene maspin. J Biol Chem, 2000; 275: 6051-6054.
28. Oshiro M. M., Watts G. S., Wozniak R. J., Junk D. J., Munoz-Rodriguez J. L., Domann F. E. and Futscher B. W. Mutant p53 and aberrant cytosine methylation cooperate to silence gene expression. Oncogene, 2003; 22: 3624-3634.
29. Kim S., Han J., Kim J. and Park C. Maspin expression is transactivated by p63 and is critical for the modulation of lung cancer progression. Cancer Res, 2004; 64: 6900-6905.
30. Spiesbach K., Tannapfel A., Mossner J. and Engeland K. TAp63gamma can substitute for p53 in inducing expression of the maspin tumor suppressor. Int J Cancer, 2004.
31. Gardiner-Garden M. and Frommer M. CpG islands in vertebrate genomes. J Mol Biol, 1987; 196: 261-282.
32. Futscher B. W., Oshiro M. M., Wozniak R. J., Holtan N., Hanigan C. L., Duan H. and Domann F. E. Role for DNA methylation in the control of cell type specific maspin expression. Nat Genet, 2002; 31: 175-179.
33. Holliday R. and Pugh J. E. DNA modification mechanisms and gene activity during development. Science, 1975; 187: 226-232..
34. Riggs A. D. X inactivation, differentiation, and DNA methylation. Cytogenet Cell Genet, 1975; 14: 9-25.
35. Jones P. A. and Laird P. W. Cancer epigenetics comes of age. Nat Genet, 1999; 21: 163-167.

36. Baylin S. B., Herman J. G., Graff J. R., Vertino P. M. and Issa J. P. Alterations in DNA methylation: a fundamental aspect of neoplasia. Adv Cancer Res, 1998; 72: 141-196.
37. Baylin S. B. and Herman J. G. DNA hypermethylation in tumorigenesis: epigenetics joins genetics. Trends Genet, 2000; 16: 168-174.
38. Domann F. E., Rice J. C., Hendrix M. J. and Futscher B. W. Epigenetic silencing of maspin gene expression in human breast cancers. Int J Cancer, 2000; 85: 805-810.
39. Maass N., Biallek M., Rosel F., Schem C., Ohike N., Zhang M., Jonat W. and Nagasaki K. Hypermethylation and histone deacetylation lead to silencing of the maspin gene in human breast cancer. Biochem Biophys Res Commun, 2002; 297: 125-128.
40. Martin K. J., Kritzman B. M., Price L. M., Koh B., Kwan C. P., Zhang X., Mackay A., O'Hare M. J., Kaelin C. M., Mutter G. L., Pardee A. B. and Sager R. Linking gene expression patterns to therapeutic groups in breast cancer. Cancer Res, 2000; 60: 2232-2238.
41. Czerwenka K. F., Manavi M., Hosmann J., Jelincic D., Pischinger K. I., Battistutti W. B., Behnam M. and Kubista E. Comparative analysis of two-dimensional protein patterns in malignant and normal human breast tissue. Cancer Detect Prev, 2001; 25: 268-279.
42. Maass N., Hojo T., Rosel F., Ikeda T., Jonat W. and Nagasaki K. Down regulation of the tumor suppressor gene maspin in breast carcinoma is associated with a higher risk of distant metastasis. Clin Biochem, 2001; 34: 303-307.
43. Streuli C. H. Maspin is a tumour suppressor that inhibits breast cancer tumour metastasis in vivo. Breast Cancer Res, 2002; 4: 137-140.
44. Mohsin S. K., Zhang M., Clark G. M. and Craig Allred D. Maspin expression in invasive breast cancer: association with other prognostic factors. J Pathol, 2003; 199: 432-435.
45. Maass N., Teffner M., Rosel F., Pawaresch R., Jonat W., Nagasaki K. and Rudolph P. Decline in the expression of the serine proteinase inhibitor maspin is associated with tumour progression in ductal carcinomas of the breast. J Pathol, 2001; 195: 321-326.
46. Futscher B. W., O'Meara M. M., Kim C. J., Rennels M. A., Lu D., Gruman L. M., Seftor R. E., Hendrix M. J. and Domann F. E. Aberrant methylation of the maspin promoter is an early event in human breast cancer. Neoplasia, 2004; 6: 380-389.
47. Sternlicht M. D., Safarians S., Rivera S. P. and Barsky S. H. Characterizations of the extracellular matrix and proteinase inhibitor content of human myoepithelial tumors. Lab Invest, 1996; 74: 781-796.
48. Reis-Filho J. S., Milanezi F., Silva P. and Schmitt F. C. Maspin expression in myoepithelial tumors of the breast. Pathol Res Pract, 2001; 197: 817-821.
49. Maass N., Hojo T., Zhang M., Sager R., Jonat W. and Nagasaki K. Maspin--a novel protease inhibitor with tumor-suppressing activity in breast cancer. Acta Oncol, 2000; 39: 931-934.
50. Duan H., Zhang H. J., Yang J. Q., Oberley L. W., Futscher B. W. and Domann F. E. MnSOD up-regulates maspin tumor suppressor gene expression in human breast and prostate cancer cells. Antioxid Redox Signal, 2003; 5: 677-688.
51. Machtens S., Serth J., Bokemeyer C., Bathke W., Minssen A., Kollmannsberger C., Hartmann J., Knuchel R., Kondo M., Jonas U. and Kuczyk M. Expression of the p53 and Maspin protein in primary prostate cancer: correlation with clinical features. Int J Cancer, 2001; 95: 337-342.
52. Zou Z., Zhang W., Young D., Gleave M. G., Rennie P., Connell T., Connelly R., Moul J., Srivastava S. and Sesterhenn I. Maspin expression profile in human prostate cancer (CaP) and in vitro induction of Maspin expression by androgen ablation. Clin Cancer Res, 2002; 8: 1172-1177.
53. Ohike N., Maass N., Mundhenke C., Biallek M., Zhang M., Jonat W., Luttges J., Morohoshi T., Kloppel G. and Nagasaki K. Clinicopathological significance and

molecular regulation of maspin expression in ductal adenocarcinoma of the pancreas. Cancer Lett, 2003; 199: 193-200.
54. Maass N., Nagasaki K., Ziebart M., Mundhenke C. and Jonat W. Expression and regulation of tumor suppressor gene maspin in breast cancer. Clin Breast Cancer, 2002; 3: 281-287.
55. Maass N., Hojo T., Ueding M., Luttges J., Kloppel G., Jonat W. and Nagasaki K. Expression of the tumor suppressor gene Maspin in human pancreatic cancers. Clin Cancer Res, 2001; 7: 812-817.
56. Sato N., Fukushima N., Matsubayashi H. and Goggins M. Identification of maspin and S100P as novel hypomethylation targets in pancreatic cancer using global gene expression profiling. Oncogene, 2004; 23: 1531-1538.
57. Fitzgerald M., Oshiro M., Holtan N., Krager K., Cullen J. J., Futscher B. W. and Domann F. E. Human pancreatic carcinoma cells activate maspin expression through loss of epigenetic control. Neoplasia, 2003; 5: 427-436.
58. Ito Y., Yoshida H., Tomoda C., Uruno T., Takamura Y., Miya A., Kobayashi K., Matsuzuka F., Matsuura N., Kuma K. and Miyauchi A. Maspin expression is directly associated with biological aggressiveness of thyroid carcinoma. Thyroid, 2004; 14: 13-18.
59. Ogasawara S., Maesawa C., Yamamoto M., Akiyama Y., Wada K., Fujisawa K., Higuchi T., Tomisawa Y., Sato N., Endo S., Saito K. and Masuda T. Disruption of cell-type-specific methylation at the Maspin gene promoter is frequently involved in undifferentiated thyroid cancers. Oncogene, 23: 2004; 1117-1124.
60. Sood A. K., Fletcher M. S., Gruman L. M., Coffin J. E., Jabbari S., Khalkhali-Ellis Z., Arbour N., Seftor E. A. and Hendrix M. J. The paradoxical expression of maspin in ovarian carcinoma. Clin Cancer Res, 2002; 8: 2924-2932.
61. Heighway J., Knapp T., Boyce L., Brennand S., Field J. K., Betticher D. C., Ratschiller D., Gugger M., Donovan M., Lasek A. and Rickert P. Expression profiling of primary non-small cell lung cancer for target identification. Oncogene, 2002; 21: 7749-7763.
62. Smith S. L., Watson S. G., Ratschiller D., Gugger M., Betticher D. C. and Heighway J. Maspin - the most commonly-expressed gene of the 18q21.3 serpin cluster in lung cancer - is strongly expressed in preneoplastic bronchial lesions. Oncogene, 2003; 22: 8677-8687.
63. Yatabe Y., Mitsudomi T. and Takahashi T. Maspin expression in normal lung and non-small-cell lung cancers: cellular property-associated expression under the control of promoter DNA methylation. Oncogene, 2004; 23: 4041-4049.
64. Son H. J., Sohn T. S., Song S. Y., Lee J. H. and Rhee J. C. Maspin expression in human gastric adenocarcinoma. Pathol Int, 2002; 52: 508-513.
65. Wang M. C., Yang Y. M., Li X. H., Dong F. and Li Y. Maspin expression and its clinicopathological significance in tumorigenesis and progression of gastric cancer. World J Gastroenterol, 2004; 10: 634-637.
66. Zheng H. C., Wang M. C., Li J. Y., Yang X. F., Sun J. M. and Xin Y. Expression of maspin and kai1 and their clinicopathological significance in carcinogenesis and progression of gastric cancer. Chin Med Sci J, 2004; 19: 193-198.
67. Ito R., Nakayama H., Yoshida K., Oda N. and Yasui W. Loss of maspin expression is associated with development and progression of gastric carcinoma with p53 abnormality. Oncol Rep, 2004; 12: 985-990.
68. Akiyama Y., Maesawa C., Ogasawara, S., Terashima M. and Masuda T. Cell-type-specific repression of the maspin gene is disrupted frequently by demethylation at the promoter region in gastric intestinal metaplasia and cancer cells. Am J Pathol, 2003; 163: 1911-1919.

69. Wada K., Maesawa C., Akasaka T. and Masuda T. Aberrant expression of the maspin gene associated with epigenetic modification in melanoma cells. J Invest Dermatol, 2004; 122: 805-811.
70. Xia W., Lau Y. K., Hu M. C., Li L., Johnston D. A., Sheng S., El-Naggar A. and Hung M. C. High tumoral maspin expression is associated with improved survival of patients with oral squamous cell carcinoma. Oncogene, 2000; 19: 2398-2403.
71. Yasumatsu R., Nakashima T., Hirakawa N., Kumamoto Y., Kuratomi Y., Tomita K. and Komiyama S. Maspin expression in stage I and II oral tongue squamous cell carcinoma. Head Neck, 2001; 23: 962-966.
72. Navarro Rde L., Martins M. T. and de Araujo V. C. Maspin expression in normal and neoplastic salivary gland. J Oral Pathol Med, 2004; 33: 435-440.
73. Sugimoto S., Maass N., Takimoto Y., Sato K., Minei S., Zhang M., Hoshikawa Y., Junemann K. P., Jonat W. and Nagasaki K. Expression and regulation of tumor suppressor gene maspin in human bladder cancer. Cancer Lett, 2004; 203: 209-215.
74. Friedrich M. G., Toma M. I., Petri S., Cheng J. C., Hammerer P., Erbersdobler A. and Huland H. Expression of Maspin in non-muscle invasive bladder carcinoma: correlation with tumor angiogenesis and prognosis. Eur Urol, 2004; 45: 737-743.
75. Wentzensen N., Wilz B., Findeisen P., Wagner R., Dippold W., von Knebel Doeberitz M. and Gebert J. Identification of differentially expressed genes in colorectal adenoma compared to normal tissue by suppression subtractive hybridization. Int J Oncol, 2004; 24: 987-994.
76. Song S. Y., Lee S. K., Kim D. H., Son H. J., Kim H. J., Lim Y. J., Lee W. Y., Chun H. K. and Rhee J. C. Expression of maspin in colon cancers: its relationship with p53 expression and microvessel density. Dig Dis Sci, 2002; 47: 1831-1835.
77. Shi H. Y., Liang R., Templeton N. S. and Zhang M. Inhibition of breast tumor progression by systemic delivery of the maspin gene in a syngeneic tumor model. Mol Ther, 2002; 5: 755-761.
78. Hurtubise A. and Momparler R. L. Evaluation of antineoplastic action of 5-aza-2'-deoxycytidine (Dacogen) and docetaxel (Taxotere) on human breast, lung and prostate carcinoma cell lines. Anticancer Drugs, 2004; 15: 161-167.
79. Lim Y. J., Lee J. K., Jang W. Y., Song S. Y., Lee K. T., Paik S. W. and Rhee J. C. Prognostic significance of maspin in pancreatic ductal adenocarcinoma. Korean J Intern Med, 2004; 19: 15-18.
80. Murakami J., Asaumi J., Maki Y., Tsujigiwa H., Kuroda M., Nagai N., Yanagi Y., Inoue T., Kawasaki S., Tanaka N., Matsubara N. and Kishi K. Effects of demethylating agent 5-aza-2(')-deoxycytidine and histone deacetylase inhibitor FR901228 on maspin gene expression in oral cancer cell lines. Oral Oncol, 2004; 40: 597-603.
81. Primeau M., Gagnon J. and Momparler R. L. Synergistic antineoplastic action of DNA methylation inhibitor 5-AZA-2'-deoxycytidine and histone deacetylase inhibitor depsipeptide on human breast carcinoma cells. Int J Cancer, 2003;103: 177-184.
82. Klus G. T., Rokaeus N., Bittner M. L., Chen Y., Korz D. M., Sukumar S., Schick A. and Szallasi Z. Down-regulation of the desmosomal cadherin desmocollin 3 in human breast cancer. Int J Oncol, 2001; 19: 169-174.
83. De Bruin A., Muller E., Wurm S., Caldelari R., Wyder M., Wheelock M. J. and Suter M. M. Loss of invasiveness in squamous cell carcinoma cells overexpressing desmosomal cadherins. Cell Adhes Commun, 1999, 7: 13-28.
84. Chidgey M. A. Desmosomes and disease. Histol Histopathol, 1997; 12: 1159-1168..
85. Tselepis C., Chidgey M., North A. and Garrod D. Desmosomal adhesion inhibits invasive behavior. Proc Natl Acad Sci U S A, 1998; 95: 8064-8069.

86. Mechanic S., Raynor K., Hill J. E. and Cowin P. Desmocollins form a distinct subset of the cadherin family of cell adhesion molecules. Proc Natl Acad Sci U S A, 1991; 88: 4476-4480.
87. Zardo G., Tiirikainen M. I., Hong C., Misra A., Feuerstein B. G., Volik S., Collins C. C., Lamborn K. R., Bollen A., Pinkel D., Albertson D. G. and Costello J. F. Integrated genomic and epigenomic analyses pinpoint biallelic gene inactivation in tumors. Nat Genet, 2002; 32: 453-458.
88. Costello J. F., Fruhwald M. C., Smiraglia D. J., Rush L. J., Robertson G. P., Gao X., Wright F. A., Feramisco J. D., Peltomaki P., Lang J. C., Schuller D. E. Yu L., Bloomfield C. D., Caligiuri M. A., Yates A., Nishikawa R., Su Huang H., Petrelli N. J., Zhang X., O'Dorisio M. S., Held W. A., Cavenee W. K. and Plass C. Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. Nat Genet, 2000; 24: 132-138.
89. Strathdee G., Davies B. R., Vass J. K., Siddiqui N. and Brown R. Cell type-specific methylation of an intronic CpG island controls expression of the MCJ gene. Carcinogenesis, 2004; 25: 693-701.
90. Strathdee G., Sim A. and Brown R. Control of gene expression by CpG island methylation in normal cells. Biochem Soc Trans, 2004; 32: 913-915.

Chapter 7

EPIGENETIC REGULATION OF THE E-CADHERIN CELL-CELL ADHESION GENE

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Abstract: Inactivation or loss of function of E-cadherin, the principal cell adhesion molecule in epithelial cells, is thought to be an important step in tumour progression and metastasis. In recent years, efforts have been made to understand how *E-cadherin* expression and function is regulated during these processes. Several mechanisms have been shown to be involved in the regulation of *E-cadherin* expression, including genetic, epigenetic and transcriptional changes. However, the complete picture of how this molecule is regulated still remains to be fully elucidated. As our understanding of how epigenetic mechanisms influence the control of gene expression expands it becomes clear that the epigenetic modification of genes involved in metastasis could influence the acquisition of malignant cell behaviour. In this chapter, we will focus our attention on the epigenetic control of the *E-cadherin* gene and discuss how this might be integrated with the known transcriptional repressors of E-cadherin. Understanding the epigenetic control of E-cadherin may help to identify new targets for drug design to block the metastatic process, the most aggressive and lethal consequence of tumour progression.

Key words: E-cadherin, epigenetic, cancer, methylation, transcriptional repression

1. WHY E-CADHERIN? AN OVERVIEW

Cancer is a disease known to involve many different steps. A wide range of molecules have been implicated in this complex disorder, and the way in which they are regulated is only just beginning to be understood. During the last 25 years, much scientific effort has been devoted to elucidate the

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common mechanisms that underlie cancer. More recently, significant advances have been made in elucidating aspects of the metastatic process. Metastasis has been related to a poor prognosis and lethality in cancer patients. Although the metastatic cascade may vary between different types of cancer, for most carcinomas the cellular and molecular mechanisms do seem to be similar (1).

The metastatic process involves a complicated deregulation of cell adhesion, extracellular matrix integrity, survival, angiogenesis, lymphangiogenesis and cell migration (2). One of the most relevant changes that occurs during this metastatic process in a large number of carcinomas is the so-called epithelial mesenchymal transition (EMT) (3). EMT involves the conversion of an epithelial cell into a mesenchymal cell characterised by a more motile, invasive and aggressive phenotype. These changes allow some tumour cells to migrate through the extracellular matrix (ECM) and colonize lymph/blood vessels in the first steps of the metastasic process.

The mechanisms that govern the process of EMT are slowly being unravelled. In particular, developmental studies have been helpful in showing that EMT during tumour progression is conceptually similar to that which takes place at defined stages of embryonic development (4, 5). In the last few years, great advances have been made in understanding this EMT process and several critical molecules have been identified. One of the key molecules in the process of EMT is E-cadherin, since the transition of phenotype commonly involves the down-regulation of E-*cadherin* (6). During development, expression of E-cadherin is maintained in all epithelial tissues, but it is silenced during the process of EMT and in established mesenchymal cells (7, 8). Indeed, loss of function studies (using functional antibodies, antisense nucleotides or transgenic knock-out mice for *E-cadherin*) have demonstrated that E-cadherin is crucial for early mouse development and the maintenance of epithelial morphology (9).

The loss of *E-cadherin* expression and/or function has been observed during the progress of most carcinomas (reviewed in 10, 11), and this has been related to the induction of EMT that frequently occurs during carcinoma invasion (3, 12). These observations, together with data gathered from other model systems, support the original idea that *E-cadherin* can be considered as an invasion suppressor gene (10). Indeed, the anti-invasive role of E-cadherin in carcinoma progression has prompted interest in understanding the mechanisms that control *E-cadherin* expression under normal and pathological circumstances.

2. REGULATION OF E-CADHERIN

During tumour progression, *E-cadherin* can be inactivated functionally by different mechanisms, including somatic mutation and the down-regulation of gene expression, or through promoter methylation and/or transcriptional repression. In this chapter we will focus our attention on the regulation of *E-cadherin* by epigenetic mechanisms. This type of regulation has historically been considered as DNA methylation of the *E-cadherin* promoter by methyltransferases (DNMTs). However, during recent years a link has started to appear between genomic inactivation, transcriptional repression and epigenetic regulation of E-cadherin during tumour progression. Thus, it is becoming increasingly clear that the regulation of E-cadherin during malignant progression is a complex mechanism in which multiple regulatory events are implicated.

2.1 Genetic Regulation

To understand the importance of the epigenetic regulation of *E-cadherin*, we must first establish what other mechanisms regulate *E-cadherin* expression, such as the genetic inactivation of *E-cadherin* during tumour progression. The human *E-cadherin* gene (*CDH1*) is located at the 16q22.1 locus and is comprised of 16 exons, spanning 99 Kb of genomic DNA (13). The mouse E-cadherin gene (*cdh1*) has a similar exon-intron organisation and is located on chromosome 8 (14). Despite the fact that the down-regulation of *E-cadherin* gene expression is observed in many carcinomas (10), inactivating mutations that may be frequent in diffuse gastric carcinomas and in lobular breast carcinomas, are rarely observed in other types of tumours (15, 16). The genetic alterations of *E-cadherin* in tumours generally involve exon-skipping and out-of-frame mutations, and in most cases mutations occur in combination with a loss of heterozygosity (LOH) of the wild type allele (15)

E-cadherin appears to be mutated in a large proportion of lobular breast tumours, in agreement with the frequent deletions in the 16q22.1 region observed in breast tumours. However, no mutations have been detected within the *E-cadherin* gene in breast ductal tumours (17-19). This is a fundamental piece of evidence for the molecular differences between these two types of breast cancer. Nevertheless, a reduced expression of *E-cadherin* has been described in both lobular and ductal breast cancer (20, 21). Another example of mutations that inactivate *E-cadherin* expression can be found in diffuse gastric carcinomas, where it is also common to observe *E-cadherin* gene mutations that are not associated with LOH (22-24).

The low number of tumours in which genetic inactivation of E-cadherin has been demonstrated, has led to the consideration that E-cadherin does not follow the classical two-hit model of tumour suppressor gene inactivation seen in many types of cancer. Moreover, it suggests that epigenetic control by methylation of the *E-cadherin* promoter or transcriptional repression could be the second hit, both in sporadic gastric carcinomas and in invasive ductal carcinomas (25-28).

2.2 Epigenetic Mechanisms of *E-cadherin* Silencing

In recent years, it has become evident that genomic regulation is not the only mechanism that governs anomalous gene expression during tumour progression. Epigenetic modifications, and in particular DNA methylation, seem to play a widespread role during carcinogenesis. Indeed, DNA methylation is the most widespread epigenetic modification studied in pathological processes. Anomalous hypermethylation of promoter CpG islands is an important means of repressing tumour suppressor genes. Such aberrant promoter methylation of tumour suppressor genes is associated with a loss of gene function that can provide selective growth advantages to neoplasic cells. This hypermethylation, coupled to sporadic genetic mutations, serves to define the heterogeneous biology of human neoplasms. DNA methylation is known to be involved in the development of human cancers, often characterised by a general hypomethylation of DNA and the local hypermethylation of CpG islands in the promoters and upstream exons of many genes (29). The hypermethylation of CpG islands is associated with the recruitment of methyl binding domain proteins (MBDs) and of histone deacetylase activity (HDACs), which together contribute to the compaction of the DNA in the promoter region and hence, to gene inactivation.

The initial studies on methylation of the *E-cadherin* promoter not only established a relationship between *E-cadherin* silencing and the methylation of CpG islands in several carcinoma cell lines, but also showed that treatment with the demethylating agent 5´-aza-2'-deoxycytidine (5´-aza) re-activated *E-cadherin* expression (30). Furthermore, methylation of the *E-cadherin* promoter was observed in primary prostate and breast tumour lesions, in contrast to normal adjacent tissues where it remained unmethylated. Shortly afterwards, endogenous transcriptional repression was correlated with *E-cadherin* promoter methylation for the first time (31). Since these two pioneering studies, a large amount of data regarding *E-cadherin* promoter methylation and tumour progression has been accumulated from a huge number of cell lines and tumours (16).

In this section we will review the hypermethylation of *E-cadherin* and its relationship to other inactivating mechanisms in selected tumour types.

2.2.1 Gastric Cancer

As commented above, hypermethylation of the *E-cadherin* promoter region may act as the second hit for E-cadherin silencing in diffuse gastric cancer. Hypermethylation of the *E-cadherin* promoter region is a frequent phenomenon in poorly differentiated, diffuse gastric carcinomas and it was identified as the main mechanism to inactivate the remaining wild-type allele in those carcinomas (32). Indeed, epigenetic inactivation of *E-cadherin* via promoter hypermethylation may be a critical early event in the development of undifferentiated tumours and significantly, it is associated with a worse prognosis (28, 33, 34). Interestingly, some analytical approaches have focused on the detection of methylation during gastric carcinogenesis. This reflects the fact that aberrant *E-cadherin* promoter methylation can be detected in serum from a substantial proportion of gastric cancer patients (35, 36). In this context, the methylation status of the *E-cadherin* promoter may also proved prognostic information for the early recurrence and survival of other pathological gastric cancers such as gastrointestinal stromal tumours (37). However, other unknown mechanisms of *E-cadherin* suppression could be involved in some diffuse gastric carcinomas, as patients have been identified in whom *E-cadherin* is down-regulated without evidence of mutations or *E-cadherin* promoter methylation (38). Interestingly, the loss of β-catenin expression in metastatic gastric cancers has recently been reported that may result from hypermethylation of the *β-catenin* promoter (39). This is undoubtedly the first suggestion of an epigenetic modification of this cadherin-associated protein in gastric cancer.

2.2.2 Breast Cancer

Another example where *E-cadherin* promoter hypermethylation may serve as the second hit during tumour progression is breast ductal carcinomas (26, 40). Indeed, methylation of the *E-cadherin* promoter has been correlated with the loss of *E-cadherin* expression in breast cancer cell lines, as well as primary ductal and lobular breast cancers (ILC) (41, 42), while it is unmethylated in normal breast tissue (31). However, the decrease of *E-cadherin* expression is not always attributable to hypermethylation, since treatment with 5′-aza fails to reactivate *E-cadherin* expression in several breast carcinoma cell lines (43). These exceptions suggest that alternative mechanisms for *E-cadherin* inactivation, such as transcriptional repression, could also be relevant in breast tumours, as will be addressed below.

Among the additional mechanisms that regulate *E-cadherin* expression in breast cancer, a single nucleotide polymorphism (SNP) at the -160 site of the

E-cadherin promoter has recently been shown to affect *E-cadherin* transcription *in vitro* (44). However, this SNP might act in concert with another unknown mechanisms to inactivate *E-cadherin* when the *CDH1* gene is not mutated (45). It has been also suggested that the loss of *E-cadherin* in breast cancer might act in concert with alterations in the adenomatous polyposis coli (APC)/β-catenin pathway to enhance oncogenic β-catenin signalling (46, 47). Indeed, some *in vitro* studies of breast cancer cell lines have shown that a decrease in E-cadherin levels can augment β-catenin oncogenic signalling (48). However, the analysis of this signalling pathway *in vitro* and in tumours revealed that alterations in the *E-cadherin* and *APC* genes do not always promote the nuclear accumulation of β-catenin in lobular tumours. This would imply that inactivation of the E-cadherin and APC proteins in ILC may have additional functional consequences, as well as affecting the oncogenic activity of β-catenin (45, 49). All these data suggest that genetic and epigenetic changes in genes other than *E-cadherin*, such as *APC*, could be implicated in the development of some lobular and ductal carcinomas. This is especially relevant when considering that alterations in APC are also frequent in breast cancer (50, 51).

2.2.3 Oesophageal Carcinomas

Advanced stages of nasopharyngeal carcinoma are also associated with reduced expression of E-cadherin and its undercoat protein β-catenin (52). The same phenomenon has been reported for oesophageal cancers (53, 54) where the loss of E-cadherin was associated with methylation of 5′ CpG islands in the *E-cadherin* promoter (55). Recently, a correlation between epigenetic modification and transcriptional repression of *E-cadherin* by the Snail transcription factor has been reported. Although Snail overexpression was unrelated to clinical and pathological factors, it reduced *E-cadherin* expression which was correlated with tumour and vascular invasion (56). However, there are still some issues that remain to be resolved when making a connection between *E-cadherin* promoter hypermethylation and oesophageal carcinomas (57-59).

2.2.4 Prostate and Bladder Cancer

The epigenetic regulation of *E-cadherin* in prostate cancer was initially investigated by Graff *et al*. (31). More recent studies have shown that methylation mainly occurs in the exon region in low-grade prostate tumours, whereas in high-grade tumours methylation can also be seen in the promoter region. Treatment with 5′-aza, restores *E-cadherin* mRNA levels in E-cadherin negative prostate cancer cell lines (60). The loss of E-cadherin in

this pathology appears to be so relevant that it has been used as a clinical and pathological marker for this disease (61). However, in several types of prostate cancer such as adenocarcinomas, *E-cadherin* gene promoter methylation is a common event but it does not appear to have any prognostic significance. Instead, decreased expression of several components of the E-cadherin/catenin complex seems to be associated with more aggressive phenotypes (62).

In bladder cancer, CpG hypermethylation is also related to *E-cadherin* gene inactivation and in bladder cancer cell lines, expression of the *E-cadherin* gene was restored after treatment with 5′-aza (63). Recently, the methylation of the *E-cadherin* gene promoter has been strongly associated with transitional cell carcinoma in situ (64), suggesting that this epigenetic mechanism may be implicated in bladder cancer. In this type of cancer, a relationship between *E-cadherin* down-regulation and constitutive Wnt/β-catenin signalling appears unlikely. However, in many urothelial cancers, decreased *E-cadherin* expression may provoke inappropriate responses to Wnt factors and is often a consequence of promoter hypermethylation (65).

2.2.5 Colorectal Cancer

The loss of *E-cadherin* expression has been documented in colorectal cancer (CC), further evidence of the widespread deregulation of cell-cell and cell-matrix adhesion molecules (66-68). Indeed more recently, allelic loss at the *E-cadherin* locus and inactivating mutations of *E-cadherin* have been implicated, albeit rarely, in human colorectal cell lines (69). The correlation of *E-cadherin* promoter hypermethylation with reduced or silent expression of this molecule was first established in ulcerative colitis associated colorectal cancer (70). Subsequently, several studies reported a similar relationship in other colorectal pathologies such as primary sporadic carcinomas (71), poorly differentiated adenocarcinomas and mucinous carcinoma of the colon (72, 73). In this latter case, epigenetic modification of *E-cadherin* is associated with lymph node metastasis and could contribute to tumourogenesis together with LOH. Interestingly, E-cadherin has been considered as a precancerous marker in CC, supporting the idea that inactivation of some tumour suppressor genes through aberrant promoter methylation may play a role in the development of colorectal tumours (74).

Irrespective of the potential involvement of alterations in E-cadherin in colorectal tumours, other molecules related to E-cadherin play a crucial role in CC, particularly β-catenin and some regulators of this protein (75). In fact, both decreased *β-catenin* and *E-cadherin* gene expression have been associated with the presence of lymph node metastases of colorectal carcinomas (76). The epigenetic control of β-catenin-related genes might be

also important since aberrant methylation of the *APC* and *HPP1(hyperplasic polyp protein 1)* genes has also been reported in colorectal adenomas (77, 78).

It is also noteworthy that epigenetic modification of other cadherin genes has also been implicated in CC. For example, *CDH13* (*H-cadherin, T-cadherin*) is frequently silenced by aberrant methylation in colorectal cancers and adenomas. Hence, it has been suggested that methylation of *CDH13* represents an early stage of multistep colorectal tumorogenesis, and that *CDH13* might also function as a tumour suppressor gene (79, 80).

2.2.6 Lung Cancer

Epigenetic control of gene expression has been associated with this pulmonary pathology. With rare exceptions, no significant differences have been found between the gene methylation profiles of different types of lung cancers and their respective derived cell lines, indicating that cell lines provide a useful model to study methylation. In a recent comparison of the gene methylation profiles of lung squamous cell carcinomas and adenocarcinomas, $p16^{INK4a}$ was more frequently methylated in squamous cell carcinomas, whereas *APC* and *CDH13* methylation was more frequent in adenocarcinomas (81). Although methylation of *E-cadherin* has not historically been related with this type of cancer, some reports have suggested that *E-cadherin* promoter methylation may have prognostic value for the outcome of patients with non-small cell lung cancer (82). Nevertheless, the prototypic cadherin gene that is methylated in lung cancer is *CDH13*, at times correlated with LOH, indicating that this molecule could be important in the genesis of lung cancer (83, 84).

2.2.7 Hepatocellular Carcinoma

Initial studies on *E-cadherin* methylation in hepatocellular carcinomas (HCC) suggested that CpG methylation of the *E-cadherin* promoter might be involved in the early pathological stages of this disease (85, 86). Aberrant DNA methylation in this chromosome region seems to participate in the precancerous stage of hepatocarcinogenesis, preceding or causing LOH (87). A more detailed study of *E-cadherin* expression in these carcinomas has detected promoter methylation in a small tumour cell population in early stages of HCC, whereas LOH was frequently found in more malignant tumours. This suggests that the down-regulation of *E-cadherin* is closely related to the progression of HCCs (88). Interestingly, in conjunction with other oncogenic signals such as c-myc, epigenetic modification of *E-*

cadherin seems to play a role in carcinogenesis without LOH arising at the *E-cadherin* locus (89).

2.2.8 Kidney Cancer

DNA methylation is known to occur in kidney tumorogenesis. For example, the *von Hippel-Lindau (VHL)* gene is inactivated by hypermethylation in a subset of clear cell renal cancers, with $p16^{INK4a}$ and *RAS-association domain family 1A (RASSF1A)* may be hypermethylated in clear cell and other histological renal cancer subtypes. In general terms, these data indicate that methylation of tumour suppressor genes might be a relatively early event in kidney tumorogenesis (90). With regards E-cadherin, there is only one report that correlates methylation of the *E-cadherin* gene promoter in renal cancer cell lines with loss of *E-cadherin* protein. Moreover, treatment of these renal cancer cells with 5´-aza restored *E-cadherin* expression in all the lines, indicating a potential role for *E-cadherin* inactivation in renal cell carcinomas (91). Additional studies will be necessary to clarify the potential involvement of E-cadherin methylation in this pathology.

2.2.9 Cervix and Endometrial Cancer

Hypermethylation of *E-cadherin* has recently been invoked to explain E-cadherin protein loss in cervical cancer cell lines and cervical cancer tissues. Interestingly, in this type of cancer *E-cadherin* methylation was correlated with a significant increase in the DNMT1 protein, and blocking *DNMT1* expression with antisense oligonucleotides led to E-cadherin protein re-expression (92). These data suggest that inactivation of the *E-cadherin* gene by promoter methylation plays an important role in cervical cancer tumourogenesis. However, no decrease in E-cadherin protein expression could be detected by immunohistochemistry in pretumour lesions, indicating that the methylation of *E-cadherin* gene could be a late event in the development of cervical cancer (93). Interestingly, it has recently been suggested that *E-cadherin/CDH13* methylation could be a prognostic marker for cervical cancer. Patients with unmethylated *E-cadherin/CDH13* in serum showed significantly better disease-free survival (94). With respect to endometrial cancer, there is only a report suggesting that methylation of the *E-cadherin* gene is associated with the acquisition of invasive properties (95). Nevertheless, alterations of molecules in the APC/β-catenin pathway are common in endometrial cancer (96, 97).

2.2.10 Skin Cancer

E-cadherin methylation has also been observed in skin cancer. Promoter hypermethylation of the *E-cadherin* gene was correlated with the invasive behaviour of certain types of skin cancer. Consequently, the frequency of *E-cadherin* promoter hypermethylation appears to be correlated with more advanced stages of squamous carcinogenesis in skin, and to a lesser extent in actinic keratosis. Interestingly, some methylation of *E-cadherin* has also been found in non-neoplasic skin, indicating the potential use of this epigenetic modification as a predictive marker (98). Recently, an analysis of *E-cadherin* methylation in epidermal keratinocyte cell lines derived from a multistage mouse skin cancer model of carcinogenesis has revealed that *E-cadherin* promoter methylation occurs concomitantly with the loss of *E-cadherin* expression and the acquisition of a mesenchymal phenotype. Interestingly, the methylation and expression patterns of the *E-cadherin* repressor *Snail* are completely inverted in this model. The methylated genes in mouse skin carcinogenesis were also hypermethylated in human cancer cell lines and primary tumours, underlining the value of this murine model of skin carcinogenesis to study aberrant DNA methylation events in cancer cells (99).

The analysis of adhesion molecules implicated in skin cancer has identified the deletion and aberrant methylation of the *CDH13* gene in relation to the loss of this molecule in other cutaneous cancers during malignant transformation (e.g., melanoma). Moreover, *CDH13* expression was restored in melanoma cell lines by treatment with 5'-aza, indicating a potential role of this molecule in the evolution of invasive melanomas (100). Down-regulation of *CDH13* in cutaneous cancers might also be related to tumour invasiveness rather than metastasis, because it is also found in basal cell carcinoma of the skin that normally lacks metastatic activity (101).

2.2.11 Oral Cancer

The relationship between *E-cadherin* methylation and oral cancer was initially analysed in oral squamous cell carcinomas (OSCC). It was demonstrated that acquisition of the invasive phenotype is a consequence of reduced expression of *E-cadherin* in the tumour due to CpG methylation of the gene's promoter (102). Significantly, *E-cadherin* down-regulation through methylation was clearly correlated with the metastatic potential in cell lines derived from OSCC. Moreover, demethylation of CpG islands in the *E-cadherin* gene could restore the expression and function of E-cadherin (103). Further studies demonstrated a correlation between *E-cadherin* methylation and poor prognosis in both primary oral tongue carcinomas and

their corresponding recurrence and nodal metastases (104). Since loss of *E-cadherin* is often concomitant with the upregulation of *N-cadherin* and the acquisition of an invasive phenotype by a phenomenon known as "cadherin switch" (See, 6, 105), it has been proposed that this dynamic process could be regulated by epigenetic modification. Indeed, methylation of 5' CpG islands in the *E-cadherin* promoter promotes its down-regulation while upregulating *N-cadherin* in OSCC through an unknown mechanism. Interestingly, treatment with a 5′-aza showed that the re-expression of *E-cadherin* is paralleled by a decrease in *N-cadherin* expression (106). This data indicates that cadherin switching and tumour progression may be controlled by an epigenetic modification. On the other hand, reduced expression of membranous β-catenin was also found frequently in invasive and metastatic areas of OSCC (107). As a result, this molecule, coupled with *E-cadherin* loss, was proposed as a potential predictive marker for this type of cancer, although further studies must be performed to corroborate this.

2.2.12 Leukaemia and Related Cancers

The role of *E-cadherin* expression in haematological malignancies is beginning to be unravelled. The analysis of samples from leukaemia patients has revealed abnormal hypermethylation of the *E-cadherin* CpG islands. In fact, both alleles of the *E-cadherin* gene were often hypermethylated in this pathology (108). Aberrant methylation of *E-cadherin* was also observed in leukaemia cell lines, acute myelogenous leukaemias and acute lymphoblastic leukaemias. The treatment of leukaemia cell lines with 5′-aza led to the expression of both E-cadherin transcripts and protein, suggesting an involvement of *E-cadherin* down-regulation in leukaemogenesis (109, 110). A role for *E-cadherin* methylation in other similar malignancies such as myeloma and monoclonal gammopathies has also been recently reported, although additional data will be needed to clarify this correlation (111, 112). Finally, silencing of *CDH13* expression by aberrant promoter methylation has also been demonstrated at early stages of chronic myeloid leukaemia and this probably influences the clinical behaviour of the disease (113).

Bearing in mind all this data, it is comprehensible that *E-cadherin* has been considered as one of the 11 candidate tumour suppressor genes to be routinely analysed in human tumours. In several types of cancer, the implication of *E-cadherin* methylation in tumourogenesis has been analysed in whole methylation screenings, together with $p16^{INK4a}$, *APC, RASSF1A, p73,* $p14^{ARF}$, *glutathione-S-transferase-pi* (*GST-pi*), *O(6)-methylguanine DNA methyltransferase (O6-MGMT)*, *human MutL homologue 1 (hMLH1), retinoid acid receptor beta (RAR-beta) and tissue inhibitor of metalloproteinase 3 (TIMP3)*. These data indicate the potential role of *E-*

cadherin epigenetic modification as a predictive marker in multiple cancer types, although further investigation should be performed to establish this correlationship.

2.3 Linking Transcriptional Repression to Epigenetic E-cadherin Silencing

2.3.1 Transcriptional Regulation of *E-cadherin*

Besides the regulation of *E-cadherin* by promoter hypermethylation and/or genetic alterations, in recent years direct transcriptional control of *E-cadherin* has emerged as an important means to regulate E-cadherin expression. The mouse *E-cadherin* promoter was initially characterised after its isolation in 1991. It was shown to be a TATA-less promoter containing several potential proximal regulatory elements, including: a CCAAT box (-65); a GC-rich region (-30 to -58); and a palindromic element (-70 to –90) composed of two adjacent E-boxes and flanked by four inverted nucleotides called 'E-pal' (114). The proximal CCAAT and GC-rich regions are required for basal *E-cadherin* expression and are recognised by CAAT-binding proteins and by constitutive AP2 and Sp1 transcription factors, respectively (114-116). The E-pal element, initially described as an epithelial-specific regulator (114), was subsequently demonstrated to act as an active repressor in E-cadherin deficient cells (115-117). Additional regulatory elements include a region proximal to the E-pal element with Ets-binding sites (117), and an epithelial specific enhancer in the first intron (116).

A comparison of the human, mouse and dog *E-cadherin* promoters revealed the conservation of the CAAT-box and GC-rich regions (118). The proximal E-box of the mouse E-pal element is also conserved in the human and dog promoters (E-box 1), and an additional E-box (E-box 3) has been found at -30. However, a further E-box (E-box 4) located downstream of the transcription initiation site in the human promoter is not present in the mouse promoter (119).

Several studies have demonstrated that the E-boxes in the proximal *E-cadherin* promoter repress its expression. Point mutations abolishing the two E-boxes in the mouse E-pal, or E-box 1 and 3 in the human promoter, produce a strong induction of *E-cadherin* promoter activity in different carcinoma cell lines deficient in E-cadherin expression. Similarly, there is evidence that the E-box 4 also represses *E-cadherin* expression (119, 120). *In vivo* footprinting analysis of the endogenous human and mouse *E-cadherin* promoters indicates that factors capable of binding to the E-boxes exist in E-cadherin deficient cells (116, 117). An important concept derived

from such studies was that the repressors that bind to the proximal E-boxes are able to overcome the positive effects of constitutive factors interacting with the basal regulatory elements of the *E-cadherin* promoter, such as AP2 and Sp1 (117).

2.3.2 A Model for Transcriptional Repression

A major breakthrough in understanding how *E-cadherin* transcription is regulated has been the identification of several *E-cadherin* repressors that bind to the E-boxes of the *E-cadherin* promoter in different systems (see Figure 1). These factors include the zinc finger factors Snail (119, 121) and Slug (120, 122), the bHLH factors, E47 (121, 123) and Twist (124), and factors of the homeodomain and zinc finger family, δEF1(Zeb1) and SIP1(Zeb2) (118, 125). These repressors silence *E-cadherin* by binding to the proximal E-boxes, although some differences are observed between the mouse and human promoters. The current evidence available suggests that at least two of the three proximal E-boxes in the mammalian *E-cadherin* promoter are functionally similar in terms of recruiting repressors, regardless of their relative location (6).

One important question that remains unresolved when considering the transcriptional repression of *E-cadherin* relates to the specific participation of these different repressors in the down-regulation of *E-cadherin* during tumour progression. Analysis of the expression of different repressors in several carcinoma cell lines has rendered some apparently contradictory results.

While Slug has been proposed as the main repressor of *E-cadherin* in breast carcinoma cell lines (120), Snail expression is directly associated to E-cadherin loss and invasiveness in primary ductal breast carcinomas (126, 127), and in hepatocarcinomas (128). Some studies have associated *E-cadherin* down-regulation with SIP-1 alone or in conjunction with Snail (118), while others found an inverse correlation between *E-cadherin* and δEF1 (associated with *Snail* expression) but not SIP1 (129). With regards to the bHLH factors, an inverse correlation has been reported between the expression of E12/E47 and *E-cadherin* in several carcinoma and melanoma cell lines (123). Furthermore, the expression of Twist has been related to the acquisition of metastatic properties (124).

Insights into the role of the different *E-cadherin* repressors have mainly been obtained from *in vitro* studies. A comparative analysis of the binding affinities for the E-pal element of *E-cadherin* repressors, Snail, Slug and E47, revealed that Snail binds with a higher affinity than the other two repressors (122). This indicates that a hierarchy might exist between the three repressors when present in the same biological context. An analysis of

the expression of the different repressors in tumour biopsies should clarify the specific participation of each factor in down-regulating *E-cadherin*. In this regard, a recent study of gastric carcinomas demonstrated an association between *E-cadherin* repression and *Snail* expression in diffuse gastric carcinomas. In intestinal type gastric carcinomas however, *SIP1* expression was linked to *E-cadherin* repression (130). While preliminary, these data suggest that the different repressors may participate specifically in silencing *E-cadherin* in different types of tumour or perhaps, at defined stages of tumour progression. A more detailed analysis of the expression of these repressors in large-scale tumour samples is clearly needed to study this hypothesis.

The process of EMT that occurs in pathological and physiological situations seem to be mechanistically similar (3, 131). Hence, important information about the possible participation of the different E-cadherin repressors in tumour progression can be obtained from developmental studies. Analysis of the expression patterns of these factors has provided important clues to clarify this issue. Snail family members are recognised as important regulators of EMT during early vertebrate development (131). Indeed, the expression pattern of *Snail* during mouse development is closely correlated with the down-regulation of *E-cadherin* expression in regions where EMT occurs. In this species, *Slug* expression is observed in the already migratory mesenchymal cells (121, 131). The pattern of *E2A* expression (encoding the E12/E47 factors) and of *E2-2* (encoding mITF2), is similar to that of *Slug* (123) (Holt, Sobrado and Cano, unpublished data). On the other hand, *Zfhx1b* (encoding SIP1) is expressed in the neuroepithelium and both the premigratory and migratory neural crest cells of specific regions, as well as in the branchial arch mesoderm (132).

In accordance with its pattern of expression, *Snail* null mice are early embryonic lethal. These embryos fail to complete gastrulation giving rise to a deficient mesodermal layer in which *E-cadherin* expression is maintained. In contrast, *Slug* null mice are viable and undergo an apparently normal program of mesoderm and neural crest cell development (131). The absence of mesoderm and neural crest defects has also been observed in null mice for the *E2A* or *E2-2A* gene. One explanation of these phenotypes lies in the potential redundancy of the class I bHLH factors encoded by both genes (E12/E47 and E2-2A/E2-2B, respectively). On the other hand, *Zfhx1b* knock-out mice are embryonic lethal at 9.5 dpc and exhibit several defects in neural crest migration or specification (132).

This information suggests that the different E-cadherin repressors establish a hierarchy during development. Thus we can envisage a prominent role for Snail in inducing EMT, a more restricted role for SIP1 in migration and specification of distinct neural crest cell populations, and a role for Slug

and E12/E47 (and E2-2 products) in the maintenance of the mesoderm. These developmental analyses strongly support the idea that the regulation of *E-cadherin* expression is similar during normal development and tumour progression (6, 121). Moreover, they support the specific and hierarchical participation of the different factors in the repression of *E-cadherin* during invasion.

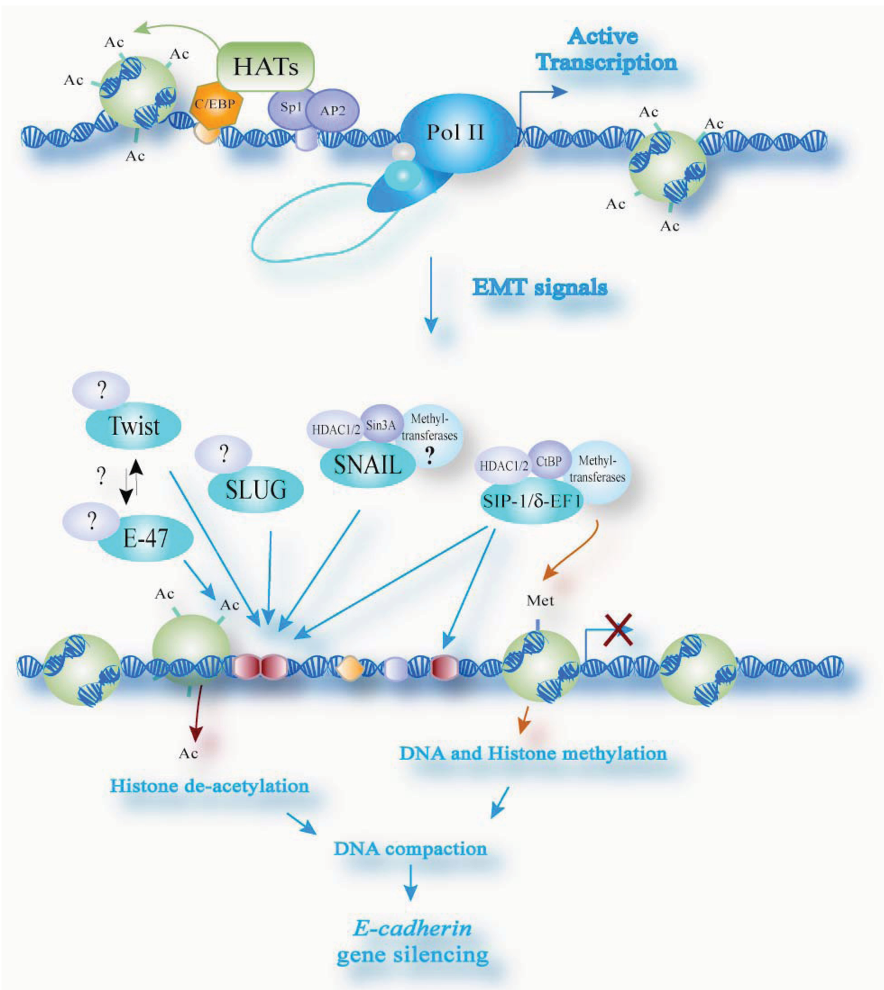

Figure 1. A model for the participation of the different E-cadherin repressors. The progression from an active to a silent methylated promoter seems to be linked to mechanisms of transcriptional repression. Snail recruits the mSin3a/HDAC1/2 complex, while ZEB-1/2 has been detected in the CtBP complex, containing several HDACs and DNMTs. In contrast, the mechanisms or molecules involved in the repression by other E-cadherin repressors, such as Slug, E47and Twist are not yet elucidated

Based on the available information we propose a model for the participation of the different *E-cadherin* repressors in tumour progression (see Figure 1). Thus, the transient expression of Snail (or SIP1) will be involved in inducing the invasion process. In contrast, other repressors, such as Slug, E47, Twist and perhaps δEF1, will be involved in the maintenance of the migratory invasive phenotype. Curiously, this model also fits with the relative binding affinities of some of the repressors (Snail, Slug and E47) determined *in vitro* for the regulatory E-boxes. It could also explain some of the apparent discrepancies found in the literature. Indeed, it may become apparent that the breast carcinoma cell lines used in different analyses represent distinct stages of tumour progression and/or invasiveness. Therefore, the expression patterns of the different repressors observed (such as Snail and Slug or SIP1) might simply reflect their distinct origins. In other words, we need to think of tumour invasion as a highly dynamic process, similar to EMT during development, whereas so far we have really only been looking at still images.

2.3.3 A Molecular Model for *E-cadherin* Methylation

As discussed in the first section of this chapter, *E-cadherin* promoter methylation is a frequent event in many carcinomas. However, the specific contribution of this epigenetic modification and more importantly, its relation to transcriptional repression in the silencing of *E-cadherin* has yet to be defined. Another important aspect to consider when analysing the participation of promoter methylation is the dynamic regulation of E-cadherin expression during tumour progression

Despite the loss of E-cadherin in primary carcinomas, E-cadherin is frequently re-expressed in secondary metastatic foci and even within lymph node metastases (133-135). This indicates that *E-cadherin* expression is dynamically regulated during tumour progression as well as during development (12, 136, 137), a fact that apparently contradicts the assumed irreversibility of DNA methylation. Analysis of *E-cadherin* methylation in breast carcinomas and in other tumour types has shown that aberrant hypermethylation of CpG islands in the promoter region of *E-cadherin* often occurs prior to invasion, indicating that it is an early event in tumorogenesis. Moreover, hypermethylation of the *E-cadherin* promoter seems to persist in invasive and metastatic lesions in breast carcinomas, but with a rather heterogeneous pattern between tumour cells. Additionally, it has been proposed that dynamic changes occur in *E-cadherin* methylation during tumour progression (138).

According to this model, tumour cells with predominantly methylated *E-cadherin* alleles would be responsible for the initiation of metastatic

behaviour. However, a decrease in methylation that would lead to the re-expression of *E-cadherin* might occur in the established metastatic lesions, and this could contribute to the survival of the cells in breast cancer. Indeed, re-expression of E-cadherin in cells within the blood stream and in secondary tissues, was suggested as a general means for cancer cells to survive (11).

As commented above, the classical "two hit hypothesis" for *E-cadherin* silencing has recently been proposed by Cheng *et al*., wherein genetic, epigenetic and transcriptional control of *E-cadherin* expression is co-ordinated in ductal breast cancer (127). In this system *Snail* expression is correlated with the silencing of *E-cadherin* and the hypermethylation of its promoter, suggesting a role for Snail in the co-ordination of both processes (26). Whether dynamic *E-cadherin* methylation also occurs in other tumour types, thereby explaining changes in E-cadherin expression during tumour progression, remains to be established. On the other hand, methylation of the *E-cadherin* promoter is not always correlated with *E-cadherin* silencing (16), indicating that additional genetic or epigenetic modifications might be required for *E-cadherin* down-regulation.

The molecular mechanisms involved in regulating *E-cadherin* promoter methylation are starting to be defined. Indeed, multiple molecular mechanisms could be involved in the epigenetic modification of the *E-cadherin* promoter. In general terms, DNA methylation and histone deacetylation are related to transcriptional repression although, the realtionship between these processes is not yet clear. HDAC activity may be linked to DNA methylation (139), but the mechanisms underlying the protection of unmethylated DNA against the action of DNMTs and HDACs remain largely unknown (140).

Two models have been suggested to link DNA methylation and histone deacetylation. The first model proposes that methyl binding domain proteins (MBDs) localise methylated CpG islands recruiting co-repressor complexes containing HDACs/DNMTs to specific promoters, thereby maintaining the repression. However, this process normally occurs when a promoter is already methylated and the MBDs maintain this methylation. Therefore these co-repressor complexes can be considered as regulators of silent/imprinted genes (141). In the second model, the machinery for transcriptional repression links local histone deacetylation with DNA methylation to repress and silence gene expression. This process seems to be more dynamic, implying the need for multiple molecules and as a consequence, being subject to many means of regulation.

While both models are compatible with the regulation of *E-cadherin* expression during malignant progression, we favour the second one where silencing can be initiated by transcriptional repression. Subsequently, this

can lead to promoter methylation and ultimately to gene silencing. The dynamic expression of E-cadherin observed during tumour progression implies that the *E-cadherin* promoter is active as long as tumour cells maintain their epithelial phenotype. However, during EMT and with the acquisition of a mesenchymal phenotype, molecular modification of *E-cadherin* most probably occurs to induce transcriptional repression and promoter methylation. Interestingly, re-expression of *E-cadherin* has been observed in the converse process of EMT, Mesenchymal-Epithelial Transition (MET), which occurs either during development and tumour progression (3). This further supports the involvement of dynamic regulatory mechanisms in the control of E-cadherin expression.

The progression from an active to a silent methylated promoter and *vice versa* seems to be linked to mechanisms of transcriptional repression. Indeed, support for this model is becoming available and several co-repressor complexes have been recently described that can be recruited to the *E-cadherin* promoter by different repressors (see Figure 1). Snail recruits the mSin3a/HDAC1/2 complex (142), while δEF1/SIP1 has been detected in the CtBP complex, containing several HDACs and DNMTs (143). This data supports a link between transcriptional repression and epigenetic modification.

In this final part of this chapter, we will focus our attention on the molecular mechanisms that regulate the E-cadherin promoter during EMT, paying particular attention to the molecules involved in E-cadherin repression and methylation. To date, there is no clear model for epigenetic regulation of E-cadherin expression during tumour progression. Thus, we will try to describe in a step-wise manner, what we consider might be occurring "epigenetically" at the E-cadherin promoter during EMT and tumour progression until gene silencing is finally achieved (see model in Figure 2).

- First act: local histone de-acetylation. We will first assume that the *E-cadherin* promoter is in an active state in an epithelial cell line (Figure 2a). It is commonly accepted that the acetylation of specific lysine residues at the N-terminal domain of histones H3 and H4 is correlated with gene transcription. In contrast, histone de-acetylation is associated with transcriptional silencing (144). Several reports have suggested that transcriptional repressor complexes could promote the de-acetylation of the *E-cadherin* promoter to repress transcription (142, 143). In our model, this could be the first step in the loss of *E-cadherin* expression and possibly as a consequence of EMT (Figure 2b).

The isolation of a CtBP co-repressor complex was the first evidence of an association between transcriptional repression and co-repressor complexes in

E-cadherin regulation. This co-repressor complex contained different methylases and HDAC activities, as well as the transcription factors δEF1 and SIP1 (143). These factors have been described as *E-cadherin* repressors (120, 125) and are known to be active in regulating the *E-cadherin* promoter (143). Hence, these CtBP complexes may participate in the down-regulation of *E-cadherin*. However, it still remains to be established whether the δEF1 and SIP1 factors do indeed play a direct or indirect role in the CtBP repressor complex.

Very recently, details of the mechanisms that underlie the transcriptional repression of *E-cadherin* by Snail were revealed. This has also provided a link between transcriptional control and epigenetic modifications of the *E-cadherin* promoter (142). The repression of the *E-cadherin* promoter by Snail involves the recruitment of a repressor complex formed by the co-repressor mSin3A, HDAC1 and HDAC2 (142). The formation of this complex is mediated by the N-terminal SNAG domain of Snail, previously thought to act as the repressor domain (103, 119, 145). The presence of this complex results in a net decrease in the amount of acetylated H3/H4 histone in the E-cadherin promoter and of H3 lysine 9 methylation, that initiates the process of chromatin compaction (142). Regarding other known repressors, the molecular mechanism responsible for Slug repression remains to be established. Nevertheless, it is worth bearing in mind that apart from the N-terminal SNAG domain, Slug contains a partially conserved domain that interacts with the CtBP co-repressor (145). This raises the possibility that Slug co-operates with different repressor complexes to Snail. Indeed, the association of Slug with CtBP1/2 corepressors and HDAC1/3 has been observed in binding assays (Bolós and Cano, unpublished data), although the functional relevance of these complexes remains to be established. The mechanisms involved in E47-mediated repression of *E-cadherin* are still unknown. In contrast to Snail and Slug, there is not evidence that this repression involves HDAC activity. Rather, the information available points to a regulatory mechanism involving heterodimerisation with either the class II bHLH factors, such as Twist, or Id regulators (E. Cubillo, H. Peinado, and A. Cano, unpublished data).

Current evidence indicates that the initial silencing of *E-cadherin* transcription requires the participation of several repressor factors (Snail, Slug, E-47, δEF1 and SIP1), which interact with specific E-boxes in the proximal promoter. The repression of gene expression may involve the local re-organisation of chromatin through the recruitment of specific co-repressor complexes (such as CtBP and mSin3A). Alternatively, it may require the heterodimerisation of repressors with specific partners. These mechanisms of repression add an additional level of complexity to our understanding of the different factors that specifically repress *E-cadherin* expression. Thus, as

well as the specific expression patterns and/or binding affinities for similar regulatory elements, repressor function might finally be regulated by the presence of and/or affinity for different co-repressors in any specific cell or tissue. In this sense, a recent study has elegantly shown that silencing of *Snail* expression can be triggered by the oestrogen receptor in breast cancer cell lines. This phenomenon involves the specific participation of the MTA3 protein in an additional repressor complex (146). The expression of *Snail* is de-repressed by a deficiency in oestrogen receptors or in the MTA3 protein, producing the suppression of *E-cadherin* expression (146).

- Act Two: local histone and DNA methylation (Figure 2b)**.** The connection between transcriptional repression, histone de-acetylation and DNA methylation is not so obvious. One key point in this process could be histone lysine methylation, since this kind of modification has emerged as an epigenetic marker to model chromatin structure and function. Histone H3 lysine 9 methylation (methH3K9) is functionally linked with transcriptional repression, while H3 lysine 4 methylation (methH3K4) is associated with active transcription (147). These modifications are carried out by histone methyltransferases (HMTs) that usually form complexes with repressors and HDACs. Hence, the initial promoter de-acetylation can lead to the concomitant methylation of histone at specific residues as an epigenetic "mark" to final DNA methylation.

The correlation of histone deacetylation and methylation in *E-cadherin* repression was firstly observed by Shi *et al*. (143). These authors confirmed that methylation of H3-Lys 9 mediated by the CtBP complex was preceded by histone deacetylation. Interestingly, they demonstrated that treatment of cells with the HDAC inhibitor, trichostatin A, augmented acetylation and produced a concomitant decrease in the methylation at Lys 9 of histone H3 in the *E-cadherin* promoter, suggesting a link between both processes. In fact, the CtBP co-repressor complex contains associated HDACs and HMTs (G9a and EuHMTase1 proteins) that could potentially be implicated in Lys 9 histone H3 methylation in the *E-cadherin* promoter (143). A specific increase in methylated K9 of histone H3 in the endogenous *E-cadherin* promoter has also been observed following histone de-acetylation and transcriptional repression by Snail (142). Moreover, it has also been shown that histone H3 lysine 9 was methylated in the *E-cadherin* promoter of *E-cadherin* deficient cells (148). In these cells, the recruitment of different MBDs suggests a potential role for this modification in *E-cadherin* repression and silencing.

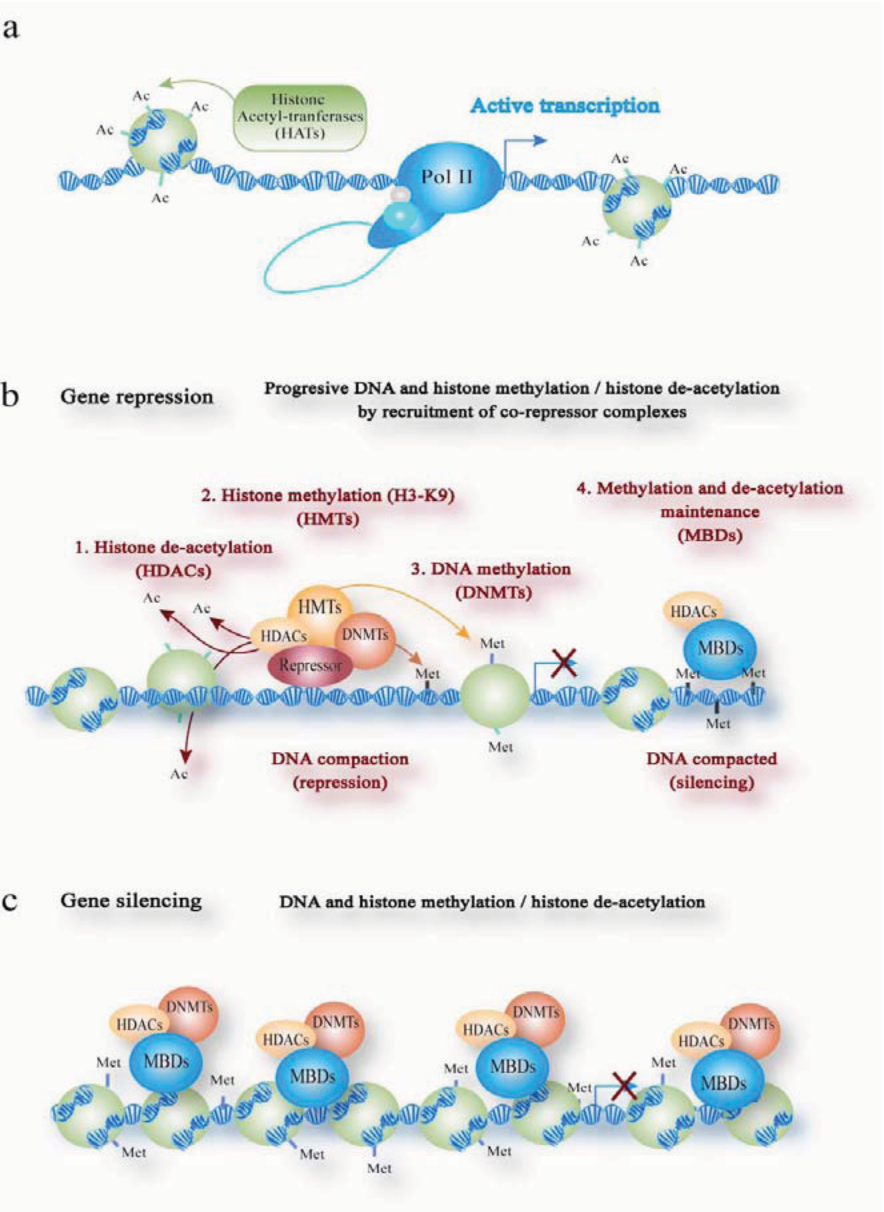

Figure 2. Proposed model for E-cadherin repression and silencing In this model, transcriptional repression is achieved through signals that attract transcriptional repressors to the E-cadherin promoter, and co-repressor complexes with HDACs, HMTs and DNMTs, leading to the initial de-acetylation and histone/DNA) methylation of promoter sequences. These changes may contribute to a local increase in DNA methylation that ends with recognition of MBD proteins, which promote and maintain E-cadherin repression, ultimately leading to gene silencing.

A link appears to exist between histone de-acetylation and histone/DNA methylation. DNMTs are responsible for methylation of CpG dinucleotides in the cell (149). Recent observations suggest that DNMTs are also involved in transcriptional regulation through the formation of complexes with HDACs, HMTs and MBDs. However, the molecular mechanism that regulate these complexes are not well understood (150). There is direct and indirect evidence in the literature to suggest that DNMTs could be responsible for methylation of the *E-cadherin* promoter in *E-cadherin* deficient cells. It has been shown that the DNA methyltransferase inhibitor, 5′-aza, can restore *E-cadherin* promoter activity and protein expression in multiple situations and cancer cell lines. Moreover, the overexpression of DNMTs leads to de novo methylation of *E-cadherin* CpG islands (151) and *E-cadherin* methylation is correlated with increased DNMT1 activity in cervical, breast and gastric cancer cell lines and tumours (92, 152, 153). Interestingly, it has recently been demonstrated that, besides histone deacetylation and K9-Histone 3 methylation, DNMT1 and DNMT3b are necessary for transcriptional silencing of the *E-cadherin* gene by DNA methylation (154).

In summary, the relationship between histone and DNA methylation in *E-cadherin* silencing is beginning to be unravelled. HMTs (G9a and EuHMTase1) and DNMTs (DNMT1 and DNMT3b) seem to be the first candidate molecules to lead this methylation-dependent repression. Nonetheless, the molecular mechanisms involved in E-cadherin repression are still to be fully elucidated, although the role of DNMTs and HMTs is undoubtedly an attractive aspect for further study.

- Final act: DNA methylation and gene silencing (Figure 2c)**.** In the model proposed, after histone de-acetylation and histone/DNA methylation, the final gene silencing of the *E-cadherin* promoter should be achieved by the participation of MBD proteins. This model is supported by evidence correlating interactions with MBD and the silent state of the *E-cadherin* promoter, as well as with promoter methylation.

In one of the first studies to demonstrate that MBD proteins can interact with *E-cadherin* promoter, Fujita et. al demonstrated that a direct interaction with the MBD1 protein led to the repression of *E-cadherin* expression (155). *In vitro*, recombinant MBD1 preferentially bound methylated DNA in the *E-cadherin* gene promoter, inhibiting transcription from methylated rather than unmethylated promoters (155). This indicated a potential role of the MBD1 protein in the maintenance of *E-cadherin* methylation. The association of other MBDs, such as MeCP2 and MBD2, with the *E-cadherin* promoter has been observed in several *E-cadherin* silent cancer cell lines (148). Interestingly, methylation of *E-cadherin* CpG islands and MeCP2 expression

have been shown to co-operate and to epigenetically down-regulate *E-cadherin* expression in colorectal cancer cells (156). However, in some cells that express *E-cadherin* an interaction between MBDs and the *E-cadherin* promoter has also been found, emphasising the complexity of this interaction (148). A relationship between DNA methylation and histone deacetylation has also been reported in another context, since MBD proteins appear to recruit co-repressor complexes with associated HDAC proteins (155, 157). These studies provide a mechanistic link between DNA methylation and histone deacetylation, further emphasising the potential activity of DNA methylation in directing transcriptional silencing within chromatin.

3. CONCLUSIONS AND PERSPECTIVES

The available information regarding the silencing of *E-cadherin* make it difficult to define a simple model in which *E-cadherin* expression is regulated by just a single genetic, epigenetic or transcriptional mechanism. It seems more likely that a combination of different mechanisms is responsible for defining the status of *E-cadherin* expression during tumour progression. As such, a modified classical "two hit hypothesis" for *E-cadherin* silencing is presently attracting great interest. In this model, genetic, epigenetic and transcriptional factors collaborate to control *E-cadherin* expression. Indeed, the modification of chromatin by the co-ordinated methylation and acetylation of DNA and/or histone has emerged as one of the principal mechanisms to regulate the transcriptional activity of different regulatory genes.

Here, we have reviewed the available information regarding these regulatory mechanisms in reference to E-cadherin. From this, we have drawn up a step-wise model of *E-cadherin* regulation, linking transcriptional to epigenetic regulation, and we propose a molecular modification of the "two hit hypothesis". In this model, transcriptional repression is achieved through signals that attract transcriptional repressors to the *E-cadherin* promoter, and co-repressor complexes with HDACs, HMTs and DNMTs, leading to the initial de-acetylation and histone/DNA methylation of promoter sequences. These changes may contribute to a local increase in DNA methylation that ends with recognition of MBD proteins, which promote and maintain *E-cadherin* repression, ultimately leading to gene silencing.

Understanding the cellular machinery implicated in the epigenetic control of epithelial genes such as *E-cadherin*, could be a key to identifying the dynamic molecular mechanisms of gene regulation during tumour progression and metastasis. However, more experimental data are needed to fully understand and link together all of these processes. The intense

scientific effort that has been made over the last few years must continue to strive for a clear model of the genetic, transcriptional and epigenetic regulation of *E-cadherin*. Once achieved, such a model will be extremely useful to identify potential new drug-targets to block the most lethal process in the tumour progression, metastasis.

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ABBREVIATIONS

EMT: Epithelial-mesenchymal Transition.
ECM: Extracellular matrix.
DNMT: DNA methyltransferases.
MBD: Methyl-binding domain proteins.
HDAC: Histone deacetylase.
5´-aza: 5´-aza-2'-deoxycytidine.
LOH: Loss of heterozygosity.
ILC: lobular breast cancers.
SNP: Single nucleotide polymorphism.
APC: Adenomatous polyposi coli.
Wnt: Wnt/Wingless factors.
CC: Colorectal cancer.
HPP1: hyperplasic polyp related protein 1.
HCC: hepatocellular carcinomas.
VHL: von Hippel-Lindau.
RASSF1A: RAS-association domain family 1A.
OSCC: oral squamous cell carcinomas.
GST-pi: glutathione-S-transferase-pi.
O6-MGMT: O(6)-methylguanine DNA methyltransferase.
hMLH1: human MutL homologue 1.
RAR-beta: retinoid acid receptor beta.
MET: Mesenchymal-Epithelial Transition.
CtBP: C-terminal binding protein.

SNAG: Snail/Gfi1 common domain.
HMT: Histone methyl-transferases

REFERENCES

1. Liotta L.A., Kohn E.C. Cancer's deadly signature. Nat Genet, 2003; 33: 10-11.
2. Bogenrieder T., Herlyn M. Axis of evil: molecular mechanisms of cancer metastasis. Oncogene, 2003; 22: 6524-6536.
3. Thiery J.P. Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer, 2002; 2: 442-454.
4. Thiery J.P. Epithelial-mesenchymal transitions in development and pathologies. Curr Opin Cell Biol, 2003; 15: 740-746.
5. Shook D., Keller R. Mechanisms, mechanics and function of epithelial-mesenchymal transitions in early development. Mech Dev, 2003; 120: 1351-1383.
6. Peinado H., Portillo F., Cano A. Transcriptional regulation of cadherins during development and carcinogenesis. Int J Dev Biol, 2004; 48: 365-375.
7. Takeichi M. The cadherins: cell-cell adhesion molecules controlling animal morphogenesis. Development, 1988; 102: 639-655.
8. Takeichi M. Morphogenetic roles of classic cadherins. Curr Opin Cell Biol, 1995; 7: 619-627.
9. Larue L., Antos C., Butz S., Huber O., Delmas V., Dominis M., Kemler R. A role for cadherins in tissue formation. Development, 1996; 122: 3185-3194.
10. Birchmeier W., Behrens J. Cadherin expression in carcinomas: role in the formation of cell junctions and the prevention of invasiveness. Biochim Biophys Acta, 1994; 1198: 11-26.
11. Takeichi M. Cadherins in cancer: implications for invasion and metastasis. Curr Opin Cell Biol, 1993; 5: 806-811.
12. Christofori G., Semb H. The role of the cell-adhesion molecule E-cadherin as a tumour-suppressor gene. Trends Biochem Sci, 1999; 24: 73-76.
13. Mansouri A., Spurr N., Goodfellow P.N., Kemler R. Characterization and chromosomal localization of the gene encoding the human cell adhesion molecule uvomorulin. Differentiation, 1988; 38: 67-71.
14. Eistetter H.R., Adolph S., Ringwald M., Simon-Chazottes D., Schuh R., Guenet J.L., Kemler R. Chromosomal mapping of the structural gene coding for the mouse cell adhesion molecule uvomorulin. Proc Natl Acad Sci U S A, 1988; 85: 3489-3493.
15. Berx G., Becker K.F., Hofler H., van Roy F. Mutations of the human E-cadherin (CDH1) gene. Hum Mutat, 1998; 12: 226-237.
16. Strathdee G. Epigenetic versus genetic alterations in the inactivation of E-cadherin. Semin Cancer Biol, 2002; 12: 373-379.
17. Berx G., Cleton-Jansen A.M., Nollet F., de Leeuw W.J., van de Vijver M., Cornelisse C., van Roy F. E-cadherin is a tumour/invasion suppressor gene mutated in human lobular breast cancers. EMBO J, 1995; 14: 6107-6115.
18. Berx G., Cleton-Jansen A.M., Strumane K., de Leeuw W.J., Nollet F., van Roy F., Cornelisse C. E-cadherin is inactivated in a majority of invasive human lobular breast cancers by truncation mutations throughout its extracellular domain. Oncogene, 1996; 13: 1919-1925.

19. Vos C.B., Cleton-Jansen A.M., Berx G., de Leeuw W.J., ter Haar N.T., van Roy F., Cornelisse C.J., Peterse J.L., van de Vijver M.J. E-cadherin inactivation in lobular carcinoma in situ of the breast: an early event in tumorigenesis. Br J Cancer, 1997; 76: 1131-1133.
20. Moll R., Mitze M., Frixen U.H., Birchmeier W. Differential loss of E-cadherin expression in infiltrating ductal and lobular breast carcinomas. Am J Pathol, 1993; 143: 1731-1742.
21. Gamallo C., Palacios J., Suarez A., Pizarro A., Navarro P., Quintanilla M., Cano A. Correlation of E-cadherin expression with differentiation grade and histological type in breast carcinoma. Am J Pathol, 1993; 142: 987-993.
22. Machado J.C., Soares P., Carneiro F., Rocha A., Beck S., Blin N., Berx G., Sobrinho-Simoes M. E-cadherin gene mutations provide a genetic basis for the phenotypic divergence of mixed gastric carcinomas. Lab Invest, 1999; 79: 459-465.
23. Guilford P., Hopkins J., Harraway J., McLeod M., McLeod N., Harawira P., Taite H., Scoular R., Miller A., Reeve A.E. E-cadherin germline mutations in familial gastric cancer. Nature, 1998; 392: 402-405.
24. Becker K.F., Atkinson M.J., Reich U., Becker I., Nekarda H., Siewert J.R., Hofler H. E-cadherin gene mutations provide clues to diffuse type gastric carcinomas. Cancer Res, 1994; 54: 3845-3852.
25. Grady W.M., Willis J., Guilford P.J., Dunbier A.K., Toro T.T., Lynch H., Wiesner G., Ferguson K., Eng C., Park J.G., Kim S.J., Markowitz S. Methylation of the CDH1 promoter as the second genetic hit in hereditary diffuse gastric cancer. Nat Genet 2000; 26: 16-17.
26. Cheng C.W., Wu P.E., Yu J.C., Huang C.S., Yue C.T., Wu C.W., Shen C.Y., Mechanisms of inactivation of E-cadherin in breast carcinoma: modification of the two-hit hypothesis of tumor suppressor gene. Oncogene, 2001; 3814-3823.
27. Machado J.C., Oliveira C., Carvalho R., Soares P., Berx G., Caldas C., Seruca R., Carneiro F., Sobrinho-Simoes M. E-cadherin gene (CDH1) promoter methylation as the second hit in sporadic diffuse gastric carcinoma. Oncogene, 2001; 20: 1525-1528.
28. Tamura G., Yin J., Wang S., Fleisher A.S., Zou T., Abraham J.M., Kong D., Smolinski K.N., Wilson K.T., James S.P., Silverberg S.G., Nishizuka S., Terashima M., Motoyama T., Meltzer S.J. E-Cadherin gene promoter hypermethylation in primary human gastric carcinomas. J Natl Cancer Inst, 2000; 92: 569-573.
29. Feinberg A.P., Tycko B. The history of cancer epigenetics. Nat Rev Cancer, 2004; 4: 143-153.
30. Yoshiura K., Kanai Y., Ochiai A., Shimoyama Y., Sugimura T., Hirohashi S. Silencing of the E-cadherin invasion-suppressor gene by CpG methylation in human carcinomas. Proc Natl Acad Sci U S A, 1995; 92: 7416-7419.
31. Graff J.R., Herman J.G., Lapidus R.G., Chopra H., Xu R., Jarrard D.F., Isaacs W.B., Pitha P.M., Davidson N.E., Baylin S.B. E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. Cancer Res, 1995; 55: 5195-5199.
32. Graziano F., Humar B., Guilford P. The role of the E-cadherin gene (CDH1) in diffuse gastric cancer susceptibility: from the laboratory to clinical practice. Ann Oncol, 2003; 14: 1705-1713.
33. Graziano F., Arduini F., Ruzzo A., Mandolesi A., Bearzi I., Silva R., Muretto P., Testa E., Mari D., Magnani M., Scartozzi M., Cascinu S. Combined analysis of E-cadherin gene (CDH1) promoter hypermethylation and E-cadherin protein expression in patients with gastric cancer: implications for treatment with demethylating drugs. Ann Oncol, 2004; 15: 489-492.

34. Graziano F., Arduini F., Ruzzo A., Bearzi I., Humar B., More H., Silva R., Muretto P., Guilford P., Testa E., Mari D., Magnani M., Cascinu S. Prognostic analysis of E-cadherin gene promoter hypermethylation in patients with surgically resected, node-positive, diffuse gastric cancer. Clin Cancer Res, 2004; 10: 2784-2789.
35. Ichikawa D., Koike H., Ikoma H., Ikoma D., Tani N., Otsuji E., Kitamura K., Yamagishi H. Detection of aberrant methylation as a tumor marker in serum of patients with gastric cancer. Anticancer Res, 2004; 24: 2477-2481.
36. Lee T.L., Leung W.K., Chan M.W., Ng E.K., Tong J.H., Lo K.W., Chung S.C., Sung J.J., To K.F. Detection of gene promoter hypermethylation in the tumor and serum of patients with gastric carcinoma. Clin Cancer Res, 2002; 8: 1761-1766.
37. House M.G., Guo M., Efron D.T., Lillemoe K.D., Cameron J.L., Syphard J.E., Hooker C.M., Abraham S.C., Montgomery E.A., Herman J.G., Brock M.V. Tumor suppressor gene hypermethylation as a predictor of gastric stromal tumor behavior. J Gastrointest Surg, 2003; 7: 1004-1014.
38. Concolino P., Papa V., Mozzetti S., Ferlini C., Pacelli F., Martinelli E., Ricci R., Filippetti F., Scambia G., Doglietto G.B. The unsolved enigma of CDH1 down-regulation in hereditary diffuse gastric cancer. J Surg Res, 2004; 121: 50-55.
39. Ebert M.P., Yu J., Hoffmann J., Rocco A., Rocken C., Kahmann S., Muller O., Korc M., Sung J.J., Malfertheiner P. Loss of beta-catenin expression in metastatic gastric cancer. J Clin Oncol, 2003; 21: 1708-1714.
40. Mielnicki L.M., Asch H.L., Asch B.B. Genes, chromatin, and breast cancer: an epigenetic tale. J Mammary Gland Biol Neoplasia, 2001; 6: 169-182.
41. Nass S.J., Herman J.G., Gabrielson E., Iversen P.W., Parl F.F., Davidson N.E., Graff J.R. Aberrant methylation of the estrogen receptor and E-cadherin 5' CpG islands increases with malignant progression in human breast cancer. Cancer Res, 2000; 60: 4346-4348.
42. Droufakou S., Deshmane V., Roylance R., Hanby A., Tomlinson I., Hart I.R. Multiple ways of silencing E-cadherin gene expression in lobular carcinoma of the breast. Int J Cancer, 2001; 92: 404-408.
43. Hajra K.M., Ji X., Fearon E.R. Extinction of E-cadherin expression in breast cancer via a dominant repression pathway acting on proximal promoter elements. Oncogene, 1999; 18: 7274-7279.
44. Li L.C., Chui R.M., Sasaki M., Nakajima K., Perinchery G., Au H.C., Nojima D., Carroll P., Dahiya R. A single nucleotide polymorphism in the E-cadherin gene promoter alters transcriptional activities. Cancer Res, 2000; 60: 873-876.
45. Sarrio D., Moreno-Bueno G., Hardisson D., Sanchez-Estevez C., Guo M., Herman J.G., Gamallo C., Esteller M., Palacios J. Epigenetic and genetic alterations of APC and CDH1 genes in lobular breast cancer: relationships with abnormal E-cadherin and catenin expression and microsatellite instability. Int J Cancer, 2003; 106: 208-215.
46. Brown A.M. Wnt signaling in breast cancer: have we come full circle? Breast Cancer Res, 2001; 3: 351-355.
47. Berx G., Van Roy F. The E-cadherin/catenin complex: an important gatekeeper in breast cancer tumorigenesis and malignant progression. Breast Cancer Res 2001; 3: 289-293.
48. Yang S.Z., Kohno N., Yokoyama A., Kondo K., Hamada H., Hiwada K. Decreased E-cadherin augments beta-catenin nuclear localization: studies in breast cancer cell lines. Int J Oncol, 2001; 18: 541-548.
49. van de Wetering M., Barker N., Harkes I.C., van der Heyden M., Dijk N.J., Hollestelle A., Klijn J.G., Clevers H., Schutte M. Mutant E-cadherin breast cancer cells do not display constitutive Wnt signaling. Cancer Res, 2001; 61: 278-284.

50. Medeiros A.C., Nagai M.A., Neto M.M., Brentani R.R. Loss of heterozygosity affecting the APC and MCC genetic loci in patients with primary breast carcinomas. Cancer Epidemiol Biomarkers Prev, 1994; 3: 331-333.
51. Jin Z., Tamura G., Tsuchiya T., Sakata K., Kashiwaba M., Osakabe M., Motoyama T. Adenomatous polyposis coli (APC) gene promoter hypermethylation in primary breast cancers. Br J Cancer, 2001; 85: 69-73.
52. Zheng Z., Pan J., Chu B., Wong Y.C., Cheung A.L., Tsao S.W. Down-regulation and abnormal expression of E-cadherin and beta-catenin in nasopharyngeal carcinoma: close association with advanced disease stage and lymph node metastasis. Hum Pathol, 1999; 30: 458-466.
53. Washington K., Chiappori A., Hamilton K., Shyr Y., Blanke C., Johnson D., Sawyers J., Beauchamp D. Expression of beta-catenin, alpha-catenin, and E-cadherin in Barrett's esophagus and esophageal adenocarcinomas. Mod Pathol, 1998; 11: 805-813.
54. Nakanishi Y., Ochiai A., Akimoto S., Kato H., Watanabe H., Tachimori Y., Yamamoto S., Hirohashi S. Expression of E-cadherin, alpha-catenin, beta-catenin and plakoglobin in esophageal carcinomas and its prognostic significance: immunohistochemical analysis of 96 lesions. Oncology, 1997; 54: 158-165.
55. Si H.X., Tsao S.W., Lam K.Y., Srivastava G., Liu Y., Wong Y.C., Shen Z.Y., Cheung A.L. E-cadherin expression is commonly downregulated by CpG island hypermethylation in esophageal carcinoma cells. Cancer Lett, 2001; 173: 71-78.
56. Takeno S., Noguchi T., Fumoto S., Kimura Y., Shibata T., Kawahara K. E-cadherin expression in patients with esophageal squamous cell carcinoma: promoter hypermethylation, Snail overexpression, and clinicopathologic implications. Am J Clin Pathol, 2004; 122: 78-84.
57. Eads C.A., Lord R.V., Kurumboor S.K., Wickramasinghe K., Skinner M.L., Long T.I., Peters J.H., DeMeester T.R., Danenberg K.D., Danenberg P.V., Laird P.W., Skinner K.A. Fields of aberrant CpG island hypermethylation in Barrett's esophagus and associated adenocarcinoma. Cancer Res, 2000; 60: 5021-5026.
58. Corn P.G., Heath E.I., Heitmiller R., Fogt F., Forastiere A.A., Herman J.G., Wu T.T. Frequent hypermethylation of the 5' CpG island of E-cadherin in esophageal adenocarcinoma. Clin Cancer Res, 2001; 7: 2765-2769.
59. Brock M.V., Gou M., Akiyama Y., Muller A., Wu T.T., Montgomery E., Deasel M., Germonpre P., Rubinson L., Heitmiller R.F., Yang S.C., Forastiere A.A., Baylin S.B., Herman J.G. Prognostic importance of promoter hypermethylation of multiple genes in esophageal adenocarcinoma. Clin Cancer Res, 2003; 9: 2912-2919.
60. Li L.C., Zhao H., Nakajima K., Oh B.R., Filho L.A., Carroll P., Dahiya R. Methylation of the E-cadherin gene promoter correlates with progression of prostate cancer. J Urol, 2001; 166: 705-709.
61. Singal R., Ferdinand L., Reis I.M., Schlesselman J.J. Methylation of multiple genes in prostate cancer and the relationship with clinicopathological features of disease. Oncol Rep, 2004; 12: 631-637.
62. Kallakury B.V., Sheehan C.E., Winn-Deen E., Oliver J., Fisher H.A., Kaufman R.P., Jr., Ross J.S. Decreased expression of catenins (alpha and beta), p120 CTN, and E-cadherin cell adhesion proteins and E-cadherin gene promoter methylation in prostatic adenocarcinomas. Cancer, 2001; 92: 2786-2795.
63. Ribeiro-Filho L.A., Franks J., Sasaki M., Shiina H., Li L.C., Nojima D., Arap S., Carroll P., Enokida H., Nakagawa M., Yonezawa S., Dahiya R. CpG hypermethylation of promoter region and inactivation of E-cadherin gene in human bladder cancer. Mol Carcinog, 2002; 34: 187-198.

64. Horikawa Y., Sugano K., Shigyo M., Yamamoto H., Nakazono M., Fujimoto H., Kanai Y., Hirohashi S., Kakizoe T., Habuchi T., Kato T. Hypermethylation of an E-cadherin (CDH1) promoter region in high grade transitional cell carcinoma of the bladder comprising carcinoma in situ. J Urol, 2003; 169: 1541-1545.
65. Thievessen I., Seifert H.H., Swiatkowski S., Florl A.R., Schulz W.A. E-cadherin involved in inactivation of WNT/beta-catenin signalling in urothelial carcinoma and normal urothelial cells. Br J Cancer, 2003; 88: 1932-1938.
66. Nigam A.K., Savage F.J., Boulos P.B., Stamp G.W., Liu D., Pignatelli M. Loss of cell-cell and cell-matrix adhesion molecules in colorectal cancer. Br J Cancer, 1993; 68: 507-514.
67. Dorudi S., Sheffield J.P., Poulsom R., Northover J.M., Hart I.R. E-cadherin expression in colorectal cancer. An immunocytochemical and in situ hybridization study. Am J Pathol,1993; 142: 981-986.
68. Kinsella A.R., Lepts G.C., Hill C.L., Jones M. Reduced E-cadherin expression correlates with increased invasiveness in colorectal carcinoma cell lines. Clin Exp Metastasis, 1994; 12: 335-342.
69. Efstathiou J.A., Liu D., Wheeler J.M.D., Kim H.C., Beck N.E., Ilyas M., Karayiannakis A.J., Mortensen N.J.M., Kmiot W., Playford R.J., Pignatelli M., Bodmer W.F. Mutated epithelial cadherin is associated with increased tumorigenicity and loss of adhesion and of responsiveness to the motogenic trefoil factor 2 in colon carcinoma cells. Proc Natl Acad Sci U S A, 1999; 96: 2316-2321.
70. Wheeler J.M., Kim H.C., Efstathiou J.A., Ilyas M., Mortensen N.J., Bodmer W.F. Hypermethylation of the promoter region of the E-cadherin gene (CDH1) in sporadic and ulcerative colitis associated colorectal cancer. Gut 2001; 48: 367-371.
71. Garinis G.A., Menounos P.G., Spanakis N.E., Papadopoulos K., Karavitis G., Parassi I., Christeli E., Patrinos G.P., Manolis E.N., Peros G. Hypermethylation-associated transcriptional silencing of E-cadherin in primary sporadic colorectal carcinomas. J Pathol, 2002; 198: 442-449.
72. Kanazawa T., Watanabe T., Kazama S., Tada T., Koketsu S., Nagawa H. Poorly differentiated adenocarcinoma and mucinous carcinoma of the colon and rectum show higher rates of loss of heterozygosity and loss of E-cadherin expression due to methylation of promoter region. Int J Cancer, 2002; 102: 225-229.
73. Kanazawa N., Oda T., Gunji N., Nozue M., Kawamoto T., Todoroki T., Fukao K. E-cadherin expression in the primary tumors and metastatic lymph nodes of poorly differentiated types of rectal cancer. Surg Today, 2002; 32: 123-128.
74. Lin S.Y., Yeh K.T., Chen W.T., Chen H.C., Chen S.T., Chiou H.Y., Chang J.G. Promoter CpG methylation of tumor suppressor genes in colorectal cancer and its relationship to clinical features. Oncol Rep, 2004; 11: 341-348.
75. Wong N.A.C.S., Pignatelli M. Beta-catenin - A Linchpin in Colorectal Carcinogenesis? Am J Pathol, 2002; 160: 389-401.
76. Garinis G.A., Spanakis N.E., Menounos P.G., Manolis E.N., Peros G. Transcriptional impairment of beta-catenin/E-cadherin complex is not associated with beta-catenin mutations in colorectal carcinomas. Br J Cancer 2003; 88: 206-209.
77. Esteller M., Sparks A., Toyota M., Sanchez-Cespedes M., Capella G., Peinado M.A., Gonzalez S., Tarafa G., Sidransky D., Meltzer S.J., Baylin S.B., Herman J.G. Analysis of adenomatous polyposis coli promoter hypermethylation in human cancer. Cancer Res, 2000; 60: 4366-4371.
78. Young J., Biden K.G., Simms L.A., Huggard P., Karamatic R., Eyre H.J., Sutherland G.R., Herath N., Barker M., Anderson G.J., Fitzpatrick D.R., Ramm G.A., Jass J.R.,

Leggett B.A. HPP1: a transmembrane protein-encoding gene commonly methylated in colorectal polyps and cancers. Proc Natl Acad Sci U S A, 2001; 98: 265-270.

79. Toyooka S., Toyooka K.O., Harada K., Miyajima K., Makarla P., Sathyanarayana U.G., Yin J., Sato F., Shivapurkar N., Meltzer S.J., Gazdar A.F. Aberrant methylation of the CDH13 (H-cadherin) promoter region in colorectal cancers and adenomas. Cancer Res, 2002; 62: 3382-3386.
80. Hibi K., Nakayama H., Kodera Y., Ito K., Akiyama S., Nakao A. CDH13 promoter region is specifically methylated in poorly differentiated colorectal cancer. Br J Cancer, 2004; 90: 1030-1033.
81. Toyooka S., Toyooka K.O., Maruyama R., Virmani A.K., Girard L., Miyajima K., Harada K., Ariyoshi Y., Takahashi T., Sugio K., Brambilla E., Gilcrease M., Minna J.D., Gazdar A.F. DNA methylation profiles of lung tumors. Mol Cancer Ther, 2001; 1: 61-67.
82. Shimamoto T., Ohyashiki J.H., Hirano T., Kato H., Ohyashiki K. Hypermethylation of E-cadherin gene is frequent and independent of p16INK4A methylation in non-small cell lung cancer: potential prognostic implication. Oncol Rep, 2004; 12: 389-395.
83. Toyooka K.O., Toyooka S., Virmani A.K., Sathyanarayana U.G., Euhus D.M., Gilcrease M., Minna J.D., Gazdar A.F. Loss of expression and aberrant methylation of the CDH13 (H-cadherin) gene in breast and lung carcinomas. Cancer Res, 2001; 61: 4556-4560.
84. Sato M., Mori Y., Sakurada A., Fujimura S., Horii A. The H-cadherin (CDH13) gene is inactivated in human lung cancer. Hum Genet, 1998; 103: 96-101.
85. Kanai Y., Ushijima S., Tsuda H., Sakamoto M., Sugimura T., Hirohashi S. Aberrant DNA methylation on chromosome 16 is an early event in hepatocarcinogenesis. Jpn J Cancer Res, 1996; 87: 1210-1217.
86. Kanai Y., Ushijima S., Hui A.M., Ochiai A., Tsuda H., Sakamoto M., Hirohashi S. The E-cadherin gene is silenced by CpG methylation in human hepatocellular carcinomas. Int J Cancer, 1997; 71: 355-359.
87. Kanai Y., Ushijima S., Tsuda H., Sakamoto M., Hirohashi S. Aberrant DNA methylation precedes loss of heterozygosity on chromosome 16 in chronic hepatitis and liver cirrhosis. Cancer Lett 2000; 148: 73-80.
88. Matsumura T., Makino R., Mitamura K. Frequent down-regulation of E-cadherin by genetic and epigenetic changes in the malignant progression of hepatocellular carcinomas. Clin Cancer Res, 2001; 7: 594-599.
89. Calvisi D.F., Ladu S., Conner E.A., Factor V.M., Thorgeirsson S.S. Disregulation of E-cadherin in transgenic mouse models of liver cancer. Lab Invest, 2004; 84: 1137-1147.
90. Dulaimi E., De C., II, Uzzo R.G., Al-Saleem T., Greenberg R.E., Polascik T.J., Babb J.S., Grizzle W.E., Cairns P. Promoter hypermethylation profile of kidney cancer. Clin Cancer Res, 2004; 10: 3972-3979.
91. Nojima D., Nakajima K., Li L.C., Franks J., Ribeiro-Filho L., Ishii N., Dahiya R. CpG methylation of promoter region inactivates E-cadherin gene in renal cell carcinoma. Mol Carcinog, 2001; 32: 19-27.
92. Chen C.L., Liu S.S., Ip S.M., Wong L.C., Ng T.Y., Ngan H.Y. E-cadherin expression is silenced by DNA methylation in cervical cancer cell lines and tumours. Eur J Cancer, 2003; 39: 517-523.
93. Narayan G., Arias-Pulido H., Koul S., Vargas H., Zhang F.F., Villella J., Schneider A., Terry M.B., Mansukhani M., Murty V.V. Frequent promoter methylation of CDH1, DAPK, RARB, and HIC1 genes in carcinoma of cervix uteri: its relationship to clinical outcome. Mol Cancer, 2003; 2: 24.

94. Widschwendter A., Ivarsson L., Blassnig A., Muller H.M., Fiegl H., Wiedemair A., Muller-Holzner E., Goebel G., Marth C., Widschwendter M. CDH1 and CDH13 methylation in serum is an independent prognostic marker in cervical cancer patients. Int J Cancer, 2004; 109: 163-166.
95. Saito T., Nishimura M., Yamasaki H., Kudo R. Hypermethylation in promoter region of E-cadherin gene is associated with tumor dedifferention and myometrial invasion in endometrial carcinoma. Cancer, 2003; 97: 1002-1009.
96. Moreno-Bueno G., Hardisson D., Sanchez C., Sarrio D., Cassia R., Garcia-Rostan G., Prat J., Guo M., Herman J.G., Matias-Guiu X., Esteller M., Palacios J. Abnormalities of the APC/beta-catenin pathway in endometrial cancer. Oncogene, 2002; 21: 7981-7990.
97. Moreno-Bueno G., Rodriguez-Perales S., Sanchez-Estevez C., Marcos R., Hardisson D., Cigudosa J.C., Palacios J. Molecular alterations associated with cyclin D1 overexpression in endometrial cancer. Int J Cancer, 2004; 110: 194-200.
98. Chiles M.C., Ai L., Zuo C., Fan C.Y., Smoller B.R. E-cadherin promoter hypermethylation in preneoplastic and neoplastic skin lesions. Mod Pathol, 2003; 16: 1014-1018.
99. Fraga M.F., Herranz M., Espada J., Ballestar E., Paz M.F., Ropero S., Erkek E., Bozdogan O., Peinado H., Niveleau A., Mao J.H., Balmain A., Cano A., Esteller M. A mouse skin multistage carcinogenesis model reflects the aberrant DNA methylation patterns of human tumors. Cancer Res, 2004; 64: 5527-5534.
100. Takeuchi T., Liang S.B., Matsuyoshi N., Zhou S., Miyachi Y., Sonobe H., Ohtsuki Y. Loss of T-cadherin (CDH13, H-cadherin) expression in cutaneous squamous cell carcinoma. Lab Invest, 2002; 82: 1023-1029.
101. Takeuchi T., Liang S.B., Ohtsuki Y. Down-regulation of expression of a novel cadherin molecule, T-cadherin, in basal cell carcinoma of the skin. Mol Carcinog, 2002; 35: 173-179.
102. Saito Y., Takazawa H., Uzawa K., Tanzawa H., Sato K. Reduced expression of E-cadherin in oral squamous cell carcinoma: relationship with DNA methylation of 5' CpG island. Int J Oncol, 1998; 12: 293-298.
103. Nakayama S., Sasaki A., Mese H., Alcalde R.E., Tsuji T., Matsumura T. The E-cadherin gene is silenced by CpG methylation in human oral squamous cell carcinomas. Int J Cancer 2001; 93: 667-673.
104. Chang H.W., Chow V., Lam K.Y., Wei W.I., Yuen A. Loss of E-cadherin expression resulting from promoter hypermethylation in oral tongue carcinoma and its prognostic significance. Cancer, 2002; 94: 386-392.
105. Hazan R.B., Qiao R., Keren R., Badano I., Suyama K. Cadherin switch in tumor progression. Ann N Y Acad Sci, 2004; 1014: 155-163.
106. Chen Q., Lipkina G., Song Q., Kramer R.H. Promoter methylation regulates cadherin switching in squamous cell carcinoma. Biochem Biophys Res Commun, 2004; 315: 850-856.
107. Kudo Y., Kitajima S., Ogawa I., Hiraoka M., Sargolzaei S., Keikhaee M.R., Sato S., Miyauchi M., Takata T. Invasion and metastasis of oral cancer cells require methylation of E-cadherin and/or degradation of membranous beta-catenin. Clin Cancer Res, 2004; 10: 5455-5463.
108. Melki J.R., Vincent P.C., Brown R.D., Clark S.J. Hypermethylation of E-cadherin in leukemia. Blood, 2000; 95: 3208-3213.
109. Farinha N.J., Shaker S., Lemaire M., Momparler L., Bernstein M., Momparler R.L. Activation of expression of p15, p73 and E-cadherin in leukemic cells by different concentrations of 5-aza-2'-deoxycytidine (Decitabine). Anticancer Res, 2004; 24: 75-78.

110. Corn P.G., Smith B.D., Ruckdeschel E.S., Douglas D., Baylin S.B., Herman J.G. E-cadherin expression is silenced by 5' CpG island methylation in acute leukemia. Clin Cancer Res, 2000; 6: 4243-4248.
111. Seidl S., Ackermann J., Kaufmann H., Keck A., Nosslinger T., Zielinski C.C., Drach J., Zochbauer-Muller S. DNA-methylation analysis identifies the E-cadherin gene as a potential marker of disease progression in patients with monoclonal gammopathies. Cancer, 2004; 100: 2598-2606.
112. Galm O., Wilop S., Reichelt J., Jost E., Gehbauer G., Herman J.G., Osieka R. DNA methylation changes in multiple myeloma. Leukemia, 2004; 18: 1687-1692.
113. Roman-Gomez J., Castillejo J.A., Jimenez A., Cervantes F., Boque C., Hermosin L., Leon A., Granena A., Colomer D., Heiniger A., Torres A. Cadherin-13, a mediator of calcium-dependent cell-cell adhesion, is silenced by methylation in chronic myeloid leukemia and correlates with pretreatment risk profile and cytogenetic response to interferon alfa. J Clin Oncol, 2003; 21: 1472-1479.
114. Behrens J., Lowrick O., Klein-Hitpass L., Birchmeier W. The E-cadherin promoter: functional analysis of a G.C-rich region and an epithelial cell-specific palindromic regulatory element. Proc Natl Acad Sci U S A, 1991; 88: 11495-11499.
115. Faraldo M.L., Rodrigo I., Behrens J., Birchmeier W., Cano A. Analysis of the E-cadherin and P-cadherin promoters in murine keratinocyte cell lines from different stages of mouse skin carcinogenesis. Mol Carcinog, 1997; 20: 33-47.
116. Hennig G., Lowrick O., Birchmeier W., Behrens J. Mechanisms identified in the transcriptional control of epithelial gene expression. J Biol Chem 1996; 271: 595-602.
117. Rodrigo I., Cato A.C., Cano A. Regulation of E-cadherin gene expression during tumor progression: the role of a new Ets-binding site and the E-pal element. Exp Cell Res, 1999; 248: 358-371.
118. Comijn J., Berx G., Vermassen P., Verschueren K., van Grunsven L., Bruyneel E., Mareel M., Huylebroeck D., van Roy F. The two-handed E box binding zinc finger protein SIP1 downregulates E- cadherin and induces invasion. Mol Cell, 2001; 7: 1267-1278.
119. Batlle E., Sancho E., Franci C., Dominguez D., Monfar M., Baulida J., Garcia De Herreros A. The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. Nat Cell Biol, 2000; 2: 84-89.
120. Hajra K.M., Chen D.Y., Fearon E.R. The SLUG zinc-finger protein represses E-cadherin in breast cancer. Cancer Res, 2002; 62: 1613-1618.
121. Cano A., Perez-Moreno M.A., Rodrigo I., Locascio A., Blanco M.J., del Barrio M.G., Portillo F., Nieto M.A. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat Cell Biol, 2000; 2: 76-83.
122. Bolós V., Peinado H., Perez-Moreno M.A., Fraga M.F., Esteller M., Cano A. The transcription factor Slug represses E-cadherin expression and induces epithelial to mesenchymal transitions: a comparison with Snail and E47 repressors. J Cell Sci, 2003; 116: 499-511.
123. Pérez-Moreno M.A., Locascio A., Rodrigo I., Dhondt G., Portillo F., Nieto M.A., Cano A. A new role for E12/E47 in the repression of E-cadherin expression and epithelial-mesenchymal transitions. J Biol Chem, 2001; 276: 27424-27431.
124. Yang J., Mani S.A., Donaher J.L., Ramaswamy S., Itzykson R.A., Come C., Savagner P., Gitelman I., Richardson A., Weinberg R.A. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell, 2004; 117: 927-939.
125. Grooteclaes M.L., Frisch S.M. Evidence for a function of CtBP in epithelial gene regulation and anoikis. Oncogene, 2000; 19: 3823-3828.

126. Blanco M.J., Moreno-Bueno G., Sarrio D., Locascio A., Cano A., Palacios J., Nieto M.A. Correlation of Snail expression with histological grade and lymph node status in breast carcinomas. Oncogene, 2002; 21: 3241-3246.
127. Cheng C.W., Wu P.E., Yu J.C., Huang C.S., Yue C.T., Wu C.W., Shen C.Y. Mechanisms of inactivation of E-cadherin in breast carcinoma: modification of the two-hit hypothesis of tumor suppressor gene. Oncogene, 2001; 20: 3814-3823.
128. Sugimachi K., Tanaka S., Kameyama T., Taguchi K., Aishima S., Shimada M., Tsuneyoshi M. Transcriptional repressor snail and progression of human hepatocellular carcinoma. Clin Cancer Res, 2003; 9: 2657-2664.
129. Guaita S., Puig I., Franci C., Garrido M., Dominguez D., Batlle E., Sancho E., Dedhar S., De Herreros A.G., Baulida J. Snail induction of epithelial to mesenchymal transition in tumor cells is accompanied by MUC1 repression and ZEB1 expression. J Biol Chem, 2002; 277: 39209-39216.
130. Rosivatz E., Becker I., Specht K., Fricke E., Luber B., Busch R., Hofler H., Becker K.F. Differential expression of the epithelial-mesenchymal transition regulators snail, SIP1, and twist in gastric cancer. Am J Pathol, 2002; 161: 1881-1891.
131. Nieto M.A. The snail superfamily of zinc-finger transcription factors. Nat Rev Mol Cell Biol, 2002; 3: 155-166.
132. van de Putte T., Maruhashi M., Francis A., Nelles L., Kondoh H., Huylebroeck D., Higashi Y. Mice lacking ZFHX1B, the gene that codes for Smad-interacting protein-1, reveal a role for multiple neural crest cell defects in the etiology of Hirschsprung disease-mental retardation syndrome. Am J Hum Genet, 2003; 72: 465-470.
133. Bukholm I.K., Nesland J.M., Borresen-Dale A.L. Re-expression of E-cadherin, alpha-catenin and beta-catenin, but not of gamma-catenin, in metastatic tissue from breast cancer patients. J Pathol, 2000; 190: 15-19.
134. Gamallo C., Palacios, J., Benito, N., Limeres, M.A., Pizarro, A., Suarez, A., Pastrana, F., Cano, A., Calero, F. Expression of E-cadherin in 230 infiltrating ductal breast carcinoma: Relationship to clinicopathological features. Int J Oncology, 1996; 9: 1207-1212.
135. Mareel M.M., Behrens J., Birchmeier W., De Bruyne G.K., Vleminckx K., Hoogewijs A., Fiers W.C., Van Roy F.M. Down-regulation of E-cadherin expression in Madin Darby canine kidney (MDCK) cells inside tumors of nude mice. Int J Cancer, 1991; 47: 922-928.
136. Behrens J., Frixen U., Schipper J., Weidner M., Birchmeier W. Cell adhesion in invasion and metastasis. Semin Cell Biol, 1992; 3: 169-178.
137. Mareel M., Bracke M., Van Roy F., Vakaet L. Expression of E-cadherin in embryogenetic ingression and cancer invasion. Int J Dev Biol, 1993; 37: 227-235.
138. Graff J.R., Gabrielson E., Fujii H., Baylin S.B., Herman J.G. Methylation patterns of the E-cadherin 5' CpG island are unstable and reflect the dynamic, heterogeneous loss of E-cadherin expression during metastatic progression. J Biol Chem 2000; 275: 2727-2732.
139. Bird A.P., Wolffe A.P. Methylation-induced repression--belts, braces, and chromatin. Cell, 1999; 99: 451-454.
140. Bird A. DNA methylation patterns and epigenetic memory. Genes Dev 2002; 16: 6-21.
141. Fournier C., Goto Y., Ballestar E., Delaval K., Hever A.M., Esteller M., Feil R. Allele-specific histone lysine methylation marks regulatory regions at imprinted mouse genes. EMBO J, 2002; 21: 6560-6570.
142. Peinado H., Ballestar E., Esteller M., Cano A. Snail Mediates E-Cadherin Repression by the Recruitment of the Sin3A/Histone Deacetylase 1 (HDAC1)/HDAC2 Complex. Mol Cell Biol, 2004; 24: 306-319.

143. Shi Y., Sawada J., Sui G., Affar el B., Whetstine J.R., Lan F., Ogawa H., Luke M.P., Nakatani Y. Coordinated histone modifications mediated by a CtBP co-repressor complex. Nature, 2003; 422: 735-738.
144. El-Osta A., Wolffe A.P. DNA methylation and histone deacetylation in the control of gene expression: basic biochemistry to human development and disease. Gene Expr, 2000; 9: 63-75.
145. Hemavathy K., Ashraf S.I., Ip Y.T. Snail/slug family of repressors: slowly going into the fast lane of development and cancer. Gene, 2000; 257: 1-12.
146. Fujita N., Jaye D.L., Kajita M., Geigerman C., Moreno C.S., Wade P.A. MTA3, a Mi-2/NuRD Complex Subunit, Regulates an Invasive Growth Pathway in Breast Cancer. Cell, 2003; 113: 207-219.
147. Noma K., Allis C.D., Grewal S.I. Transitions in distinct histone H3 methylation patterns at the heterochromatin domain boundaries. Science, 2001; 293: 1150-1155.
148. Koizume S., Tachibana K., Sekiya T., Hirohashi S., Shiraishi M. Heterogeneity in the modification and involvement of chromatin components of the CpG island of the silenced human CDH1 gene in cancer cells. Nucleic Acids Res 2002; 30: 4770-4780.
149. Bestor T.H. The DNA methyltransferases of mammals. Hum Mol Genet, 2000; 9: 2395-2402.
150. El-Osta A. DNMT cooperativity--the developing links between methylation, chromatin structure and cancer. Bioessays, 2003; 25: 1071-1084.
151. Graff J.R., Herman J.G., Myohanen S., Baylin S.B., Vertino P.M. Mapping patterns of CpG island methylation in normal and neoplastic cells implicates both upstream and downstream regions in de novo methylation. J Biol Chem, 1997; 272: 22322-22329.
152. Girault I., Tozlu S., Lidereau R., Bieche I. Expression analysis of DNA methyltransferases 1, 3A, and 3B in sporadic breast carcinomas. Clin Cancer Res, 2003; 9: 4415-4422.
153. Etoh T., Kanai Y., Ushijima S., Nakagawa T., Nakanishi Y., Sasako M., Kitano S., Hirohashi S. Increased DNA methyltransferase 1 (DNMT1) protein expression correlates significantly with poorer tumor differentiation and frequent DNA hypermethylation of multiple CpG islands in gastric cancers. Am J Pathol, 2004; 164: 689-699.
154. Kawasaki H., Taira K. Induction of DNA methylation and gene silencing by short interfering RNAs in human cells. Nature, 2004; 431: 211-217.
155. Fujita N., Takebayashi S., Okumura K., Kudo S., Chiba T., Saya H., Nakao M. Methylation-Mediated Transcriptional Silencing in Euchromatin by Methyl-CpG Binding Protein MBD1 Isoforms. Mol Cell Biol, 1999; 19: 6415-6426.
156. Darwanto A., Kitazawa R., Maeda S., Kitazawa S. MeCP2 and promoter methylation cooperatively regulate E-cadherin gene expression in colorectal carcinoma. Cancer Sci, 2003; 94: 442-447.
157. Nan X., Ng H.H., Johnson C.A., Laherty C.D., Turner B.M., Eisenman R.N., Bird A. Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature, 1998; 393: 386-389.

Chapter 8

EPIGENETIC DISRUPTION OF THE SLIT-ROBO INTERACTIONS IN HUMAN CANCER

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Abstract: During the development of the nervous system, guidance cues are required to correctly direct the developing axons. These cues are highly conserved in evolution and may have diverse functions and receptors. The Slit proteins are members of these cues and along with their roundabout (robo) receptors, they act as repulsive cues for robo-expressing axons preventing them from crossing or re-crossing the midline. There is increasing evidence that the slit-robo interactions are not limited to axon guidance. The repulsive effect on axons due to slit-robo binding is mirrored in the immune system as well as in breast tumour cells; Slit proteins act as inhibitors of cell migration and invasion. Our group recently demonstrated that both *SLIT* and *ROBO* genes are inactivated in human cancers by promoter region CpG island hypermethylation with the subsequent silencing of gene expression. Restoring expression after treatment with a demethylating agent, provided further evidence that promoter hypermethylation was responsible for silencing SLIT-ROBO genes in several human cancers. Whilst *Robo1* homozygous mutant mice die at birth due to incomplete lung development, the heterozygous mice show increased predisposition to tumour development concurrent with the inactivation of the remaining wild type *Robo1* allele by promoter region CpG island hypermethylation. *SEMA3B,* another axon guidance molecule, was recently demonstrated to be epigenetically inactivated in human cancers and suppressed tumour growth. Hence, evidence is accumulating for the role of axon guidance molecules in human cancer development. Unlike mutational inactivation, epigenetic inactivation is a reversible event. This presents new and exciting opportunities for clinical management of cancer. In addition, promoter hypermethylation of genes is increasingly being developed as molecular biomarkers for non-invasive screens for early detection of cancer. Furthermore, the *SLIT(s)* gene products are secretary proteins, which may also lead to the development of novel therapeutic approaches. This chapter

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summarises the literature on SLIT-ROBO gene families in relation to human diseases.

Key words: epigenetic, cancer, SLIT and ROBO genes, axon guidance cues, Drosophila genes.

1. CHARACTERISATION OF THE *ROBO* AND *SLIT* GENES

The *Roundabout* (*Robo*) gene was initially identified in *Drosophila* in a large scale mutant screen for the identification of genes that control midline crossing of axons. In mutant *robo*, the tight control over axons crossing the midline is lost and they become able to cross and re-cross the midline (1). The human *ROBO1* was later identified by homology to the fly *robo* gene (2) (Figure 1). Another isoform of *ROBO1* was cloned independently by CpG island rescue PCR and was designated *DUTT1* (Deleted in U-Twenty Twenty SCLC cell line).

Figure 1. Cladograms of human (h), *Drosophila melanogaster* (d) and *C. elegans* Slits and robo genes, based on the ClustalW alignment of the full length of the proteins.

DUTT1 was considered a potential tumour suppressor gene since it resides in a region of high frequency of hemi- and homozygous deletions in cancer (3) (Figure 2). The NCBI RefSeq database gives the designation *ROBO1A* for *ROBO1* and *ROBO1B* for *DUTT1*, which is an alternatively spliced isoform of *ROBO1*. *ROBO1* gene resides at chromosome 3p12.3, according to the May 2004 freeze of the human genome at the UCSC genome browser (http://genome.ucsc.edu/). *ROBO1A* consists of 30 exons coding for 1,652 amino acids and occupies about 1 MB of genomic

sequence. *ROBO1B* is an alternatively spliced isoform that consists of 29 exons coding for 1,613 amino acids. Unlike *ROBO1A*, the promoter region of *ROBO1B* contains a CpG island.

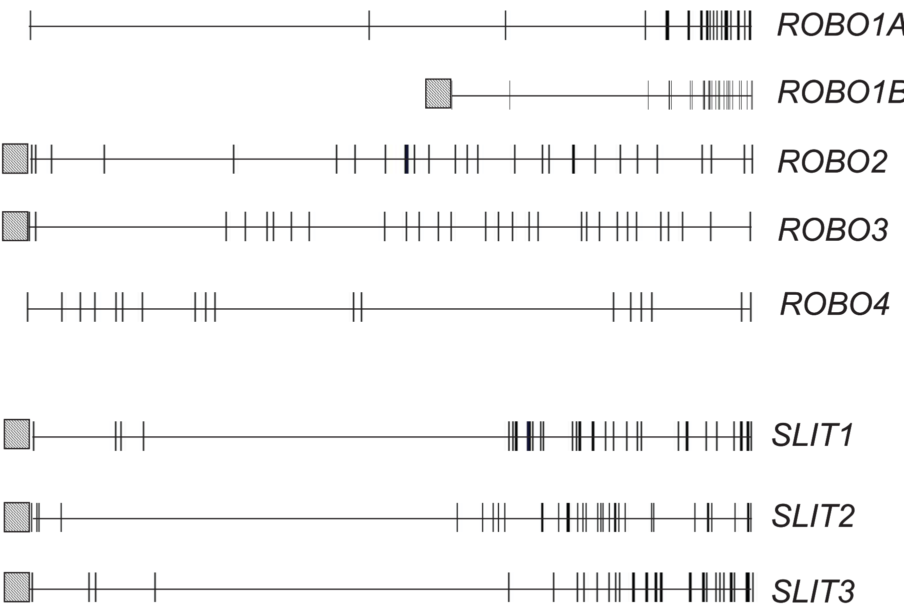

Figure 2. Comparison of the genomic organization of the SLIT and ROBO genes in human. Indicates the presence of a CpG island.

The protein structure of ROBO1 classifies it as a member of the immunoglobulin superfamily (Figure 3) which includes members of the NCAM and L1 families of receptors. *ROBO1* codes for a transmembrane protein with the ectodomain containing 5 immunoglobulin domains (Ig) and 3 fibronectin type III domains (FN3). The cytoplasmic domain contains 4 highly conserved regions or motifs that are designated CC0, CC1, CC2 and CC3. (Figure 3) The Tyrosine residue in CC1 is a phosphorylation target for Abelson (Abl) Tyrosine Kinase (4) and the motif is a preferred binding site for DCC (netrin-1 receptor) (5). The CC2 (LPPPP) is a consensus binding site for the Ena-VASP homology (EVH1) domain of *Drosophila* Enabled or its human homologue MENA/ENAH (4). The CC3 motif can serve as a binding site for srGAPs (slit-robo gtpases activating proteins) regulating the activity of the Rho family of small guanosine triphosphotases (GTPases) (6). The CC3 motif is also required for Abl binding to Robo (7,8). A cDNA probe against both isoforms reveals a broad pattern of expression in embryonic and adult human tissues and cancer cell lines as determined by

northern blotting (3). The ROBO1A/B protein can also be detected in adult mouse tissues although it appears that Robo1A expression is restricted to embryonic developmental stages while Robo1B expression continues throughout adulthood (9).*ROBO2* is telomeric to *ROBO1* at 3p12.3, separated by about 1 MB of genomic DNA. The gene consists of 28 exons coding for 1,493 amino acids (Figure 2). The gene occupies about 170 kb of genomic sequence and contains a 5' CpG island. *ROBO2* was initially partially cloned by Kidd *et al*. (1998) (2). The full length gene was cloned later by a systematic sequencing approach of size-fractionated large-size cDNAs from foetal brain and designated KIAA1568 (10). ROBO2 has overall 38% similarity to ROBO1 (Figure 1). ROBO2 also consists of 5 Ig and 3 FN3 domains in the extracellular domain and the four conserved intracellular CC motifs (Figure 3). In addition to being expressed in embryonic tissues, *Robo2* can be detected (10) in adult spleen, thymus, liver, kidney, and in human ovaries and brain (Dallol and Latif unpublished observation). *Robo2*$^{-/-}$ mice usually die after birth (11).

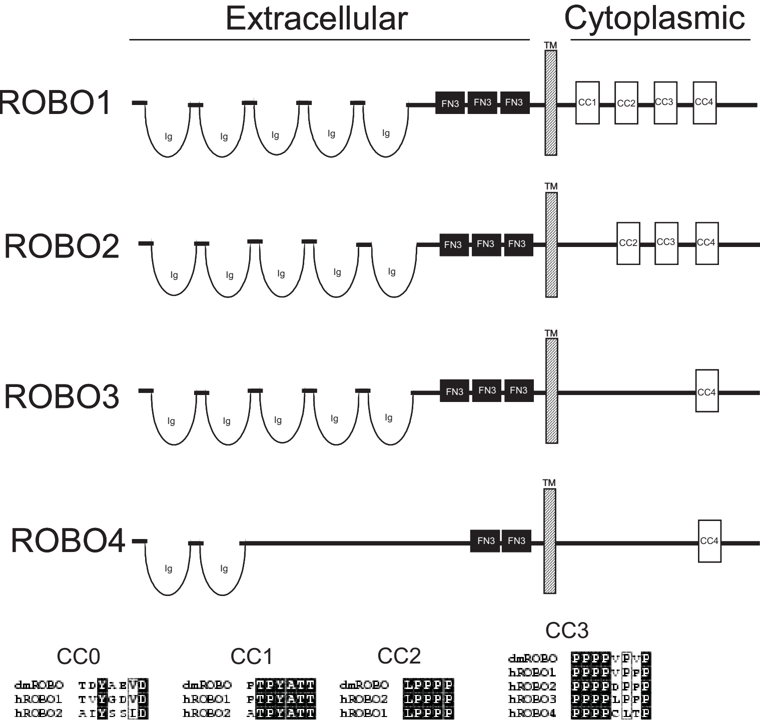

Figure 3. Comparison of the protein and domain structure of the ROBO genes in human. Also shown the ClustalW alignments of the conserved cytoplasmic CC motifs in different species. Ig is immunglobulin motifs, FN3 is fibronectin type III motif, TM is the transmembrane region.

ROBO3 or *RIG-1* (retinoblastoma inhibiting gene-1) is mapped to chromosome 11q24.2. It consists of 29 exons coding for 1,546 amino acids (Figure 2). The gene itself occupies about 16 kb of the genome. *ROBO3* was identified as an upregulated gene in the mouse *Rb1*-deficient embryos (12). ROBO3 is a divergent member of the ROBO family, it has 33% overall similarity to ROBO1 and 30% similarity to ROBO2 (Figure 1). However, ROBO3 shares the domain structures of both ROBO1 and ROBO2 (5+3 Ig+FN3 domains) but it is missing 3 cytoplasmic CC motifs which may indicate different interacting partners or post-translational modifications (Figure 3). *ROBO3* expression is controlled by RB and PAX-2 and is specifically expressed during embryonic development (13). *Robo3*$^{-/-}$ mice die few hours after birth without a clear cause (14)

ROBO4 is the smallest member of the ROBO family with only 18 exons coding for 1,008 amino acids (Figure 2). Similar to *ROBO1* and *ROBO2*, *ROBO4* is telomeric to ROBO3 at chromosome 11q24.2, separated by only about 3 kb of genomic DNA. *ROBO4* occupies about 14 kb of the genome. The gene was cloned using bioinformatics approaches to search for endothelial-specific genes (15). Unlike the other members of the ROBO family, ROBO4 has only 2 Ig and 2 FN3 domains in its ectodomain (Figure 3). These differences are reflected in its reduced overall similarity with ROBO1 or ROBO2 (22% and 16%, respectively; Figure 1). ROBO4 is the closest member of the ROBO family to ROBO3 with 30% overall similarity. Based on its expression pattern, ROBO4 is implicated in angiogenesis since it appears to be specifically expressed in the vasculature system (16,17).

dRobo is a repulsive guidance receptor on growth cones that binds to what was an unknown midline ligand. This ligand was later identified in *Drosophila* as the Slit protein. The mammalian Slit proteins (Slit1, Slit2 and Slit3) were later identified and found that they share a common domain structure and sequence homology with *Drosophila* Slit (43.5%, 43% and 41.1% between dSlit and Slit-1, Slit2 and Slit3, respectively; Figure 1) (18-20). The Slit proteins also have between 60-66% overall similarity with one another (18). Slit proteins were found to have a conserved function as ligands for the Robo receptors in controlling axon migration. All three vertebrate Slit proteins contain a N-terminus signal peptide, four tandem leucine-rich repeats (LRR), Nine EGF-like motifs (EGF), an Agrin-Laminin-Perlecan-Slit (ALPS) spacer between EGF6 and EGF7, and a cysteine-knot that is usually found in several secreted growth factors (Figure 4) (18). All the SLIT genes have CpG islands in their promoter regions. Human *SLIT1* is located at chromosome 10q24.1. *SLIT1* consists of 37 exons coding for 1,535 amino acids (Figure 2). The gene occupies about 185 kb of the genome. In addition to its expression during embryonic stages, *SLIT1* is specifically expressed in the adult brain (21). *SLIT2* is located at

chromosome 4p15.2. It is coded for by 37 exons over about 365 kb of the genome (Figure 2). The protein consists of 1,530 amino acids. Unlike *SLIT1*, *SLIT2* is expressed in a variety of adult tissues (21,22). *SLIT3* occupies about 600 kb of chromosome 5q34-5q35.1 and it consists of 36 exons coding for 1,524 amino acids (Figure 2). *SLIT3* has a similar pattern of expression to *SLIT2* (21). $Slit1^{-/-}$ mice appear grossly normal while the *Slit2* homozygous deficiency is lethal (23). $Slit3^{-/-}$ mice are viable but their morbidity and mortality increase as they pass 30 postnatal days. In addition, $Slit3^{-/-}$ mutant mice develop congenital diaphragmatic hernia caused by abnormal bundling of collagen in the central tendon (24,25).

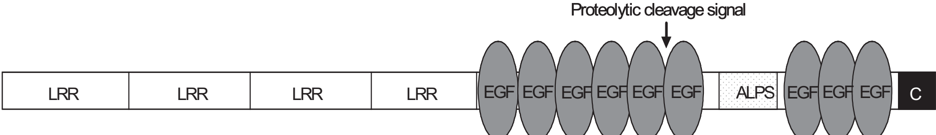

Figure 4. The domain organisation of SLIT2. All SLIT proteins share a similar domain structure containing an N-terminal signal peptide, four tandem leucine-rich repeats (LRR), EGF-like repeats (EGF), a conserved Agrin-Laminin-Perlecan-Slit (ALPS) and a carboxy-terminal cysteine knot (C). However, the presence of a proteolytics cleavage signal is not confirmed in SLIT1 and SLIT3.

The SLIT proteins are secreted proteins but remain to a large extent associated with the cell membrane. However, SLIT2 protein (190 kDa) also contains a conserved proteolytics cleavage site that generates a membrane-associated N-terminus fragment about 140 kDa in size. The C-terminus 55-60 kDa fragment is more diffusible with about 1:1 distribution between secreted:membrane-associated (18). SLIT2 can bind to both rRobo1 and rRobo2. dSlit can also bind dRobo1. These interactions are conserved since SLIT2 can bind the dRobo and dSlit can bind either rRobo1 and rRobo2 (18). SLIT1 and SLIT3 can also bind ROBO1-3 but it is not clear whether ROBO4 is a SLIT receptor or not (16,17). The SLIT-ROBO interactions require the first two Ig domains of the ROBO receptors (26) and the LRR domains of SLIT (27). SLIT2 can also bind the other axon guidance molecules namely Netrin-1 (18). In addition, binding of SLIT2 to Laminin-1 (18) and Glypican-1 (28) has been demonstrated.

The expression patterns of both Slit2 and Robo1 may also indicate that the former is a ligand to the latter. Using western blotting, Clark *et al.* (2002) detected Robo1 protein in the mouse embryonic brain, heart, muscle, lung, kidney, eye and liver. With the exception of the adult liver, Robo1 expression is maintained in adult tissues, albeit at lower levels (9). When immunohistochemistry was performed to detect Robo1 in the embryonic

lungs, it was found that Robo1 and Robo2 can be detected in the mesenchyme and embryonic epithelia (9,29). Expression was more restricted to the large airways in the bronchial epithelia of the newborn mouse (9,29). In adult lungs, Robo1 is detectable at high levels in epithelial cells lining the bronchia but at low or none detectable in the adjacent mesenchyme (30). In the embryonic lung, Slit2 expression is detectable in the mesenchyme and on the cells adjacent to the bronchial lumen, an indication that Slit2 is secreted into the airway lumen (29). Expression of Slit2 but not slit3 in the peri-luminar array becomes more evident in the newborn mice (29). Expression of Slit2 in adjacent sites to where Robo1 is being expressed is an indication of the requirement of the slit-robo interactions in lung development.

Slit2$^{-/-}$ mouse embryos suffer from major kidney abnormalities (11). The E18.5 (just before birth) embryos exhibit a condition called hydroureters in which the collecting ducts and ureters are grossly dilated. In addition, the region where the new nephrons are being generated extends into the interior of the mutant kidney. The cause for such abnormalities is the formation of supernumerary ureteric buds (UB). The precise control of ureteric bud formation, which insures that only one is formed, is a crucial step in normal kidney development. *Robo2*$^{-/-}$ embryos exhibit a similar case of supernumerary ureteric bud formations but to a lesser extent. The site of the Slit2-Robo2 interaction is in the nephrogenic mesenchyme. It is also where the signal for promoting UB formation is generated. This signal is the glial-derived neurotrophic factor (GDNF) and its interaction with its RET receptor tyrosine kinase. Therefore, Slit2/Robo2 interaction may act as a counter-balance for the growth-promoting effects of GDNF/RET interaction.

Robo1$^{-/-}$ embryos do not exhibit detectable abnormalities in kidney development which indicates that Slit2-Robo2 interaction and not Slit2/Robo1 interaction is important for kidney development (11). The latter interaction maybe more important for correct lung development.

2. HEMI- AND HOMOZYGOUS DELETIONS AT THE *SLIT-ROBO* LOCI IN HUMAN DISEASES

By use of hemi and homozygosity mapping, cytogenetic analysis and functional studies, 3p12 region has been shown to be important for the development of several common sporadic cancers including, lung, breast, kidney, ovarian, cervical and head and neck cancer (31,32). In addition, several overlapping homozygous deletions and cases of LOH have been reported at 3p12.3. An 8 MB homozygous deletion in the U2020 small cell lung carcinoma cell line flanked by microsatellite markers D3S1284 and D3S1276 was initially reported (33-35). Analysis of this region using the

May 2004 assembly of the human genome at UCSC (http://genome.ucsc.edu/) reveals that this deletion is around 12.3 Mb in length and harbours in addition to *ROBO1* and *ROBO2*, *GBE1*, *CONTACTIN3* and *PPP4R2*. A 1 MB homozygous deletion overlapping the U2020 deletion was reported in SCLC tumour sample (36). A breast tumour line (HCC38) was also found to harbour a homozygous deletion spanning a region of 4.2 MB containing only *ROBO1* and *ROBO2* loci (37). A further deletion, 110 kb in size was characterized from another SCLC cell line, NCI-H2196, this deletion overlaps the homozygous deletions characterized in the HCC38 and the U2020 tumour cell lines. This homozygous deletion maps at the D3S1274 microsatellite marker at 3p12.3 in intron 2 of *ROBO1B* (37). Both *ROBO3* and *ROBO4* are located at chromosome 11q24.2. There are no reported LOH or homozygous deletions for this region in human cancers. Therefore genetic mapping short-listed *ROBO1* as a possible candidate tumour suppressor gene.

SLIT1 is located at chromosome 10q24.1. Deletions in this region have been implicated in hereditary spastic paraplegia, split hand/split foot malformation, and familial temporal lobe epilepsy (38,39,39,40). One could hypothesise that genetic alterations in the brain-specific *SLIT1* locus may be associated with other neurodegenerative disorders yet to be identified. Reports linking 10q24.1 chromosome region with carcinogenesis are not common. The *SLIT2* gene was mapped to chromosome 4p15.2. Microsatellite markers from this region show LOH in 63% of mesothelioma (41), 60% of SCLC (41), and 25% of NSCLC (41). Around 63% of breast tumours also show LOH at 4p15.1–15.3 (42). Deletions of the 4p15.1–15.3 region have also been reported to occur in colorectal carcinoma, (43) invasive cervical cancer (44), head and neck squamous cell carcinoma (45), and bladder cancer (46). LOH in the *SLIT3* region at chromosome 5q34-5q35.1 have been reported in malignant ovarian germ cell tumours (47). Furthermore, LOH at 5q34 –q35 is associated with the progression of urinary bladder cancer (48). In addition, genetic variation in *SLIT3* is also suggested to be associated with schizophrenia in Chinese populations (49)

3. SLIT-ROBO INTERACTIONS IN INFLAMMATION

The SLIT-ROBO family of genes was initially identified as key molecules in mediating attractive or repulsive cues to guide axons during the development of the nervous system (50). The expression patterns of the SLIT-ROBO family in non-neural adult tissues may indicate other functions that could be interpreted in the context of cancer development. One such

function is the ability to control cell migration. After establishing that rat slit2 and slit3 and their receptors robo1 and robo2 are expressed in non-neuronal tissues, and only slit1 is brain-specific, Wu *et al.* (2001) went on to show that Slit2 was able to block SDF-1 and f-Met-Leu-Phe (fMLP)-induced chemotaxis of lymphocytes isolated from lymph nodes or differentiated HL-60 cells in a dose-dependent manner. This inhibitory effect is Robo-dependent since a dominant negative fragment of Robo containing only the extracellular fragment abolished the inhibitory effect of slit2 on leukocytes chemotaxis (21). The endothelial-specific ROBO4 can also act as a receptor for SLIT2 and inhibits vascular endothelial growth factor (VEGF) or serum-induced cell migration in human microvascular endothelial cells HMVEC and or fibroblast growth factor(FGF)-induced migration in HEK293 expressing exogenous ROBO4 (16). However, it was later shown that a soluble fragment of ROBO4 is able to block endothelial cell growth and migration independently of slit binding (17). In a contradiction to all of the above studies, Wang *et al.* (2003) found that slit2 actually promotes endothelial cell migration and that the slit-robo interaction plays a role in promoting tumour angiogenesis (22). This would represent the first and only evidence of Slit having pro-migratory properties, as all previous studies of Slits in neurons and leukocytes have shown them to be repulsive factors. Such studies have shown that SLIT2 can act as a negative regulator for chemokine-induced leukocytes chemotaxis *in vitro*. The *in vivo* evidence for an anti-inflammatory role for SLIT2 was shown when it was successfully used to treat experimental crescentic glomerulonephritis in rats (51). Since chronic inflammation is one of the causes of tumour development, the anti-inflammatory role for SLIT2 may be a protective role against tumour development.

4. SLIT-ROBO INTERACTIONS IN CANCER

Xian *et a.l* (2001) generated mice with a targeted deletion of exon 2 of *dutt1/robo1* that eliminates the first Ig domain of the molecule (30). This deletion mimics the naturally occurring homozygous deletion of *ROBO1* in NCI-H2196 SCLC cell line. The homozygous mutant mice usually die at birth of respiratory failure due to delayed lung maturation. The surviving litter mates usually die within 7 months of their first year as they accumulate various lung abnormalities including hyperplasia (30). *Robo1*$^{+/-}$ heterozygous mice survive beyond the first year of birth. However, these mice exhibit a significant increase in predisposition to tumours that are often invasive, compared with wild-type controls (52). We have shown that overexpression of *SLIT2* in breast (53), colorectal (54) and glioma (55) cancer cell lines

reduced their ability to form colonies. In addition SLIT2-conditioned media caused significant increase in breast cancer cell death (53) and induced apoptosis in colorectal cell lines (54). MCF-7 breast carcinoma cell lines stably expressing *SLIT2* have reduced ability to grow in soft-agar compared to vector-only transfected controls.

The potential role for the slit-robo interactions in affecting tumour invasion and metastasis has been recently suggested (56). SLIT2 was found to have an antagonising role on the pro-migration and pro-invasion effects of the CXCR4 chemokine receptor and its chemokine ligand, CXCL12. Aberrant expression and activation of the CXCL12/CXCR4 has been demonstrated to be associated with tumour invasion and metastasis in several cancer types (57). Prasad *et al.* (56) demonstrated that both *ROBO1* and *ROBO2* are expressed at cell surfaces of breast cancer cells, in addition to showing by immunohistochemistry that breast cancer tissues express *ROBO1* and *ROBO2* at various degrees. Treatment with SLIT2 inhibited the CXCL12/CXCR4-induced breast cancer cell migration, invasion or adhesion. These effects were not mediated by direct binding of SLIT2 to CXCR4 or reducing the binding ability of CXCL12 to CXCR4. Instead, SLIT2 was found to inhibit the activity of Src kinase, which is a key player in controlling focal adhesion dynamics. This SLIT2-mediated inhibition antagonised the CXCL12-induced tyrosine-phosphorylation and subsequent activation of RAFTK, FAK and Paxillin. The PI3-Kinase activity was also reduced subsequently to SLIT2 treatment, independently of PTEN activity. The CXCL12-induced activities of the matrix metalloproteinases MMP-2 and MMP-9 were also inhibited upon SLIT2 treatment. The inhibition of the p44/42 MAP Kinase activity without affecting the p38 MAP kinase or JNK kinase activities following SLIT2 treatment may indicate that SLIT2 can counteract the effects of other cytokines growth factors that use the same signalling pathway (56) In support of this study, tumours forming spontaneously in *Robo*$^{+/-}$ mice are often found to be invasive which may further implicate slit-robo interaction in controlling invasion and metastasis (52).

How does ROBO1/2 interaction with SLIT2 inhibit cellular migration and invasion? SLIT2 can antagonise the tyrosine phosphorylation of proteins in the focal adhesion pathway. Abelson tyrosine kinase (Abl), which is related to the Src family of tyrosine kinase can antagonise Robo function by phosphorylating several tyrosine residues in the cytoplasmic domain (4). One could speculate that binding of ROBO1/2 to SLIT2 can trigger the dephosphorylation of ROBO1/2 hence, activating the receptors. The link between Robo and the cytoskeleton has already been established by demonstrating that Robo1 interacts with a novel family of RhoGAP proteins, designated the slit-roboGAP (srGAPs) (6). GAPs (GTPases activating

proteins) negatively regulate the activity of the RhoGTPases such as CDC42, RAC1 or RhoA and therefore reduce actin polymerisation, filopodia and lamellipodia formation and the subsequent reduction in cell movement. Slit binding to Robo increases the interaction between the SH3 domain of the srGAPs and the CC3 motif in Robo. This interaction may interfere with Abl binding and the subsequent phosphorylation of Robo1, hence maintaining Robo1 in the active state. The active state of Robo1 in this case entails the increase in the intrinsic GTPase activity of at least Cdc42, resulting in its inactivation and the subsequent decrease in actin polymerisation and reduction in cell motility and migration.

5. EPIGENETICS IN CANCER

Inactivation of tumour suppressor genes can occur by genetic and/or epigenetic mechanisms. The genetic alterations include hemi- or homozygous deletion, and mutations. The epigenetic silencing of genes is achieved through interplay between histone modifications and DNA methylation and their effect on the chromatin structure and promoter accessibility. The nucleosomes, the building blocks of the chromatin consist of DNA wrapped around histones. Post-translational modifications of these histones (acetylation, methylation or phosphorylation) mediate the histone code. For example, hypoacetylation of histone 3 and histone 4 are associated with heterochromatic and transcriptionally inactive regions of the genome. In mammals, the major target for DNA methylation (addition of a methyl group to the 5'-carbon of cytosine) is a cytosine located next to a guanine forming what is known as a CpG dinucleotide. The non-random distribution of this CpG dinucleotide often results in its clustering in CpG islands. A typical CpG island is >200 base pairs with a GC content >50% and an observed:expected ratio of CpG ≥0.6. CpG islands are often found in the 5'-regions of house-keeping genes or to a lesser extent in tissue-specific regulated genes. Most CpG islands are unmethylated in normal cells with few exceptions that include imprinted genes. Introduction of bisulphite treatment of genomic DNA expedited the discovery of novel tumour suppressor genes that are silenced by CpG island hypermethylation (58). In this method, the chemical treatment results in the conversion of unmethylated cytosines to uracils leaving the methylcytosines unconverted. Polymerase chain reaction (PCR)-based assays were developed to discriminate between methylated and unmethylated alleles in a relatively easy fashion (59). Tumour-specific hypermethylation of promoters have been described for an increasing number of tumour suppressor genes (60,61). In fact, the recently discovered tumour suppressor gene, *RASSF1A*,

is almost exclusively inactivated by tumour-specific hypermethylation of its promoter region (62-64). It was also demonstrated that genes silenced by promoter hypermethylation can be reactivated by treatment with the demethylating drug 5-aza-2'-deoxycytidine, either alone or in combination of an histone deacetylases (HDAC) inhibitor such as trichostatin A. Therefore, unlike genetic inactivation, which is irreversible, epigenetic inactivation of tumour suppressor genes is a reversible process. This fact renders tumours treatable by DNA demethylating agents (65) and/or HDAC inhibitors (66).

6. INACTIVATION OF THE *SLIT-ROBO* GENES IN HUMAN CANCER

Chromosome 3p deletions are a frequent event in human cancers. The region 3p21.3 harbours the *RASSF1* tumour suppressor gene. We and others have demonstrated that Isoform A of *RASSF1* is frequently inactivated in most forms of childhood and adult cancers by promoter region CpG island hypermethylation. The other region of interest is 3p12. LOH in this region is a frequent event in human carcinoma which indicated that there could be at least one tumour suppressor gene in this region. Following the publication of the human genome sequence, it was apparent that the region at 3p12 exhibiting the most deletions and rearrangements in cancer, harbours only two genes, *ROBO1* and *ROBO2*. *ROBO1* was of particular interest since it is homozygously deleted in three lung and breast cancer cell lines. Therefore, we performed a comprehensive mutation screen of the full coding region of the gene in lung and breast cancers (67). Seven germ-line nucleotide substitutions were found, only two resulted in amino-acid substitutions. Expression analysis of the *ROBO1B* gene in a panel of cancer cell lines demonstrated loss of expression in a breast tumour cell line, and expression was restored after treatment with 5-azacytidine. This finding, along with the absence of inactivating somatic mutations suggested that *ROBO1B* maybe inactivated by an epigenetic mechanism in a subset of tumours, namely by hypermethylation of its promoter region. Hypermethylation analysis of the 5' CpG island in a variety of tumour cell lines and primary tumours demonstrated that *ROBO1B* is inactivated by hypermethylation of its promoter region in some breast, kidney, lung and colorectal cancers (Table 1) (67). Furthermore, majority of tumours with *ROBO1B* methylation also underwent allelic loss at the 3p12.3 region. Recently Xian *et al.*, demonstrated that the wild type *Robo1* allele in tumours from *Robo1*$^{+/-}$ mice was silenced by hypermethylation (52). This observation is similar to what has been shown in *Hic1*$^{+/-}$ mice. These mice showed increased

predisposition to various cancers which was associated with the hypermethylation of the remaining allele in the resulting tumour (68,69). *ROBO1B* inactivation follows to a large extent the Knudson two-hit hypothesis for tumour suppressor genes (70). Considering the high level of LOH and overlapping homozygous deletions in the 3p12 region detected in human cancers, it was hypothesized that haploinsufficiency of ROBO1 receptor coupled with haploinsufficiency or inactivation of it ligands can be detrimental for tumour progression and metastasis. Therefore, it was logical to perform the analysis of *SLIT2* mutation and expression status in human cancers. *SLIT2* was the most obvious choice among the three human *SLIT* genes since LOH and rearrangements at its genomic locus at 4p15 were detected in a significant proportion of human cancers (41-44). A comprehensive screen for mutations in *SLIT2* coding region in lung and breast cancers revealed, in addition to polymorphisms, a couple of missense substitutions in one of the EGF-like domains.[53] Expression analysis revealed a significant proportion of tumour cell lines with reduced or absent *SLIT2* expression (53-55). Analysis of the *SLIT2* promoter region in these tumour cell lines revealed the presence of extensive hypermethylation of *SLIT2* 5' CpG Island. Lack or reduced expression of *SLIT2* correlated with CpG hypermethylation as treatment with the demethylating agent 5'-azacytidine restored *SLIT2* expression. Screening various human cancer types for the presence of *SLIT2* methylation using a combination of CoBRA (Cobined Bisulphite and Restriction Analysis), MSP (Methylation-Sensitive PCR) or direct sequencing of bisulphite-modified DNA of the promoter region demonstrated that *SLIT2* promoter region CpG island is extensively methylated in a significant proportion of lung, breast, colorectal and glioma tumours and less frequently in kidney, neuroblastoma and Wilm's tumours (53-55,71) (Table 1).

Table 1. Levels of epigenetic inactivation of ROBO1, SLIT1, SLIT2 and SLIT3 in human cancers. %Methylation is percentage of methylated samples in a cohort of samples. * indicates the detection of low levels of SLIT2 methylation in paired normal mucosa corresponding to the tumour analysed. SLIT1 expression is brain-specific therefore analysis of its methylation status was attempted only in glioma. (-) indicates not analysed.

Cancer Type	***ROBO1*** **%Methylation**	***SLIT1*** **%Methylation**	***SLIT2*** **%Methylation**	***SLIT3*** **%Methylation**	**References**
Sporadic Breast cancer					
cell lines	19		59	41	53,67,72
Primary tumours	20		43	16	
Paired non-malignant tissue	0		14	-	
Sporadic NSCLC					
cell lines	20		77	33	53,67
Primary tumours	4		53	-	
Paired non-malignant tissue	0		13	-	
Sporadic SCLC					
cell lines	20		55	0	53,67,72
Primary tumours	0		36	-	
Paired non-malignant tissue	0		8	-	
Colorectal cancer					
cell lines	-		83	33	54,67,72
Primary tumours	19		72	37	
Paired normal mucosa	0		*	0	
Glioma					
cell lines	-	83	71	29	55,72
Primary tumours	-	10	59	35	
Normal Brain	-	0	0	0	

Table 1 (cont.)

Cancer Type	*ROBO1* %Methylation	*SLIT1* %Methylation	*SLIT2* %Methylation	*SLIT3* %Methylation	References
Neuroblastoma					
cell lines	-		25	-	71
Primary tumours	-		29	-	
Blood controls	-		2	-	
Clear Cell Renal Carcinoma					
cell lines	-		75	-	71
Primary tumours	18		25	-	
Paired non-malignant tissue	0		8	-	
Wilms' tumours	-		38	-	71
Paired non-malignant tissue	-		0	-	

Subsequently, *SLIT2* expression as detected by quantitative real-time RT-PCR demonstrated that where tumours exhibit *SLIT2* methylation, gene expression is reduced in relation to non-methylated tumours or normal tissues (53-55). It is worth noting the detection of some *SLIT2* methylation in a number of adjacent histologically normal matched tissues and mucosa (53-55). This may indicate that *SLIT2* inactivation is an early step in tumour progression. However, considering the accumulating evidence for the role of *SLIT2* in inhibiting migration and chemotaxis, another possibility is that *SLIT2* methylation in histologically normal tissues maybe caused by the presence of invading tumour cells. This is consistent with the findings that robo1+/- mice exhibit increased frequency of spontaneous invasive tumours (52). The hypermethylation status of both *SLIT1* and *SLIT3* was also investigated (Table 1) (72). *SLIT1* expression is brain-specific, therefore, only glioma samples were analysed. *SLIT1* was frequently methylated in glioma tumour cell lines but was infrequently methylated in glioma tumours, therefore, *SLIT1* may play a role in late gliomagenesis. *SLIT3* inactivation, however, was more common but remained less frequent than the incidence of *SLIT2* methylation (72). So far no correlation was found between SLIT-ROBO inactivation and clinicopathological characteristics of tumour analysed. However, we have demonstrated that *SLIT2* hypermethylation can be detected in ductal carcinoma in situ (DCIS) from breast cancer patients and in sputum from lung cancer patients (Dickinson and Latif unpublished observation).

7. THE INVOLVEMENT OF OTHER AXON GUIDANCE EFFECTORS IN CANCER

The Slits are members of a conserved group of axon guidance cues. The other members include the netrins, the semaphorins and the ephrins. The slits, semaphorins, and ephrins generally act as repellents while the netrins are considered to be generally attractants during the neural and mesodermal cell migration. Each family of guidance cues have their own transmembrane receptors; the slits have the robos, the UNC-5/DCC act as a receptor for the netrins; Neuropilin and plexin receptors bind to the semaphorins; The ephrins are a ligand for the Eph receptors (73). There is accumulating evidence that implicates the axon guidance cues in tumour development. One of the best characterised is *SEMA3B*. *SEMA3B* is located at chromosome 3p21.3. This region exhibits a high frequency of hemi- and homozugous deletions in several types of cancers (74). *SEMA3B* is frequently inactivated by a combination of promoter region hypermethylation and allele loss in NSCLC (75) The down-regulation of

SEMA3B was found to be associated with acquiring a metastatic phenotype in lung cancer cell lines (76). The gene has tumour-suppression activity in NSCLC[77] and ovarian cancer cell lines (78) in addition to its ability to induce apoptosis in lung and breast cancer cell lines (77,79). *SEMA3B* antagonises the binding of the tumour-promoting VEGF to neuropilins receptors (79). *SEMA3F* is another semaphorin that is located at 3p21.3. As with *SEMA3B*, *SEMA3F* exhibits tumour suppressor ability (80). Loss or delocalization of *SEMA3F* from the cell membrane to a cytoplasmic location correlates with advanced tumour stage (81-83). Abrogation of *SEMA3A* expression appears to be responsible for VEGF-induced tumour growth (84). *DCC* or Deleted in Colorectal Cancer is thought to be a tumour suppressor gene in colorectal cancer based on its high frequency of allele loss and down-regulation of its expression (85). Despite the lack of inactivating mutations or significant tumour-specific hypermethylation of its promoter region (86), and lack of cancer predisposition in *DCC* mutant mice (87), *DCC* is emerging as an important conditional TSG. DCC/UNC5 function as dependence receptors, i.e., they emit a survival signal when bound to their ligand, Netrin-1. However, when the Netrin-1 is unbound, the receptors emit pro-apoptotic signals (88,89).

8. FUTURE DIRECTIONS

Robo1$^{+/-}$ mice show an increased predisposition to invasive tumours. The wild-type allele in the resulting tumours is heavily methylated at the promoter CpG island. This results in substantial reduction of *Robo1* protein expression in these tumours. Hence at least in mice, *Robo1* acts as a classical tumour suppressor gene where inactivation of both alleles is required for tumour development. *Slit2* homozygosity is embryonic lethal, so the following questions are raised; Are *Slit2*$^{+/-}$ mice also prone to increased spontaneous and or induced tumour formation? Would the wild-type allele in the resulting tumours be inactivated by methylation? Answers to the above questions will further our understanding of the role of these proteins in cancer development. One could generate double mutant *Slit2*$^{+/-}$/*Robo1*$^{+/-}$ mice which could serve as a model for the situation often observed in human cancers with the hypermethylation of *SLIT2* and the loss of heterozygozity of *ROBO1* allele.

It is possible to utilize the RNA interference to knockdown the expression of the different *slits* and *robos* in primary and untransformed mammalian cell lines and analyse the resulting phenotype for increased tumourigenesity and spontaneous transformation. The same approach could be used to dissect the roles of the different SLIT and ROBO proteins on the

phenotype of cell lines derived from different tissue background. We could look at synergism or even antagonism between the different robo and soluble slit proteins in response to specific stimuli. Large proteomic screens could be carried out to identify further SLIT(s) and ROBO(s) associated proteins. This will help in determining the pathways in which these genes may play critical roles in relation to human cancers.

Expression analysis of the slit-robo genes and the subsequent analysis of their promoter regions for hypermethylation in a larger cohort of samples from different tumours could help in identifying associations with clinicopathological stages. Our preliminary results demonstrate *SLIT2* methylation in ductal carcinoma in situ (DCIS) from breast cancer patients. This may indicate that, at least in breast cancer, *SLIT2* hypermethylation maybe an early event. So far, the highest frequency of *SLIT2* methylation was found in colorectal carcinomas. For colorectal tumour development, there is a comprehensively characterised adenoma-carcinoma sequence (90), therefore one could analyse *SLIT2* methylation in early to late adenomas following the possible increase in methylation and the progression of the disease. Increasingly, methylation profiling is being developed as a non-invasive screening tool for early cancer detection that can be used for screening populations at risk. *SLIT2* was also frequently methylated in lung cancer, one could analyse *SLIT2* methylation in sputum from lung cancer patients, as well as sputum from individuals at risk of developing lung cancer (smokers versus non-smokers). The *SLIT2* gene resides at 4p15.2, this region undergoes frequent loss of heterozygosity in several other cancers that so far have not been analysed for *SLIT2* methylation, including, cervical, head and neck and bladder cancer.

Slit-robo family of genes was first discovered in *Drosophila* in which many candidate tumour suppressor genes have been identified (91,92). Until recently this resource has been overlooked, but in recent years there have been reports on tumour suppressor genes that were first identified in *Drosophila* that are also inactivated in human cancers and can cause tumour susceptibility in mice. The fly model therefore presents exciting opportunities to identify novel tumour suppressor genes that have relevance to human cancer. The recent publication of the protein interaction map of the fly proteome will aid in determining the functional pathways regulated by the candidate tumour suppressor genes (93).

REFERENCES

1. Seeger M., Tear G., Ferres-Marco D. and Goodman C. S. Mutations affecting growth cone guidance in Drosophila: genes necessary for guidance toward or away from the midline. Neuron, 1993; 10: 409-426.
2. Kidd T., Brose K., Mitchell K. J., Fetter R. D., Tessier-Lavigne M., Goodman C. S. and Tear, G. Roundabout controls axon crossing of the CNS midline and defines a novel subfamily of evolutionarily conserved guidance receptors. Cell, 1998; 92: 205-215.
3. Sundaresan V., Roberts I., Bateman A., Bankier A., Sheppard M., Hobbs C., Xiong J., Minna J., Latif F., Lerman M. and Rabbitts P. The DUTT1 gene, a novel NCAM family member is expressed in developing murine neural tissues and has an unusually broad pattern of expression. Mol. Cell Neurosci., 1998; 11: 29-35.
4. Bashaw G. J., Kidd T., Murray D., Pawson T. and Goodman, C. S. Repulsive axon guidance: Abelson and Enabled play opposing roles downstream of the roundabout receptor. Cell, 2000; 101: 703-715.
5. Stein E. and Tessier-Lavigne M. Hierarchical organization of guidance receptors: silencing of netrin attraction by slit through a Robo/DCC receptor complex. Science, 2001; 291: 1928-1938.
6. Wong K., Ren X. R., Huang Y. Z., Xie Y., Liu G., Saito H., Tang H., Wen L., Brady-Kalnay S. M., Mei L., Wu J. Y., Xiong W. C. and Rao Y. Signal transduction in neuronal migration: roles of GTPase activating proteins and the small GTPase Cdc42 in the Slit-Robo pathway. Cell, 2001, 107: 209-221.
7. Emerson M. M. and Van Vactor D. Robo is Abl to block N-Cadherin function. Nat. Cell Biol., 2002; 4: E227-E230.
8. Rhee J., Mahfooz N. S., Arregui C., Lilien J., Balsamo J. and VanBerkum M. F. Activation of the repulsive receptor Roundabout inhibits N-cadherin-mediated cell adhesion. Nat. Cell Biol., 2002; 4: 798-805.
9. Clark K., Hammond E. and Rabbitts P. Temporal and spatial expression of two isoforms of the Dutt1/Robo1 gene in mouse development. FEBS Lett., 2002, 523: 12-16.
10. Nagase T., Kikuno R., Nakayama M., Hirosawa M. and Ohara O. Prediction of the coding sequences of unidentified human genes. XVIII. The complete sequences of 100 new cDNA clones from brain which code for large proteins in vitro. DNA Res., 2000; 7: 273-281.
11. Grieshammer U., Le M., Plump A. S., Wang F., Tessier-Lavigne M. and Martin G. R. SLIT2-mediated ROBO2 signaling restricts kidney induction to a single site. Dev. Cell, 2004; 6: 709-717.
12. Yuan S. S., Cox L. A., Dasika G. K. and Lee E. Y. Cloning and functional studies of a novel gene aberrantly expressed in RB-deficient embryos. Dev. Biol., 1999, 207: 62-75.
13. Yuan S. S., Yeh Y. T. and Lee E. Y. Pax-2 interacts with RB and reverses its repression on the promoter of Rig-1, a Robo member. Biochem. Biophys. Res. Commun., 2002, 296: 1019-1025.
14. Sabatier C., Plump A. S., Le M., Brose K., Tamada A., Murakami F., Lee E. Y. and Tessier-Lavigne M. The divergent Robo family protein rig-1/Robo3 is a negative regulator of slit responsiveness required for midline crossing by commissural axons. Cell, 2004; 117: 157-169.
15. Huminiecki L., Gorn M., Suchting S., Poulsom R. and Bicknell R. Magic roundabout is a new member of the roundabout receptor family that is endothelial specific and expressed at sites of active angiogenesis. Genomics, 2002; 79: 547-552.

16. Park K. W., Morrison C. M., Sorensen L. K., Jones C. A., Rao Y., Chien C. B., Wu J. Y., Urness L. D. and Li D. Y. Robo4 is a vascular-specific receptor that inhibits endothelial migration. Dev. Biol., 2003; 261: 251-267.
17. Suchting S., Heal P., Tahtis K., Stewart L. M. and Bicknell, R. Soluble Robo4 receptor inhibits in vivo angiogenesis and endothelial cell migration. FASEB J., 2004.
18. Brose K., Bland K. S., Wang K. H., Arnott D., Henzel W., Goodman C. S., Tessier-Lavigne M. and Kidd T. Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance. Cell, 1999; 96: 795-806.
19. Itoh A., Miyabayashi T., Ohno M. and Sakano S. Cloning and expressions of three mammalian homologues of Drosophila slit suggest possible roles for Slit in the formation and maintenance of the nervous system. Brain Res. Mol. Brain Res., 1998, 62: 175-186.
20. Li H. S., Chen J. H., Wu W., Fagaly T., Zhou L., Yuan W., Dupuis S., Jiang Z. H., Nash W., Gick C., Ornitz D. M., Wu J. Y. and Rao Y. Vertebrate slit, a secreted ligand for the transmembrane protein roundabout, is a repellent for olfactory bulb axons. Cell, 1999; 96: 807-818.
21. Wu J. Y., Feng L., Park H. T., Havlioglu N., Wen L., Tang H., Bacon K. B., Jiang Z., Zhang X. and Rao Y. The neuronal repellent Slit inhibits leukocyte chemotaxis induced by chemotactic factors. Nature, 2001; 410: 948-952.
22. Wang B., Xiao Y., Ding B. B., Zhang N., Yuan X., Gui L., Qian K. X., Duan S., Chen Z., Rao Y. and Geng J. G. Induction of tumor angiogenesis by Slit-Robo signaling and inhibition of cancer growth by blocking Robo activity. Cancer Cell, 2003; 4: 19-29.
23. Plump A. S., Erskine L., Sabatier C., Brose K., Epstein C. J., Goodman C. S., Mason C. A. and Tessier-Lavigne M. Slit1 and Slit2 cooperate to prevent premature midline crossing of retinal axons in the mouse visual system. Neuron, 2002; 33: 219-232.
24. Liu J., Zhang L., Wang D., Shen H., Jiang M., Mei P., Hayden P. S., Sedor J. R. and Hu H. Congenital diaphragmatic hernia, kidney agenesis and cardiac defects associated with Slit3-deficiency in mice. Mech. Dev., 2003; 120: 1059-1070.
25. Yuan W., Rao Y., Babiuk R. P., Greer J. J., Wu J. Y. and Ornitz D. M. A genetic model for a central (septum transversum) congenital diaphragmatic hernia in mice lacking Slit3. Proc. Natl. Acad. Sci. U.S.A, 2003; 100: 5217-5222.
26. Liu Z., Patel K., Schmidt H., Andrews W., Pini A. and Sundaresan V. Extracellular Ig domains 1 and 2 of Robo are important for ligand (Slit) binding. Mol. Cell Neurosci., 2004; 26: 232-240.
27. Battye R., Stevens A., Perry R. L. and Jacobs J. R. Repellent signaling by Slit requires the leucine-rich repeats. J. Neurosci., 2001; 21: 4290-4298.
28. Liang Y., Annan R. S., Carr S. A., Popp S., Mevissen M., Margolis R. K. and Margolis R. U. Mammalian homologues of the Drosophila slit protein are ligands of the heparan sulfate proteoglycan glypican-1 in brain. J. Biol. Chem., 1999; 274: 17885-17892.
29. Anselmo M. A., Dalvin S., Prodhan P., Komatsuzaki K., Aidlen J. T., Schnitzer J. J., Wu J. Y. and Kinane, T. B. Slit and robo: expression patterns in lung development. Gene Expr. Patterns., 2003; 3: 13-19.
30. Xian J., Clark K. J., Fordham R., Pannell R., Rabbitts T. H. and Rabbitts P. H. Inadequate lung development and bronchial hyperplasia in mice with a targeted deletion in the Dutt1/Robo1 gene. Proc. Natl. Acad. Sci. U.S.A, 2001; 98: 15062-15066.
31. Todd S., Franklin W. A., Varella-Garcia M., Kennedy T., Hilliker C. E., Jr., Hahner L., Anderson M., Wiest J. S., Drabkin H. A. and Gemmill R. M. Homozygous deletions of human chromosome 3p in lung tumors. Cancer Res., 1997; 57: 1344-1352.
32. Kok K., Naylor S. L. and Buys C. H. Deletions of the short arm of chromosome 3 in solid tumors and the search for suppressor genes. Adv. Cancer Res., 1997; 71: 27-92.

33. Drabkin H. A., Mendez M. J., Rabbitts P. H., Varkony T., Bergh J., Schlessinger J., Erickson P. and Gemmill R. M. Characterization of the submicroscopic deletion in the small-cell lung carcinoma (SCLC) cell line U2020. Genes Chromosomes. Cancer, 1992; 5: 67-74.
34. Latif F., Tory K., Modi W. S., Graziano S. L., Gamble G., Douglas J., Heppell-Parton, A. C., Rabbitts, P. H., Zbar, B., and Lerman, M. I. Molecular characterization of a large homozygous deletion in the small cell lung cancer cell line U2020: a strategy for cloning the putative tumor suppressor gene. Genes Chromosomes. Cancer, 1992; 5: 119-127.
35. Rabbitts P., Bergh J., Douglas J., Collins F. and Waters J. A submicroscopic homozygous deletion at the D3S3 locus in a cell line isolated from a small cell lung carcinoma. Genes Chromosomes. Cancer, 1990; 2: 231-238.
36. Todd S., Franklin W. A., Varella-Garcia M., Kennedy T., Hilliker C. E., Jr., Hahner L., Anderson M., Wiest J. S., Drabkin H. A. and Gemmill R. M. Homozygous deletions of human chromosome 3p in lung tumors. Cancer Res., 1997; 57: 1344-1352.
37. Sundaresan V., Chung G., Heppell-Parton A., Xiong J., Grundy C., Roberts I., James L., Cahn A., Bench A., Douglas J., Minna J., Sekido Y., Lerman M., Latif F., Bergh J., Li H., Lowe N., Ogilvie D. and Rabbitts P. Homozygous deletions at 3p12 in breast and lung cancer. Oncogene, 1998; 17: 1723-1729.
38. Kang Y. S., Cheong H. M., Moon Y., Lee I. B., Kim S. M., Kim H. S., Jun S. Y., Jung S. K., Kim J. S., Choi J. H., Cho H. E., Son J. S., Min N. Y. and Lee K. H. Molecular genetic characterization of a Korean split hand/split foot malformation (SHFM). Mol. Cells, 2004; 17: 397-403.
39. Meijer I. A., Cossette P., Roussel J., Benard M., Toupin S. and Rouleau G. A. A novel locus for pure recessive hereditary spastic paraplegia maps to 10q22.1-10q24.1. Ann. Neurol., 2004; 56: 579-582.
40. Lo N. C., Cusano R., Gigli G. L., Forabosco P., Valente M., Ravazzolo R., Diomedi M. and Seri M. Genetic heterogeneity in inherited spastic paraplegia associated with epilepsy. Am. J. Med. Genet., 2003; 117A: 116-121.
41. Shivapurkar N., Virmani A. K., Wistuba I. I., Milchgrub S., Mackay B., Minna J. D. and Gazdar A. F. Deletions of chromosome 4 at multiple sites are frequent in malignant mesothelioma and small cell lung carcinoma. Clin. Cancer Res., 1999; 5: 17-23.
42. Shivapurkar N., Sood S., Wistuba I. I., Virmani A. K., Maitra A., Milchgrub S., Minna J. D. and Gazdar A. F. Multiple regions of chromosome 4 demonstrating allelic losses in breast carcinomas. Cancer Res., 1999; 59: 3576-3580.
43. Shivapurkar N., Maitra A., Milchgrub S. and Gazdar A. F. Deletions of chromosome 4 occur early during the pathogenesis of colorectal carcinoma. Hum. Pathol., 2001; 32: 169-177.
44. Sherwood J. B., Shivapurkar N., Lin W. M., Ashfaq R., Miller D. S., Gazdar A. F. and Muller C. Y. Chromosome 4 deletions are frequent in invasive cervical cancer and differ between histologic variants. Gynecol. Oncol., 2000; 79: 90-96.
45. Pershouse M. A., El Naggar A. K., Hurr K., Lin H., Yung W. K. and Steck P. A. Deletion mapping of chromosome 4 in head and neck squamous cell carcinoma. Oncogene, 1997; 14: 369-373.
46. Polascik T. J., Cairns P., Chang W. Y., Schoenberg M. P. and Sidransky, D. Distinct regions of allelic loss on chromosome 4 in human primary bladder carcinoma. Cancer Res., 1995; 55: 5396-5399.
47. Faulkner S. W. and Friedlander M. L. Molecular genetic analysis of malignant ovarian germ cell tumors. Gynecol. Oncol., 2000; 77: 283-288.

48. von Knobloch R., Bugert P., Jauch A., Kalble T, and Kovacs G. Allelic changes at multiple regions of chromosome 5 are associated with progression of urinary bladder cancer. J. Pathol., 2000; 190: 163-168.
49. Shi Y., Zhao X., Yu L., Tao R., Tang J., La Y., Duan Y., Gao B., Gu N., Xu Y., Feng G., Zhu S., Liu H., Salter H. and He L. Genetic structure adds power to detect schizophrenia susceptibility at SLIT3 in the Chinese Han population. Genome Res., 2004; 14: 1345-1349.
50. Brose K. and Tessier-Lavigne M. Slit proteins: key regulators of axon guidance, axonal branching, and cell migration. Curr. Opin. Neurobiol., 2000; 10: 95-102.
51. Kanellis J., Garcia G. E., Li P., Parra G., Wilson C. B., Rao Y., Han, S., Smith C. W., Johnson R. J., Wu J. Y. and Feng L. Modulation of inflammation by slit protein in vivo in experimental crescentic glomerulonephritis. Am. J. Pathol., 2004; 165: 341-352.
52. Xian J., Aitchison A., Bobrow L., Corbett G., Pannell R., Rabbitts T. and Rabbitts P. Targeted disruption of the 3p12 gene, Dutt1/Robo1, predisposes mice to lung adenocarcinomas and lymphomas with methylation of the gene promoter. Cancer Res., 2004; 64: 6432-6437.
53. Dallol A., Da Silva N. F., Viacava P., Minna J. D., Bieche I., Maher E. R. and Latif F. SLIT2, a human homologue of the Drosophila Slit2 gene, has tumor suppressor activity and is frequently inactivated in lung and breast cancers. Cancer Res., 2002; 62: 5874-5880.
54. Dallol A., Morton D., Maher E. R. and Latif F. SLIT2 axon guidance molecule is frequently inactivated in colorectal cancer and suppresses growth of colorectal carcinoma cells. Cancer Res., 2003; 63: 1054-1058.
55. Dallol A., Krex D., Hesson L., Eng C., Maher E. R. and Latif, F. Frequent epigenetic inactivation of the SLIT2 gene in gliomas. Oncogene, 2003; 22: 4611-4616.
56. Prasad A., Fernandis A. Z., Rao Y. and Ganju R. K. Slit protein-mediated inhibition of CXCR4-induced chemotactic and chemoinvasive signaling pathways in breast cancer cells. J. Biol. Chem., 2004; 279: 9115-9124.
57. Balkwill F. The significance of cancer cell expression of the chemokine receptor CXCR4. Semin.Cancer Biol., 2004; 14: 171-179.
58. Wang R. Y., Gehrke C. W. and Ehrlich M. Comparison of bisulfite modification of 5-methyldeoxycytidine and deoxycytidine residues. Nucleic Acids Res., 1980; 8: 4777-4790.
59. Clark S. J., Harrison J., Paul C. L. and Frommer M. High sensitivity mapping of methylated cytosines. Nucleic Acids Res., 1994; 22: 2990-2997.
60. Herman J. G. and Baylin S. B. Gene silencing in cancer in association with promoter hypermethylation. N. Engl. J. Med., 2003; 349: 2042-2054.
61. Esteller M. CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. Oncogene, 2002; 21: 5427-5440.
62. Agathanggelou A., Honorio S., Macartney D. P., Martinez A., Dallol A., Rader J., Fullwood P., Chauhan A., Walker R., Shaw J. A., Hosoe S., Lerman M. I., Minna J. D., Maher E. R. and Latif F. Methylation associated inactivation of RASSF1A from region 3p21.3 in lung, breast and ovarian tumours. Oncogene, 2001; 20: 1509-1518.
63. Dammann R., Schagdarsurengin U., Strunnikova M., Rastetter M., Seidel C., Liu L., Tommasi S., and Pfeifer, G. P. Epigenetic inactivation of the Ras-association domain family 1 (RASSF1A) gene and its function in human carcinogenesis. Histol. Histopathol., 2003; 18: 665-677.
64. Dammann R., Li C., Yoon J. H., Chin P. L., Bates S. and Pfeifer G. P. Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. Nat. Genet., 2000; 25: 315-319.

65. Ruter B., Wijermans P. W. and Lubbert M. DNA methylation as a therapeutic target in hematologic disorders: recent results in older patients with myelodysplasia and acute myeloid leukemia. Int. J. Hematol., 2004; 80: 128-135.
66. Villar-Garea A. and Esteller M. Histone deacetylase inhibitors: understanding a new wave of anticancer agents. Int. J. Cancer, 2004; 112: 171-178.
67. Dallol A., Forgacs E., Martinez A., Sekido Y., Walker R., Kishida T., Rabbitts P., Maher E. R., Minna J. D. and Latif F. Tumour specific promoter region methylation of the human homologue of the Drosophila Roundabout gene DUTT1 (ROBO1) in human cancers. Oncogene, 2002; 21: 3020-3028.
68. Chen W., Cooper T. K., Zahnow C. A., Overholtzer M., Zhao Z., Ladanyi M., Karp J. E., Gokgoz N., Wunder J. S., Andrulis I. L., Levine A. J., Mankowski J. L. and Baylin S. B. Epigenetic and genetic loss of Hic1 function accentuates the role of p53 in tumorigenesis. Cancer Cell, 2004; 6: 387-398.
69. Chen W. Y., Zeng X., Carter M. G., Morrell C. N., Chiu Yen R. W., Esteller M., Watkins D. N., Herman J. G., Mankowski J. L. and Baylin S. B. Heterozygous disruption of Hic1 predisposes mice to a gender-dependent spectrum of malignant tumors. Nat. Genet., 2003; 33: 197-202.
70. Knudson A. G., Jr., Hethcote H. W. and Brown B. W. Mutation and childhood cancer: a probabilistic model for the incidence of retinoblastoma. Proc. Natl. Acad. Sci. U.S.A, 1975; 72: 5116-5120.
71. Astuti D., Da Silva N. F., Dallol A., Gentle D., Martinsson T., Kogner P., Grundy R., Kishida T., Yao M., Latif F. and Maher E. R. SLIT2 promoter methylation analysis in neuroblastoma, Wilms' tumour and renal cell carcinoma. Br.J.Cancer, 2004; 90: 515-521.
72. Dickinson R. E., Dallol A, Bieche I., Krex D., Morton D., Maher E. R. and Latif F. Epigenetic inactivation of SLIT3 and SLIT1 genes in human cancers. Br. J. Cancer 2004; (In Press).
73. Yu T. W. and Bargmann C. I. Dynamic regulation of axon guidance. Nat. Neurosci., 2001; 4 Suppl: 1169-1176.
74. Lerman M. I. and Minna J. D. The 630-kb lung cancer homozygous deletion region on human chromosome 3p21.3: identification and evaluation of the resident candidate tumor suppressor genes. The International Lung Cancer Chromosome 3p21.3 Tumor Suppressor Gene Consortium. Cancer Res., 60: 6116-6133, 2000.
75. Kuroki T., Trapasso F., Yendamuri S., Matsuyama A., Alder H., Williams N. N., Kaiser L. R. and Croce C. M. Allelic loss on chromosome 3p21.3 and promoter hypermethylation of semaphorin 3B in non-small cell lung cancer. Cancer Res., 2003; 63: 3352-3355.
76. de Lange R., Dimoudis N. and Weidle U. H. Identification of genes associated with enhanced metastasis of a large cell lung carcinoma cell line. Anticancer Res., 2003; 23: 187-194.
77. Tomizawa Y., Sekido Y., Kondo M., Gao B., Yokota J., Roche J., Drabkin H., Lerman M. I., Gazdar A. F. and Minna J. D. Inhibition of lung cancer cell growth and induction of apoptosis after reexpression of 3p21.3 candidate tumor suppressor gene SEMA3B. Proc. Natl. Acad. Sci. U.S.A, 2001; 98: 13954-13959.
78. Tse C., Xiang R. H., Bracht T. and Naylor S. L. Human Semaphorin 3B (SEMA3B) located at chromosome 3p21.3 suppresses tumor formation in an adenocarcinoma cell line. Cancer Res., 2002; 62: 542-546.
79. Castro-Rivera E., Ran S., Thorpe P. and Minna J. D. Semaphorin 3B (SEMA3B) induces apoptosis in lung and breast cancer, whereas VEGF165 antagonizes this effect. Proc. Natl. Acad. Sci. U.S.A, 2004; 101: 11432-11437.

80. Xiang R. H., Hensel C. H., Garcia D. K., Carlson H. C., Kok K., Daly M. C., Kerbacher K., van den B. A., Veldhuis P., Buy, C. H. and Naylor S. L. Isolation of the human semaphorin III/F gene (SEMA3F) at chromosome 3p21, a region deleted in lung cancer. Genomics, 1996; 32: 39-48.
81. Brambilla E., Constantin B., Drabkin H. and Roche J. Semaphorin SEMA3F localization in malignant human lung and cell lines: A suggested role in cell adhesion and cell migration. Am. J. Pathol., 2000; 156: 939-950.
82. Nasarre P., Constantin B., Rouhaud L., Harnois T., Raymond G., Drabkin H. A., Bourmeyster N. and Roche J. Semaphorin SEMA3F and VEGF have opposing effects on cell attachment and spreading. Neoplasia., 2003, 5: 83-92.
83. Xiang R., Davalos A. R., Hensel C. H., Zhou X. J., Tse C. and Naylor S. L. Semaphorin 3F gene from human 3p21.3 suppresses tumor formation in nude mice. Cancer Res., 2002; 62: 2637-2643,.
84. Catalano A., Caprari P., Rodilossi S., Betta P., Castellucci M., Casazza A., Tamagnone L. and Procopio A. Cross-talk between vascular endothelial growth factor and semaphorin-3A pathway in the regulation of normal and malignant mesothelial cell proliferation. FASEB J., 2004; 18: 358-360.
85. Fearon E. R., Cho K. R., Nigro J. M., Kern S. E., Simons J. W., Ruppert J. M., Hamilton S. R., Preisinger A. C., Thomas G. and Kinzler K. W. Identification of a chromosome 18q gene that is altered in colorectal cancers. Science, 1990; 247: 49-56.
86. Sato K., Tamura G., Tsuchiya T., Endoh Y., Usuba O., Kimura W. and Motoyama, T. Frequent loss of expression without sequence mutations of the DCC gene in primary gastric cancer. Br. J. Cancer, 2001; 85: 199-203.
87. Fazeli A., Dickinson S. L., Hermiston M. L., Tighe R. V., Steen R. G., Small C. G., Stoeckli E. T., Keino-Masu K., Masu M., Rayburn H., Simons J., Bronson R. T., Gordon J. I., Tessier-Lavigne M. and Weinberg R. A. Phenotype of mice lacking functional Deleted in colorectal cancer (Dcc) gene. Nature, 1997; 386: 796-804.
88. Llambi F., Causeret F., Bloch-Gallego E. and Mehlen P. Netrin-1 acts as a survival factor via its receptors UNC5H and DCC. EMBO J., 2001; 20: 2715-2722.
89. Mazelin L., Bernet A., Bonod-Bidaud C., Pays L., Arnaud S., Gespach C., Bredesen D. E., Scoazec J. Y. and Mehlen P. Netrin-1 controls colorectal tumorigenesis by regulating apoptosis. Nature, 2004; 431: 80-84.
90. Fearon E. R. and Vogelstein B. A genetic model for colorectal tumorigenesis. Cell, 1990; 61: 759-767.
91. Bilder D. Epithelial polarity and proliferation control: links from the Drosophila neoplastic tumor suppressors. Genes Dev., 2004; 18: 1909-1925.
92. St John M. A. and Xu T. Understanding human cancer in a fly? Am. J. Hum. Genet., 1997; 61: 1006-1010.
93. Giot L., Bader J. S., Brouwer C., Chaudhuri A., Kuang B., Li, Y., Hao Y. L., Ooi C. E., Godwin B., Vitols E., Vijayadamodar G., Pochart P., Machineni H., Welsh M., Kong Y., Zerhusen B., Malcolm R., Varrone Z., Collis A., Minto M., Burgess S., McDaniel L., Stimpson E., Spriggs F., Williams J., Neurath K., Ioime N., Agee M., Voss E., Furtak K., Renzulli R., Aanensen N., Carrolla S., Bickelhaupt E., Lazovatsky Y., DaSilva A., Zhong J., Stanyon C. A., Finley R. L., Jr., White K. P., Braverman M., Jarvie T., Gold S., Leach M., Knight J., Shimkets R. A., McKenna M. P., Chant J. and Rothberg J. M. A protein interaction map of Drosophila melanogaster. Science, 2003; 302: 1727-1736.

Chapter 9

MOLECULAR MECHANISMS OF THE METASTASIS-ASSOCIATED GENE FAMILY OF COREGULATORS: ROLE IN CANCER AND INVASION

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Abstract: Localized cancer, before it metastasizes, can be cured by surgery. The high mortality rate associated with most cancers, however, is due to the propensity of tumors to metastasize while the primary tumor is small and undetected. Metastasis, which occurs through a complex series of events, involves various gene products that dictate the progression of a cancer from a precursor lesion, to localized disease, and finally to metastatic disease. The expression of certain genes or alterations in gene structure or gene products may result in the progression of benign tumor cells to an invasive and metastatic state. Thus, the process of cancer metastasis requires, among other steps, changes in signaling pathways, activation of target gene products, enhanced cell survival, and increased epithelial-to-mesenchymal transition. A proper understanding of the progression of tumors to the metastatic stage and of the events that occur in highly malignant cells is important in the development of new therapeutic approaches for the diagnosis, treatment, and prognosis of highly progressive tumors. The molecular mechanisms that cause a cancer to exhibit more malignant behavior are widely believed to involve the deregulation of genetic and epigenetic cascades. We will here highlight the discovery and emerging significance of one family of regulators or chromatin modifiers, namely, the metastasis-associated antigens.

Key words: Metastasis-associated protein 1 (MTA1), coactivators, corepressors, estrogen receptor, metastasis

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1. IDENTIFICATION OF *MTA1*

The metastasis-associated gene 1 *(MTA1)* was initially identified in the highly metastatic 1376NF rat mammary adenocarcinoma system by differential screening of the complementary DNA (cDNA) library (1). MTA1 expression closely correlated with the aggressiveness of several human cancers. MTA1 expression was not limited to tumors, however: several normal mouse tissues and organs also showed detectable levels of MTA1 (2).

1.1 MTA Family

MTA1 is part of a family of genes and gene products that has been highly conserved throughout evolution and includes the metastasis-associated 1–like protein (*MTA1-L1*) (3), *MTA2* (4), and *MTA3*. The cDNAs of *MTA1*, *2* and *3* share an approximately 30% homology at the gene level and a 60% homology at the protein level. The *MTA1* gene is located on chromosome 14q32.3 in humans and chromosome 12F in mice (5, 6). The *MTA2* gene is located on chromosome 19B in mice, whereas the *MTA3* gene is located on chromosome 2p22.1 in humans (6). In *Drosophila* melanogaster, the single MTA-like protein shows a 50% homology in its amino acid sequence with all three MTA isoforms.

Structural analysis showed that *MTA1*, *2* and *3* contain *GATA*-like putative leucine zipper domains; the SANT domains, which are similar to the DNA-binding domain of myb-related proteins; a single SH3 binding motif; and two highly acidic regions. MTA1 and MTA2 contained two potential nuclear localization signals, but MTA3 contained only one. MTA1 and MTA2 are predominantly located in the nucleus, whereas MTA3 is located in both the nucleus and the cytoplasm. A recent study has shown that MTA1 can also be located in the cytoplasm (7).

1.2 Zymogen Granule Protein ZG29p, an MTA1 Gene Derivative

The gene encoding the 29-kDa protein ZG29p was identified by immunoscreening with a polyclonal antibody against purified pancreatic zymogen granules (8). Sequence analysis revealed that this protein is encoded by the *MTA1* gene. However, the transcription start site of this protein was different from that of MTA1. That is, it appears that ZG29p is encoded by the last seven exons of MTA1, according to the genome database (accession no. AF450245) and published data. Around the translation start

codon of ZG29p, two introns are present; this codon contains a putative TATA box and two consensus DNA-binding motifs for pancreas-specific transcription factor 1, termed E-box (CANNTG) and B-box (TGGGA). Binding of this factor to the pancreas consensus element is reported to initiate the transcription of *ZG29p* from an intron within the *MTA1* gene. *ZG29* cDNA is 96% homologus to the C-terminal part of MTA1, except for an insertion of 12 amino acids at position 129, which corresponds to position 592 in the MTA1 protein. This insertion overlaps but does not interfere with the second nuclear localization signal in the region.

Interestingly, despite the presence of two putative nuclear localization signals, ZG29p neither translocates to the nucleus nor stays in the cytoplasm but instead localizes predominantly in the soluble fraction of zymogen granules and, to a small extent, in the Golgi complex and rough endoplasmic reticulum. ZG29p may also act as a helper protein in granule formation. ZG29p was shown to interact with amylase mediated by SH3 binding domains, which are involved in the sorting of amylase to the granule membrane (8, 9).

1.3 Discovery of MTA1s, a Naturally Occurring Variant of MTA1

Recently, a second naturally occurring variant of MTA1 was discovered (10). This variant, termed MTA1s for the short version of MTA1, is an N-terminal truncated form of MTA1 that is generated by alternative splicing at a cryptic splice site in exon 14, the deletion of 47 base-pair nucleotides, and a frameshift involving the addition of 33 novel amino acids. These amino acids showed almost no homology with any proteins included in the genome database, except for a very conserved LXILL nuclear receptor–binding motif. MTA1s predominantly localized in the cytoplasm and inhibit the transactivating functions of estrogen receptor α (ERα by sequestering it in the cytoplasm in breast cancer cells. MTA1s-sequestered ERα remains functional, however, and contributes to the hyperactivation of the mitogen-activated protein kinase (MAPK) pathway. MAPK is known to participate in the aggressive behavior of breast cancer cells; because MTA1s activates the MAPK pathway, MTA1s-linked stimulation of MAPK may contribute to the aggressiveness of the overexpression of MTA1s in breast cancer cells.

Using a yeast two-hybrid screen with full-length MTA1s as a bait to clone MTA1s-binding proteins, Mishra *et al.* (11) identified casein kinase I-γ_2 (CKI-γ_2) as an MTA1s-interacting protein (11). Casein kinase I is a family of serine/threonine kinases ubiquitously expressed in eukaryotes (including yeast), humans, and plants. MTA1s interacts with CKI-γ_2 both *in vitro* and *in vivo* and colocalizes in the cytoplasm. In addition, CKI-γ_2 was found to

phosphorylate MTA1s on Ser 321 and to further potentiate the ER corepressive function of MTA1s (11). Interestingly, the impairment of the nuclear transactivation functions of ERα by MTA1s was found to be reversed by a mutant MTA1s that was possibly deficient in the CKI phosphorylation site (serine residue at position 321). Together, these findings offer new insights about the potential regulation of MTA1s' function by a ubiquitously expressed estrogen-responsive CKI-γ_2 and suggest that the extranuclear effects of estrogen might be important in regulating the functions of MTA1s in human mammary epithelial and cancer cells.

1.4 Identification of MTA1 as a Component of the NuRD Complex

Xue *et al*. (12) in 1998 identified MTA1 as a subunit of the nuclear remodeling and deacetylation (NuRD) complex. The NuRD complex is a multisubunit complex containing nucleosome-dependent ATPase subunits and histone deacetylase (HDAC) activity (13). MTA2 later was also found to be a subunit of the NuRD complex (4). Recently, Fujita *et al*. (14) reported that MTA3 is a subunit of the NuRD complex. Dynamic changes in chromatin structure play an important role in transcriptional regulation and are closely linked with the acetylation and deacetylation of histones. Histone acetylation occurs on the amino-terminal tails of lysine residues, thus neutralizing the positive charge of the histone tails and reducing their affinity for DNA. Hyperacetylated chromatin is generally associated with transcriptional activation, and hypoacetylated chromatin is generally associated with transcriptional repression (15). Two groups of enzymes, namely histone acetyltransferases and HDACs, regulate the acetylation state of histones. These enzymes, therefore, are an important link between the chromatin structure and the transcriptional outcome. MTA family members are exclusive; that is, they form distinct complexes in which only one MTA molecule is present, which may be the basis for the functional specialization of MTA family members.

2. DEREGULATION OF MTA FAMILY MEMBERS IN CANCER

The morbidity and mortality of cancer patients predominantly result from the tumor invasion and metastasis of neoplastic cells of primary tumors to distant organ sites. Because distant metastasis is an important factor

affecting prognosis, identifying metastasis-associated genes remains a key tool in preventing metastasis. Using a differential cDNA library screen to identify genes associated with metastasis, Toh *et al*. (1) were the first to describe the *MTA1* gene, whose expression correlated with metastatic potential in a metastatic rat mammary adenocarcinoma system. Indeed, rat *MTA1* messenger RNA (mRNA) expression was found to be four-fold higher in the highly metastatic rat cell line MTLn3 than in the nonmetastatic cell line MTC4.

Similarly, mRNA expression levels of the human *MTA1* gene were higher in cell lines that were highly invasive and metastatic than in noninvasive and nonmetastatic cell lines. Invasive human breast carcinomas also overexpressed the *MTA1* gene, compared with the surrounding normal tissue. In addition, overexpression of the *MTA1* gene closely correlated with tumor invasion and lymph node metastasis in colorectal and gastric carcinomas (16): *MTA1* gene expression was assessed in 36 colorectal and 34 gastric carcinomas and in corresponding normal tissues by semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR) (16). The *MTA1* gene was overexpressed in 14 of 36 colorectal carcinomas (39%) and 13 of 34 gastric carcinomas (38%). Clinical pathologic correlations showed that tumors overexpressing the *MTA1* gene exhibited significantly higher rates of vascular involvement and invasion of and metastasis to lymph nodes. These data suggest that overexpression of the MTA1 gene may be a useful indicator of the malignant potential of colorectal and gastric carcinomas.

MTA1 mRNA expression was also assessed in 47 esophageal squamous cell carcinomas by RT-PCR analysis; overexpression of MTA1 mRNA was observed in 16 of 47 (34%) of these carcinomas (17). A significantly higher frequency of advential invasion and lymph node metastasis and a higher rate of lymphatic involvement were observed in tumors overexpressing MTA1 mRNA, which suggests that the MTA1 gene plays an important role in the invasion and metastasis in esophageal carcinoma (17). Recently, Toh *et al*. (18) performed immunohistochemical studies to examin MTA1 protein and acetylation levels of histone H4 in 70 cases of esophageal carcinoma. Overexpression of the MTA1 protein was observed in 30 of 70 cases (43%); these tumors invaded deeper into the esophageal wall and were associated with significantly higher rates of lymph node metastasis, a higher pathologic stage, more lymphatic involvement, and a poorer prognosis. Interestingly, the acetylation levels of histone H4 inversely correlated with the depth of cancer invasion, pathologic stage, and prognosis. These data showed that overexpression of the MTA1 protein and the acetylation levels of histone H4 might be a useful predictor of the malignant potential of esophageal carcinomas (18).

Similarly, Iguchi *et al*. (19) examined *MTA1* mRNA expression in 23 pancreatic cancer samples by using RT-PCR. Overexpression of *MTA1* mRNA was observed in 12 of 23 pancreatic tumors (52%), and these tumors were associated with a higher incidence of lymph node metastasis. These results suggest that *MTA1* is involved in the progression and lymph node metastasis of pancreatic cancer.

MTA1 mRNA expression and mutation was also studied in 20 primary ovarian carcinomas, 20 corresponding lymph nodes, and 8 normal ovarian samples by using RT-PCR (20). *MTA1* overexpression was observed in 7 of 7 (100%) of the primary ovarian carcinomas with metastasis but only in 5 of 13 (42%) of those without metastasis. In addition, *MTA1* overexpression was observed in 6 of 7 lymph nodes (86%) with metastasis but only 3 of 13 (23%) without metastasis. No mutation of *MTA1* was found, and *MTA1* mRNA expression positively correlated with lymph node metastasis.

Sasaki *et al*. (21) used RT-PCR to examine *MTA1* mRNA expression in 30 thymoma samples. *MTA1* mRNA was found to be significantly higher in samples of stage IV thymoma than in samples of either stage I or stage II thymoma. These results showed that *MTA1* gene expression is closely related to invasiveness in thymoma.

MTA1 mRNA expression was likewise examined in 74 non-small cell lung carcinoma samples by using RT-PCR (22). *MTA1* mRNA was found to be significantly higher in non-small cell lung carcinoma tissues and in the associated lymph node metastases than in normal lung tissues. These data suggest that *MTA1* gene expression is closely related to the invasiveness and metastasis of non-small cell lung carcinomas.

Using DNA microarray, immunoblot, and immunohistochemical analyses, Hofer *et al*. (23) reported selective overexpression of MTA1 in metastatic prostate cancer but not in clinically localized prostate cancer or benign prostatic tissue. Expression of MTA1 in a wide range of prostatic tissue suggested that its level may be a useful tissue biomarker.

MTA1 mRNA was also examined by RT-PCR analysis in 33 hepatocellular carcinoma and paired nontumor liver tissues (24). Overexpression of the *MTA1* gene was observed in 14 of 33 hepatocellular carcinomas (42%) [compared with the paired-nontumor samples], and the disease-free survivalrate associated with the *MTA1*-overexpressing samples was found to be significantly lower than the rate associated with samples with low *MTA1* expression. In another study, Moon *et al*. (7) studied MTA1 expression and its potential relationship to ERα expression and metastasis in 45 hepatocellular carcinomas by using immunohistochemical analysis. Overexpression of MTA1 was observed in 31 of 45 hepatocellular carcinoma samples, with MTA1 expressed predominantly in the nucleus or cytoplasm of the carcinomas. In addition, strong MTA1 expression was

observed in 19 of 20 hepatocellular carcinoma specimens with vascular invasion (95%), and MTA1 expression also significantly correlated with large tumor size. Interestingly, MTA1 expression inversely correlated with the nuclear localization ERα. These results suggest that MTA1 expression is closely related to vascular invasion and the growth of hepatocellular carcinoma.

Mazumdar *et al*. (2) reported that the overexpression of MTA1 in noninvasive breast cancer MCF-7 cells significantly enhanced the ability of these cells to form large colonies on soft agar and increased colony formation compared with the vector-transfected control cells. Overexpression of MTA1 in MCF-7 cells was also found to be associated with an enhanced metastatic potential in Boyden-chamber assays. Recently, Mahoney *et al*. (25) reported that forced expression of MTA1 cDNA in human immortalized keratinocyte HaCaT cells enhanced the migration and invasion of these cells and that MTA1 expression was induced by epidermal growth factor receptor (EGFR) activation, which was essential but not sufficient for EGFR-dependent cell survival in the anchorage-independent state. These authors also observed that overexpression of MTA1 enhanced the expression of Bcl-xL, a member of the antiapoptotic Bcl-2 family, and that inhibition of EGFR activation resulted in decreased MTA1 expression. It remains to be determined whether MTA1 regulation of Bcl-xL is mediated by its HDAC activity.

Similarly, Hofer *et al*. (26) reported that overexpression of green fluorescent fusion protein–tagged MTA1 in PANC-1 pancreatic carcinoma cells facilitated the development of the motile, invasive, and metastatic phenotype of PANC-1 cells by altering their cellular cytoskeleton organization and that MTA1 overexpression may be important in the progression of pancreatic cancer. In addition, inhibition of MTA1 protein expression with antisense phosphorothioate oligonucleotides resulted in growth inhibition of the highly invasive human breast cancer cell line MDA-MB-231, which expresses very high levels of the *MTA1* gene; these results were thought to suggest that the MTA1 protein plays a role in cellular signaling processes important for the progression and growth of cancer cells (27). Taken together, the results of Mazumdar *et al.* (2), Mahoney *et al*. (25), Hofer *et al*. (26), and Nawa *et al*. (27) suggest that overexpression of MTA1 promotes the invasive and metastatic phenotypes in different cancer cell lines.

Expression of the variant MTA1s was also found to be four-fold higher in ER-negative tumors than in ER-positive tumors. Interestingly, in MTA1s-overexpressing ER-negative tumors, ER was found to be located in the cytoplasm (10). These findings suggest that MTA1s overexpression

contributes to hormone independence by sequestering ER and stimulating the MAPK pathway in the cytoplasm.

There is no evidence of a direct role of MTA2, another member of the MTA family, in malignancy. However, a role for MTA2 in regulating p53-mediated growth arrest and apoptosis was reported by Luo *et al*. (28). Cotransfection of MTA2 effectively abrogated p53-mediated growth inhibition and also impaired the stimulation of p21 gene expression by p53. Overexpression of p53-induced apoptosis and cotransfection of both p53 and MTA2 significantly reduced the rate of apoptosis, and treatment with trichostatin A, an inhibitor of HDAC, effectively prevented MTA2-mediated downregulation of apoptosis. The functional interaction between p53 and MTA2 and the deacetylation of p53 may both play important roles in tumor progression.

MTA3 has been shown to modulate the metastatic potential of breast cancer cells by regulating the levels of Snail, a master regulator of epithelial-mesenchymal transition; Snail is an important phenotypic characteristic of highly motile and invasive cancer cells (14). MTA3 expression was found to be closely associated with the ER status, and downregulation of ER or MTA3 led to increased Snail expression and decreased E-cadherin levels.

An interesting recent study conducted by Fujita *et al*. (29) identified a role for MTA3 in B-lymphocyte differentiation. MTA3 was found to be expressed in germinal-center B cells, a distinct subset of lymphocytes, and correlated with the expression pattern of BCL-6, a master regulator of B-cell differentiation. MTA3 physically interacted with BCL-6 in B-lymphocyte cell lines, and knockdown of MTA3 by RNA-mediated inhibition resulted in concomitant alterations in the cell type–specific transcriptional program in B-lymphocytes. Acetylation of BCL-6, which affects the ability of BCL-6 to repress transcription, regulated the interaction with MTA3. Additionally, exogenous expression of BCL-6 in a plasma cell line resulted in reprogramming of its cellular differentiation to that of a B lymphocyte in an MTA3-dependent manner. The findings of Fujita *et al*. (29) suggest that MTA3 plays an important role in the determination of B-cell fate.

Taken together, the studies discussed in this section suggest the regulatory role of MTA family members in the invasiveness and metastatic potential of cancer cells.

3. REGULATION OF ER TRANSACTIVATION BY MTA FAMILY MEMBERS

Although previous studies have suggested a role for MTAl expression in the metastasis of various tumors, the targets of its action have largely

remained elusive. Mazumdar *et al*. (2) reported that heregulin, a natural ligand for HER3 and HER4, promoted a hormone-independent phenotype and repressed the ER transactivation functions in breast cancer cells. Heregulin also stimulated MTA1 expression, promoted histone deacetylation, and enhanced the HDAC complex. Because gene expression is closely associated with chromatin modification, these authors speculated that MTA1 repression of ER transactivation was mediated by chromatin modification (2). Heregulin promoted the interaction of MTA1 with endogenous ER and the association of

MTA1 or HDAC with ER target gene promoters. Together, these data imply that ER transactivation is a nuclear target of MTA1 and that HDAC complexes are involved in MTA1-mediated ER transcriptional repression (Figure 1).

To further understand the role of MTA1 cellular functions in breast cancer cells, other authors used a yeast two-hybrid screen with the MTA1 C-terminal domain as bait to identify several MTA1-interacting proteins, such as MAT1 (ménage á trois 1), MTA1-interacting coactivator (MICoA) and nuclear receptor inhibitory factor 3 (NRIF3) (29-31).

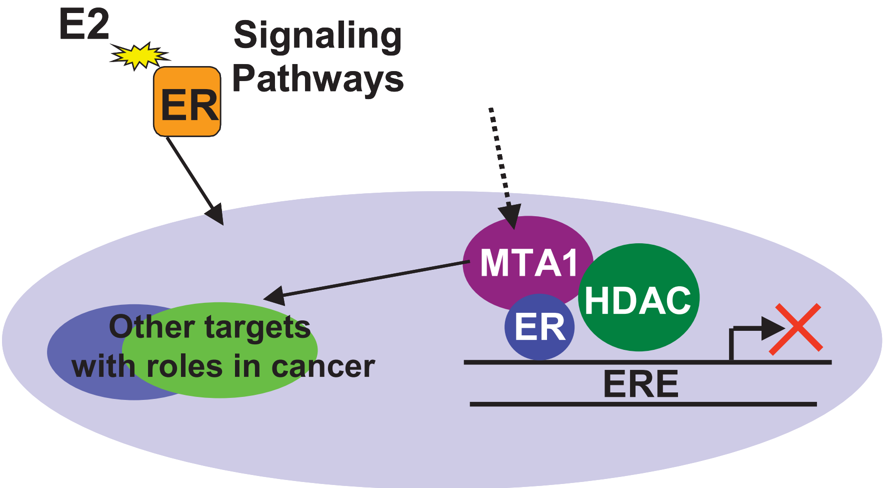

Figure 1. MTA1 regulation of ERα transactivation functions.

3.1 MAT1

MAT1 is an assembly or targeting factor for cyclin-dependent kinase–activating kinase (CAK) that functionally interacts with general transcription factor IIH, a known inducer of the transactivation functions of ER. MTA1

inhibited CAK stimulation of ER transactivation, and this inhibition was partially relieved by the HDAC inhibitor trichostatin A (30). These findings suggest that MTA1 inhibits CAK-induced ER transactivation by recruiting HDAC to ER transcriptional complexes and by altering the chromatin structure. In addition, overexpression of MTA1 prevented the CAK complex from phosphorylating ER, suggesting that the transactivation functions of ER might be influenced by the regulatory interactions between CAK and MTA1 in breast cancer cells.

3.2 MICoA

The previously uncharacterized protein MICoA was identified as an MTA1-binding protein in a yeast two-hybrid screen and was found to be a *bona fide* coactivator of ERα transactivation functions (31). MICoA cooperates with other ER coactivators, stimulates ERα-transactivation functions, and associates with the endogenous ER and its target gene promoters. MTA1 repressed MICoA-mediated stimulation of ER response element–mediated transcription in the presence of ER and ER variants with naturally occurring mutations, such as D351Y and K303R, and interfered with the association of MICoA with the ER target gene chromatin. These findings suggest that MTA1 and MICoA transmodulate each other's functions and that potential deregulation of MTA1 contributes to the functional inactivation of the ER pathway, presumably by derecruitment of MICoA from ER target promoter chromatin.

3.3 NRIF3

Recently, NRIF3, a known nuclear receptor coregulator, was identified as an MTA1-binding protein and was shown to interact with MTA1 both *in vitro* and *in vivo* (32). NRIF3 functioned as an ER coactivator, hyperstimulated ER transactivation functions, and associated with the endogenous ER and its target gene promoter. NRIF3-mediated stimulation of ER response element–driven transcription was repressed by MTA1, which was also found to interfere with the association between NRIF3 and the ER target gene chromatin. Deregulation of NRIF3 enhanced the responsiveness of breast cancer cells to estrogen-induced stimulation of growth and anchorage independence. Furthermore, NRIF3 was found to be an estrogen-inducible gene that activated the ER associated with the ER response element in the NRIF3 gene promoter (32). These data suggest that NRIF3 is an estrogen-inducible gene and that regulatory interactions between MTA1 and NRIF3 play an important role in modulating the sensitivity of breast cancer cells to estrogen.

3.4 Role of MTA1s and MTA3

MTA1s-mediated repression of ER transactivation functions was found to be potentiated by the phosphorylation of MTA1s at Ser 321 by casein kinase I-γ, a binding protein of MTA1s (11). Recent studies have identified the role of ER in the regulation of epithelial-to-mesenchymal transition via MTA3, but the role of upstream determinants of ER regulation of MTA3 and the underlying molecular mechanisms of this regulation remain unknown. There are, however, a few clues to both. For example, dynamic changes in the levels of nuclear coregulators were found to influence the ER regulation of the *MTA3* gene (33). Under basal conditions, MTA1 and HDACs interact with the functional ER element half-site in the MTA3 promoter; on estrogen stimulation, these corepressors are derecruited, resulting in concomitant recruitment of ER and increased MTA3 transcription and expression. Inactivation of the MTA1 pathway stimulates ER to upregulate MTA3 expression, whereas ER knockdown enhances MTA1's association with the MTA3 gene. Modulation of ER transactivation functions by corepressors (i.e., MTA1 and MTA1s) or coactivators (i.e., AIB1 and PELP1/MNAR) altered ER recruitment to the MTA3 chromatin, MTA3 transcription, and expression of downstream epithelial-mesenchymal transition components (Figure 2). These data provide novel insights into the transregulation of the *MTA3* gene and reveal new roles for the upstream determinants in modifying the functions of MTA3 and cell differentiation in breast cancer cells.

Recently, Fujita *et al*. (34) identified a small sequence element containing an SP1 site near a consensus ER response element half-site in the MTA3 promoter. The SP1 site was found to be essential for the productive binding of ERα to MTA3 chromatin, and the knock down of either SP1 or ERαby RNA-mediated inhibition led to loss of the MTA3 transcript in multiple breast cancer cell lines, suggesting a requirement for both transcription factors in the expression of endogenous MTA3. These results suggest that the local chromatin architecture influences the ability of ERα to selectively bind to the MTA3 promoter in an SP1-dependent fashion.

Taken together, these data suggest that different members of the MTA family elicit different responses to ER action either by inhibiting ER transactivation (as with MTA1 and MTA1s) or by indirectly regulating ER target genes (as with MTA3) and play an important role in regulating the ER pathway.

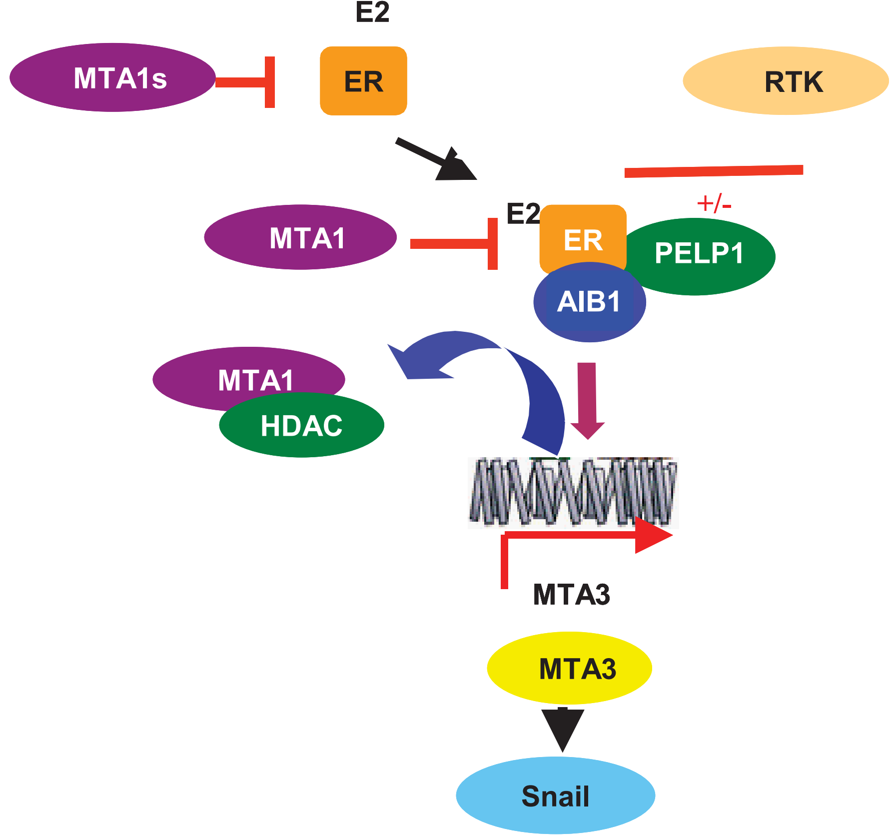

Figure 2. Upstream regulators of the MTA3 pathway.

4. ROLE OF MTA1 IN MOUSE MAMMARY GLAND

Although MTA1 represses the ligand-dependent transactivation functions of ER in breast cancer cells and although upregulation of MTA1 has been observed in various human breast tumors, the role of MTA1 in tumorigenesis in a physiologically relevant animal system remains unknown. Recently, Rozita *et al*. (35) examined the role of MTA1 in mammary gland development in transgenic mice that express MTA1 under the control of the mouse-mammary-tumor-virus-promoter long terminal repeat. Mammary glands of virgin transgenic mice exhibited extensive side branching and precocious differentiation because of increased proliferation of ductal and alveolar epithelial cells, resembled glands of wild-type mice in

midpregnancy, and inappropriately expressed beta-casein, cyclin D1, and beta-catenin protein. Dysregulation of MTA1 in mammary epithelium and cancer cells triggered downregulation of the progesterone receptor B isoform and upregulation of the progesterone receptor A isoform, resulting in an imbalance in the native ratio of progesterone receptor A and B isoforms. MTA1 expression also increased the expression of the progesterone receptor A target genes *Bcl-XL* and cyclin D1 in the mammary gland of virgin mice, which subsequently showed delayed involution. Interestingly, 30% of MTA1 transgenic females developed focal hyperplastic nodules, and approximately 7% exhibited mammary tumors within 18 months. This study established for the first time a potential role for MTA1 in mammary gland development and tumorigenesis (35). The authors suggested that the underlying mechanism involves the upregulation of progesterone receptor A and its targets, Bcl-XL and cyclin D1.

5. CONCLUSION

In conclusion, the past decade has witnessed one of the most exciting periods to date in terms of an increase in our understanding of the MTA family of coregulators in the cancer biology of metastasis. In particular, although much work remains to be done, we have begun to learn more about the importance of chromatin modifiers in the regulation of epithelial proliferation and in the pathogenesis of cancer. Much of the biologic research on chromatin modifiers and cancer has focused on understanding the physical modifications of nucleosome complexes, histones, and critical transcription complexes and their targets. Although this research has been very fruitful, it is now clear that gaining a more complete understanding of key regulatory pathways depends on conducting both physiologically relevant *in vivo* studies in laboratory animals Since MTA family members are expressed at an easily detectable level in several normal tissues, it will be also important to start delineating the basis of functional deregulation of the MTA coregulators during tumorigenesis.

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REFERENCES

1. Toh Y., Pencil S. D., Nicolson G. L. A novel candidate metastasis-associated gene, mta1, differentially expressed in highly metastatic mammary adenocarcinoma cell lines. cDNA cloning, expression, and protein analyses. J Biol Chem, 1994; 269:22958-22963.
2. Mazumdar A., Wang R. A., Mishra S. K., Adam L., Bagheri-Yarmand R., Mandal M., Vadlamudi R. K., Kumar R. Transcriptional repression of oestrogen receptor by metastasis-associated protein 1 corepressor. Nat Cell Biol, 2001; 3:30-37.
3. Futamura M., Nishimori H., Shiratsuchi T., Saji Nakamura Y., Tokino T. Molecular cloning, mapping, and characterization of a novel human gene, MTA1-L1, showing, homology to a metastasis-associated gene, MTA1. J Hum Genet, 1999; 44:52-56.
4. Zhang Y., Ng H. H, Erdjument-Bromage H., Tempst P., Bird A., Reinberg D. Analysis of the NuRD subunits reveals a histone deacetylase core complex and a connection with DNA methylation. Genes Dev, 1999; 13:1924-1935.
5. Cui Q., Takiguchi S., Matsusue K., Toh Y., Yoshida M. A. Assignment of the human metastasis-associated gene 1 (MTA1) to human chromosome band 14q32.3 by fluorescence in situ hybridization. Cytogenet Cell Genet, 2001; 93:139-140.
6. Cui Q., Matsusue K., Toh Y., Kono A., Takiguchi S. Assignment of the metastasis-associated gene (Mta1) to mouse chromosome band 12F and the metastasis-associated gene 2 (Mta2) to mouse chromosome band 19B by fluorescence in situ hybridization. Cytogenet Cell Genet, 2001; 94:246-247.
7. Moon W. S., Chang K., Tarnawski A S. Overexpression of metastatic tumor antigen 1 in hepatocellular carcinoma: relationship to vascular invasion and estrogen receptor-alpha. Hum Pathol 2004; 35:424-429.
8. Kleene R., Zdzieblo J., Wege K., Kern H. F. A novel zymogen granule protein (ZG29p) and the nuclear protein MTA1p are differentially expressed by alternative transcription initiation in pancreatic acinar cells of the rat. J Cell Sci, 1999; 112:2539-2548.
9. Kleene R., Classen B., Zdzieblo J., Schrader M. SH3 binding sites of ZG29p mediate an interaction with amylase and are involved in condensation-sorting in the exocrine rat pancreas. Biochemistry, 2000; 39:9893-9900.
10. Kumar R., Wang R. A., Mazumdar A., Talukder A. H., Mandal M., Yang Z., Bagheri-Yarmand R., Sahin A., Hortobagyi G., Adam L., Barnes C. J., Vadlamudi R.K. A naturally occurring MTA1 variant sequesters oestrogen receptor-alpha in the cytoplasm. Nature, 2002; 418:654-657.
11. Mishra S. K., Yang Z., Mazumdar A., Talukder A. H., Larose L., Kumar R. Metastatic tumor antigen 1 short form (MTA1s) associates with casein kinase I-γ2, an estrogen-responsive kinase. Oncogene, 2004; 23:4422-4429.
12. Xue Y., Wong J., Moreno G. T. , Young M. K., Cote J, Wang W. NURD, a novel complex with both ATP-dependent chromatin-remodeling and histone deacetylase activities. Mol Cell, 1998; 2:851-861.
13. Bowen N. J., Fujita N., Kajita M., Wade P. A. Mi-2/NuRD: multiple complexes for many purposes. Biochim Biophys Acta, 2004; 1677:52-57.
14. Fujita N., Jaye D. L., Kajita M., Geigerman C., Moreno C. S., Wade P. A. MTA3, a Mi-2/NuRD complex subunit, regulates an invasive growth pathway in breast cancer. Cell, 2003; 113:207-219.
15. Brownell J. E., Zhou J., Ranalli T., Kobayashi R., Edmondson D. G., Roth S. Y., Allis C. D. Tetrahymena histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation. Cell, 1996; 84:843-851.

16. Toh Y., Oki E., Oda S., Tokunaga E., Ohno S., Maehara Y., Nicolson G. L., Sugimachi K. Overexpression of the MTA1 gene in gastrointestinal carcinomas: correlation with invasion and metastasis. Int J Cancer, 1997; 74:459-463.
17. Toh Y., Kuwano H., Mori M., Nicolson G. L., Sugimachi K. Overexpression of metastasis-associated MTA1 mRNA in invasive oesophageal carcinomas. Br J Cancer, 1999; 79:1723-1726.
18. Toh Y., Ohga T., Endo K. Adachi E., Kusumoto H., Haraguchi M., Okamura T., Nicolson G. L. Expression of the metastasis-associated MTA1 protein and its relationship to deacetylation of the histone H4 in esophageal squamous cell carcinomas. Int J Cancer, 2004; 110:362-367.
19. Iguchi H., Imura G., Toh Y., Ogata Y. Expression of MTA1, a metastasis-associated gene with histone deacetylase activity in pancreatic cancer. Int J Oncol, 2000; 16:1211-1214.
20. Yi S., Guangqi H., Guoli H. The association of the expression of MTA1, nm23H1 with the invasion, metastasis of ovarian carcinoma. Chin Med Sci J, 2003; 18:87-92.
21. Sasaki H., Yukiue H., Kobayashi Y., Nakashima Y., Kaji M., Fukai I., Kiriyama M., Yamakawa Y., Fujii Y. Expression of the MTA1 mRNA in thymoma patients. Cancer Lett, 2001; 174:159-163.
22. Sasaki H., Moriyama S., Nakashima Y., Kobayashi Y., Yukiue H., Kaji M., Fukai I., Kiriyama M., Yamakawa Y., Fujii Y. Expression of the MTA1 mRNA in advanced lung cancer. Lung Cancer, 2002; 35:149-154.
23. Hofer M. D., Kuefer R., Varambally S., Li H., Ma J., Shapiro G. I., Gschwend J. E., Hautmann R. E., Sanda M. G., Giehl K., Menke A., Chinnaiyan A. M., Rubin M. A. The role of metastasis-associated protein 1 in prostate cancer progression. Cancer Res 2004; 64:825-829.
24. Hamatsu T., Rikimaru T., Yamashita Y., Aishima S., Tanaka S., Shirabe K., Shimada M., Toh Y., Sugimachi K. The role of MTA1 gene expression in human hepatocellular carcinoma. Oncol Re,p 2003; 10:599-604.
25. Mahoney M. G., Simpson A., Jost M., Noe M., Kari C., Pepe D., Choi Y. W., Uitto J., Rodeck U. Metastasis-associated protein (MTA)1 enhances migration, invasion, and anchorage-independent survival of immortalized human keratinocytes. Oncogene, 2002; 21:2161-2170.
26. Hofer M. D., Menke A., Genze F., Gierschik P., Giehl K. Expression of MTA1 promotes motility and invasiveness of PANC-1 pancreatic carcinoma cells. Br J Cancer, 2004; 90:455-462.
27. Nawa A., Nishimori K., Lin P., Maki Y., Moue K., Sawada H., Toh Y., Fumitaka K., Nicolson G. L. Tumor metastasis-associated human MTA1 gene: its deduced protein sequence, localization, and association with breast cancer cell proliferation using antisense phosphorothioate oligonucleotides. J Cell Biochem, 2000; 79:202-212.
28. Luo J., Su F., Chen D., Shiloh A., Gu W. Deacetylation of p53 modulates its effect on cell growth and apoptosis. Nature, 2000; 408:377-381.
29. Fujita N., Jaye D. L., Geigerman C., Akyildiz A., Mooney M. R., Boss J. M., Wade P. A. MTA3 and the Mi-2/NuRD complex regulate cell fate during B lymphocyte differentiation. Cell, 2004; 119:75-86.
30. Talukder A. H., Mishra S. K., Mandal M., Balasenthil S., Mehta S., Sahin A. A., Barnes C. J., Kumar R. MTA1 interacts with MAT1, a cyclin-dependent kinase-activating kinase complex ring finger factor, and regulates estrogen receptor transactivation functions. J Biol Chem, 2003; 278:11676-11685.
31. Mishra S. K., Mazumdar A., Vadlamudi R. K., Li F., Wang R. A., Yu W., Jordan V. C., Santen R. J., Kumar R. MICoA, a novel metastasis-associated protein 1 (MTA1)

interacting protein coactivator, regulates estrogen receptor-alpha transactivation functions. J Biol Chem, 2003; 278:19209-19219.
32. Talukder A. H., Gururaj A., Mishra S. K., Vadlamudi R. K., Kumar R. Metastasis-associated protein 1 interacts with NRIF3, an estrogen-inducible nuclear receptor coregulator. Mol Cell Biol, 2004; 24:6581-6591.
33. Mishra S. K., Talukder A. H., Gururaj A. E., Yang Z., Singh R. R., Mahoney M. G., Franci C., Vadlamudi R. K., Kumar R. Upstream determinants of estrogen receptor-alpha regulation of metastatic tumor antigen 3 pathway. J Biol Chem, 2004; 279:32709-32715.
34. Fujita N., Kajita M., Taysavang P., Wade P. A. Hormonal regulation of MTA3 transcription in breast cancer cells. Mol Endocrinol, 2004 Sep 9 [Epub ahead of print].
35. Bagheri-Yarmand R., Talukder A. H., Wang R. A., Vadlamudi R. K., Kumar R. Metastasis-associated protein 1 deregulation causes inappropriate mammary gland development and tumorigenesis. Development, 2004; 131: 3469- 3479.

Chapter 10

THE MOLECULAR MECHANISMS FOR BREAST CANCER METASTASIS SUPPRESSOR 1 ACTION IN CANCER METASTASIS

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Abstract: Recent advances in drug discoveries and understanding of epigenetic regulation of gene expression have brought histone deacetylases (HDACs) in spotlight. **B**reast **C**ancer **M**etastasis **S**uppressor **1** (BRMS1), a gene shown to functionally suppress metastasis of breast cancer and melanoma, is a member of mSin3-HDAC complex. This chapter reviews the emerging understanding of the molecular mechanisms of BRMS1 action and possible players involved in it. New evidence of BRMS1-family of proteins and their possible role in histone code is discussed.

Key words: BRMS1, HDAC, Sin3, histone code, metastasis suppressor

1. INTRODUCTION

Metastasis of cancers such as breast cancer is a sequential-multi-step event and a result of complex genetic aberrations (1). Since a metastasis competent cell needs to be able to successfully complete each and every step of the metastasis cascade, ability to block any one of the steps of the metastatic cascade could block the spread of cancer. Metastasis suppressor genes **(MSGs)** suppress spread and growth at a secondary site without altering tumor formation (2). To date there are at least twelve metastasis suppressor genes functionally characterized. ***Nm23***, ***KAI-1***, ***KISS-1, TXNIP***, ***CRSP3, VDUP1***, ***MKK4***, Src-suppressed C kinase substrate (***SSeCKS***) the likely rodent ortholog of human Gravin/AKAP12, ***RhoGDI2***, ***E-cadherin*** (encoded by *CAD1*), ***Drg-1*** (a.k.a *RTP, cap43* and *rit42*), Tissue inhibitors

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of metalloproteases (***TIMPs***) and ***BRMS1*** (3-23). **Br**east Cancer **M**etastasis **S**uppressor **1** (BRMS1) is a very promising member of the MSGs family (24-30, 32-35).

2. IDENTIFICATION OF BRMS1

Evidence from clinical material has revealed regarding alteration of genetic material on chromosome 11q13-q14 in approximately two-thirds of late-stage, metastatic breast carcinomas. To test the hypothesis that chromosome 11 encodes metastasis suppressor gene(s), a normal, human neomycin-tagged chromosome 11 was introduced into metastatic, breast carcinoma cell line, MDA-MB-435 [435] by microcell-mediated chromosome transfer (24). Differential display comparing the resultant **metastasis-suppressed** hybrid, termed *neo11/435 or neo11 hybrid* with 435 identified BRMS1 (up regulated) in the neo11 hybrid (25).

3. CHARACTERIZATION OF BRMS1

By fluorescent *in situ* hybridization (FISH), BRMS1 maps to chromosome 11q13.1-13.2. BRMS1 mRNA is widely expressed in normal tissues, including human breast (from reduction mammoplasty). It is also present in multiple species including human, cow, rabbit, zebrafish, mouse, drosophila (Genbank database, NCBI). MDA-MB-231 [231], another metastatic breast carcinoma cell line and MDA-MB-435 express very low levels of BRMS1 mRNA as compared to other aggressive breast carcinoma cell lines (25, 26). A real time-PCR (RTQ) demonstrated inverse correlation of BRMS1 expression with acquisition of metastatic potential (competence) in human melanoma cells suggesting importance of BRMS1 in more than one cancer types (26). BRMS1 expression correlated to metastatic potential in lineage related human bladder cancer cell lines. RTQ analysis of trophoblasts isolated using LASER capture microdissection demonstrates that expression levels of BRMS1 are higher in term than in early placentas (7-9 weeks). Also studies of metastasis-associated genes in hepatocellular carcinoma revealed lower expression of *BRMS1* in metastatic tumors compared with the primaries (31).

3.1 BIOCHEMICAL CHARACTERIZATION

BRMS1 encodes a protein of 246 amino acids (Mr approx. 28.5 kDa). The predicted protein bears several putative phosphorylation sites, a putative bipartite NLS (nuclear localization sequence; confirmed by cell fractionation and immunofluorescence studies), two coiled-coil motifs and imperfect leucine zipper motifs and a glutamic acid-rich region (25). No DNA binding domains are identifiable. Exogenously added, recombinant epitope-tagged BRMS1 does not dimerize with epitope-tagged BRMS1 expressed in metastatic cells.

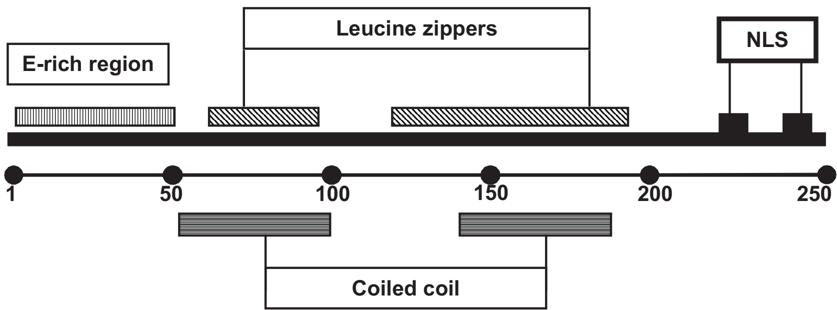

Figure 1. Predicted domain organization of BRMS1 protein. BRMS1 protein (246 aa) shows presence of several interesting domains such as N-terminal glutamic acid (E) rich region, leucine zippers, coiled coil. domains and nuclear localization sequences (NLS).

3.2 FUNCTIONAL CHARACTERIZATION

3.2.1 Suppression of Metastasis in Mouse Models of Cancer Metastasis

BRMS1 cDNA suppressed metastasis, without affecting tumorigenicity or invasion in two unrelated highly metastatic breast carcinoma cell lines, MDA-MB-435 and MDA-MB-231. Metastasis-suppression was expression-dependent i.e., cells expressing higher BRMS1 levels demonstrated greater reduction in metastatic potential (25, 28). There were no apparent differences in the levels of other known metastasis suppressor genes, in BRMS1 transfectants. Neither were parameters like MMP production, growth rate or adhesive properties to extracellular matrix components altered. BRMS1 transfectants of metastatic breast cancer cell lines do not show any significant changes in invasion, adhesion to extracellular matrix,

motility, and levels of Matrix Metalloproteinases (MMPs) etc. as compared to the metastatic parental cell line (25, 28). Thus BRMS1 causes very subtle changes in the cell physiology. Murine ortholog of *BRMS1* (*Brms1*), bears a substantial identity with the human counterpart. *Brms1* was tested for suppression of metastasis of mouse mammary carcinoma cell line 66cl4 in syngeneic BALB/c mice. Transfection with *Brms1* did not inhibit 66cl4 primary tumor formation but significantly suppressed its metastatic capability. This suggests that the murine ortholog functions similar to BRMS1 (27).

Stable transfectants of BRMS1 in the human melanoma cell lines MelJuSo and C8161.9 did not alter the tumorigenicity of either cell line, but instead significantly suppressed metastasis. While orthotopically growing tumors continued to express BRMS1, expression was lost in lung metastases. Cumulatively the studies suggest that BRMS1 functions as a metastasis suppressor in more than one tumor type (26).

3.2.2 Effect on Cell-to-cell Communication

BRMS1-expressing 435 (as well as 231 and C8161.9) cells demonstrated restored gap junctional intercellular communication compared to the vector-only transfectants, accompanied by decreased expression of connexin-32 (Cx-32) and increased expression of Cx-43 (29). Connexins are the protein subunits of gap junctions and the expression pattern in BRMS1 transfectants more closely mimics normal breast tissue. Taken together, these results suggested that gap junctional communication and the connexin expression profile may contribute to the metastatic potential. Since most breast cancers have propensity to metastasize to bones, studies on cell-to-cell communication between 435 carcinoma cells and a human osteoblastic cell line, hFOB1.19 were performed to determine whether breast carcinoma cells can form gap junctions with bone cells. It was revealed that 435 cells displayed greater cell-to-cell communication with hFOB 1.19 cells than with themselves. Transfection of BRMS1 into 435 cells increased homotypic gap junctional communication but did not significantly affect heterotypic communication with hFOBs. Thus, heterotypic communication of BRMS1 transfectants with hFOB cells was reduced relative to homotypic communication. In contrast, parental 435 cells displayed greater heterotypic communication with hFOBs relative to homotypic communication. This suggests that that there are differences in the relative homotypic and heterotypic GJIC of metastasis-capable and BRMS1 expressing metastasis - suppressed cell lines (32). This implies that BRMS1 may play a role in regulating breast cancer metastasis to bone.

3.2.3 Effect on uPA Expression

BRMS1 expression inversely correlated with that of urokinase-type plasminogen activator (uPA). uPA is a pro-metastatic gene that is regulated at least in part by NFκB. It was observed that BRMS1 down-regulated uPA expression by reducing NFκB binding activity in metastatic MDA-MB-231 breast cancer and C8161 melanoma cells. Suppression of both constitutive and tumor necrosis factor (TNF)-induced NFκB activation by BRMS1 was observed in MDA-MB-231 and C8161. This was possibly due to inhibition of IκBα degradation and phosphorylation. Screen of a dot-blot breast cancer profiling array containing normalized cDNA from 50 breast tumor and corresponding normal tissues revealed reduced BRMS1 mRNA expression in breast tumors compared to their matched normal breast tissue samples (paired *t*-test, $p<.0001$) while the uPA gene expression pattern showed the reverse trend ($p<.01$) (33).

3.2.4 Effect on Intracellular Ca^{+2} Levels

Based on the observation that BRMS1-transfected cells showed differential growth patterns, DeWald *et al.* studied signaling pathways that might be affected by BRMS1 restoration. MDA-MB-435 Cells were radio-labeled with myo-[3H] inositol and PtdIns head groups and analyzed by HPLC. While parental and vector-only transfectant patterns were virtually identical. 435-BRMS1 cells showed a significant (<10% of control), and selective, diminishment of PtdIns (4,5)P2. Precursor, PtdIns (4)P and PtdIns (3)P, levels remained relatively unchanged between 435 and 435-BRMS1. PtdIns (3,4,5)P3 levels were undetectable in both 435 and 435BRMS1 even when stimulated by exogenous insulin or PDGF. PtdIns (4,5)P2 results were confirmed by immunofluorescence using a monoclonal antibody specific to PtdIns (4,5)P2. Ins(1,4,5)P3 was measured in 435 and 435-BRMS1 cells and steady state levels in 435BRMS1 were 50% lower than in 435 cells. Also *BRMS1*-transfected cells had lower intracellular calcium levels than parental 435 cells. While the gross morphology of 435-BRMS cells is similar to 435, F-actin was more readily apparent in the 435-BRMS1 cells. Taken together, these results implicate signaling through phosphoinositides is altered by BRMS1 (34).

3.2.5 Partners of BRMS1

Two-dimensional proteomic and mass spectrometry (LC-tandem MS and MALDI-TOF) analysis performed to identify proteins differentially expressed between highly metastatic MDA-MB-435 cells and metastasis-

suppressed BRMS1-transfected MDA-MB-435 cells identified differential expression for annexin I and alpha B-crystallin. Furthermore, both proteins were expressed *in vivo* in lungs containing metastasized MDA-MB-435 cells but not expressed in normal lung tissue of athymic mice. These results suggest that annexin I and alpha B-crystallin are important cellular proteins that are down regulated through BRMS1 mediated metastasis suppression (35).

3.2.6 Involvement in mSin3-HDAC Complex

By yeast two-hybrid screens and co-immunoprecipitation, BRMS1 was found to interact with retinoblastoma binding protein 1 (RBBP1 or RBP1) and at least seven members of the mSin3 histone deacetylase (HDAC) complex in human breast and melanoma cell lines. BRMS1 co-immunoprecipitated enzymatically active HDAC proteins. GAL4-DB-BRMS1 fusion repressed transcription when recruited to a Gal4 *cis*-element containing promoter -luciferase reporter assay. Size exclusion chromatography showed that BRMS1 exists in large mSin3 complex(es) of approximately 1.4-1.9 MDa, but also forms smaller complexes with HDAC1. Deletion analyses show that the carboxyl-terminal 42 amino acids of BRMS1 are not critical for interaction with much of the mSin3 complex and that BRMS1 appears to have more than one binding point to the complex (30).

3.3 BRMS1-FAMILY OF PROTEINS

After the discovery of BRMS1 gene, various GenBank searches and reports from various groups indicated that there are proteins such as SAP45 or mSDS3 and MGC11296 coded at loci distinct than BRMS1. These proteins share substantial homology with BRMS1.

Purification of mSin3A complex from K562 erythroleukemia cells and identified three new mSin3A-associated proteins (SAP): SAP180, SAP130, and SAP45. SAP180 is 40% identical to a previously identified mSin3A-associated protein, RBP1. SAP45 is homologous to mSDS3, the human ortholog of the SDS3p component of the *Saccharomyces cerevisiae* Sin3p-Rpd3p corepressor complex. Co-immunoprecipitation and gel filtration data suggested that the new SAPs are, at the very least, components of the same mSin3A complex. SAP45 repressed transcription when tethered to DNA. Furthermore, repression correlated with mSin3A binding, suggesting that SAP45 is a component of functional mSin3A$^-$corepressor complexes. It appears that these SAPs function in the assembly and/or enzymatic activity of the mSin3A complex or in mediating interactions between the mSin3A

complex and other regulatory complexes. Also it was found that SAP45 binds to the HDAC-interaction domain (HID) of mSin3A (36).

Recently using mass spectrometry, a novel component of the mSin3A/p33 (ING1b)/HDAC1 complex, p40 has been identified. p40 bears homology to both yeast Sds3, a component of yeast histone deacetylase complexes, and its mammalian homologue mSds3. The p40-associated complex purified from human cells shows a strong histone deacetylase activity. When tethered to a Gal-DNA binding domain, the Gal-p40 is able to significantly repress transcription of a Gal-luciferase promoter. Overexpression of p40 in human cells can significantly inhibit cell growth. Thus, p40 may be critically involved in transcription repression of cell growth-associated gene expression by recruiting the HDAC1 deacetylase complex (37).

Cyclin-dependent kinase 5 (Cdk5), a serine/threonine kinase that displays kinase activity predominantly in neurons, is activated by two non-cyclin activators, p35 or p39. mSds3, an essential component of mSin3-histone deacetylase (HDAC) corepressor complex and the homolog of BRMS1 was found to physically and functionally interaction with Cdk5-p35 complex. mSds3 binds to p35 both *in vitro* and *in vivo*, enabling active Cdk5 to phosphorylate mSds3 at serine-228. An mSds3 S228A mutant retains mSin3 binding activity but its dimerization was not greatly enhanced by p35 when compared to wild-type. Notably, p35 over-expression augments mSds3-mediated transcriptional repression *in vitro*. Interestingly, mutational studies reveal that the ability of exogenous mSds3 to rescue cell growth and viability in mSds3 null cells correlates with its ability to be phosphorylated by Cdk5 (38).

4. DISCUSSION

BRMS1 has been shown to be functionally responsible for metastasis suppression in mouse models of breast cancer as well as melanoma metastasis. There has been a surge of discoveries from the cell and cancer biology field as well as basic signaling, transcription regulation fields that unravel lot of interesting roles of BRMS1. One of the major finding is BRMS1 is a member of mSin3-HDAC complex. That implies that it may be a very important player in the process of histone deacetylation based transcriptional regulation or “histone Code”. The BRMS1 family members, mSDS3 (SAP45) and P40 have also been strongly shown to be involved in the same or similar Sin3-HDAC complex(es) (36-40).

The mSin3A corepressor complex contains 7 to 10 tightly associated polypeptides and is utilized by many transcriptional repressors. Much of the

corepressor function of mSin3A derives from associations with the histone deacetylases HDAC1 and HDAC2; however, the contributions of the other mSin3A-associated polypeptides remain largely unknown (36).

The "histone code" guides many aspects of chromosome biology including the equal distribution of chromosomes during cell division. Specific factors and their precise roles in highly orchestrated process of chromosome segregation still remain under investigated. Germ-line or somatic deletion of mSds3, generates a cell-lethal condition associated with rampant aneuploidy, defective karyokinesis, and consequently, a failure of cytokinesis. mSds3-deficient cells fail to deacetylate and methylate pericentric heterochromatin histones and to recruit essential heterochromatin-associated proteins, resulting in aberrant associations among heterologous chromosomes *via* centromeric regions and consequent failure to properly segregate chromosomes. Thus mSds3 and its associated mSin3/HDAC components are postulated to play a central role in initiating the cascade of pericentric heterochromatin-specific modifications necessary for the proper distribution of chromosomes during cell division in mammalian cells (40). BRMS1's role in this process is still under investigation.

BRMS1 has been demonstrated to interact with retinoblastoma binding protein, RBBP1 (RBP1). RBP1 was shown to regulate the cell cycle (G_0/G_1) by targeting the E2F regulated promoters. These findings have great relevance to cancer as HDAC inhibitors are currently tested in several clinical trials. It should be noted that though these set of findings imply that BRMS1 and its family members may play a role in cell cycle and cell division, the exact relevance of this with respect to control of metastatic process is still enigmatic. Also it is still not clear whether the homologs of BRMS1 have any direct role in metastasis.

Recently a number of HDAC inhibitors are in clinical trials (41-46). The above mentioned role of BRMS1 appears to be crucial in understanding the reasoning behind their mechanism of action and predicting their disease relevance.

Other mechanisms discussed above are cell-cell communication changes, suppression of uPA expression *via* reduction of NFkB activity. Annexin I down-regulation is strongly associated with aggressive metastatic phenotype and clearly suggest possible mechanism(s) through which BRMS1 may function to suppress metastasis. The drop in Ca^{+2} levels through PLC signaling in presence of BRMS1 will regulate cell survival. It is interesting to note that these mechanisms are connected to some kind of upstream event, possibly transcription regulation. May be all the suggested genes such as uPA, Connexins, some enzymes of the phosphoinositide pathway are regulated by one or more BRMS1-mSin3-HDAC complexes.

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REFERENCES

1. Steeg P. S., Metastasis suppressors alter the signal transduction of cancer cells. Nat Rev Cancer, 2003; 1:55-63.
2. Shevde L. A. et al., Metastasis suppressor genes: an evolving paradigm. Cancer Lett, 2003; 198:1-20.
3. Hartsough M. T., Morrsson D. K., Salerno M., Palmieri D., Outas T., Mair M., Patrick J., Steeg P. S. Nm23-H1 metastasis suppressor phosphorylation of kinase suppressor of ras via a histidine protein kinase pathway. J Biol Chem, 2002; 277:32389-32399.
4. Odintsova E., Sugiura T. and Berditchevski F. Attenuation of EGF receptor signaling by a metastasis suppressor, the tetraspanin CD82/KAI-1. Current Biol ,2000; 10:1009-1012.
5. Kotani M., Detheux M., Vandenbogaerde A., Communi D., Vanderwinden J. M., LePoul E., Brezillon S., Tyldesley R., Suarez-Huerta N., Vandeput F., Blanpain C, Schiffmann S. N., Vassart G. and Parmentier M. The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. J Biol Chem, 2001; 276:34631-34636.
6. Muir A. I., Chamberlain L., Elshourbagy N. A., Michalovich D., Moore D. J., Calamari A., Szekeres P. G., Sarau H. M., Chambers J. K., Mordock P., Steplewski K., Shabon U., Miller J. E., Middleton S. E., Darker J. G., Larminie C. G. C., Wilson S., Bergsma D. J., Emson P., Faull R., Pilpott K. L., Harrison D. C. AXOR12: A novel human G protein-coupled receptor, activated by the peptide KiSS-1. J Biol Chem, 2001; 276:28969-28975.
7. Ohtaki T., Shintani Y., Honda S., Matsumoto H., Hori A., Kanehashi K., Torao Y., Kumano S., Takatsu Y., Matsuda Y., Ishibashi Y., Watanabe T., Asada M., Yamada T., Suenaga M., Kitada C., Usuki S., Kurokawa T., Onda H., Nishimura O. and Fujino M. Metastasis suppressor gene KiSS1 encodes peptide ligand of a G-protein coupled receptor. Nature, 2001; 411:613-317.
8. Goldberg S. F., Miele M. E., Hatta N., Takata M., Paquette-Straub C. A., Freeman L. P. and Welch D. R. Melanoma metastasis suppression by chromosome 6: Evidence for a pathway regulated by CRSP3 and TXNIP. Cancer Res, 2003; 63:432-440.
9. Nakamura H., Nakamura K. and Yodoi J. Redox regulation of cellular activation. Ann Revs Immunol, 1997; 15:351-369.
10. Nishiyama A., Matsui M., Iwata S., Hirota K., Masutani H., Nakamura H., Takagi Y., Sono H., Gon Y. and Yodoi J. Identification of thioredoxin-binding protein-

2/vitaminD(3) up-regulated protein 1 as a negative regulator of thioredoxin function and expression. J Biol Chem, 1999; 264:21645-21650.

11. Yamanaka H., Maehira F., Oshiro M., Asato T., Yanagawa Y., Takei H. and Nakashima Y. A possible interaction of thioredoxin with VDUP1 in HeLa cells detected in a yeast two-hybrid system. Biochem Biophys Res Comm, 2000; 271:796-800.
12. Naar A. M., Beaurang P. A., Zhou S., Abraham S., Solomon W. and Tijan R., Composite co-activator ARC mediates chromatin-directed transcriptional activation. Nature, 1999; 398:828-832.
13. Taatjes D. J., Naar A. M., Andel F., III, Nogales E. and Tijan R. Structure, function, and activator-induced conformations of the CRSP coactivator. Science, 2002; 295:1058-1062.
14. Kauffman E. C., Robinson V. L., Stadler W. M., Sokoloff M. H. and Rinker-Schaeffer C. W. Metastasis suppression: the evolving role of metastasis suppressor genes for regulating cancer cell growth at the secondary site. J Urol, 2003; 169:1122-1133.
15. Nam J. S., Ino Y., Sakamoto M. and Hirohashi S. Src family kinase inhibitor PP2 restores the E-cadherin/catenin cell adhesion system in human cancer cells and reduces cancer metastasis. Clin Cancer Res, 2002; 8:2430-2436.
16. Boettenr B. and VanAelst L. The role of RhoGTPases in disease development. Gene, 2002; 286: 155-174.
17. Shiozaki H., Oka H., Inoue M., Tamura S. and Monden M. E-cadherin mediated adhesion system in cancer cells. Cancer, 1996; 77(8 Supl): 1605-1613.
18. Kurdistani S. K., Aritzi P., Reimer C. L., Sugrue M. M., Aaronson S. A., Lee S. W. Inhibition of tumor cell growth by RTP/rit42 and its responsiveness to p53 and DNA damage. Cancer Res, 1998; 58: 4439-4444.
19. Guan R. J., Ford H. L., Fu Y., Li Y., Shaw L. M., Pardee A. B. Drg-1 as a differentiation-related, putative metastasis suppressor gene in human colon cancer. Cancer Res, 2000; 60:749-755.
20. Bandopadhyay S., Pai S. K., Gross S. C., Hirota S., Miura K., Saito K., Coomes T., Hayashi S., Watabe M., Watabe K. The Drg-1 gene suppresses tumor metastasis in prostate cancer. Cancer Res, 2003; 63:1731-1736.
21. Egeblad M. and Werb Z. New functions for the matrix metalloproteinases in cancer progression. Nature Revs Cancer, 2002; 2:161-174.
22. Jiang Y. F., Goldberg I. D. and Shi Y. E. Complex roles of tissue inhibitors of metalloproteinases in cancer. Oncogene, 2002; 21:2245-252.
23. Chang C. and Werb Z. The many faces of metalloproteases: cell growth, invasion, angiogenesis and metastasis. Trends in Cell Biol, 2001; 11:S37-S43.
24. Phillips K. K., Welch D. R., Meiel M. E., Lee J. H., Wei L. L., Weissman B. E. Suppression of MDA-MB-435 breast carcinoma cell metastasis following the introduction of human chromosome 11. Cancer Res, 1996; 56:12222-12226.
25. Seraj M. J., Samant R. S., Verderame M. F., Welch D. R. Functional evidence for a novel human breast carcinoma metastasis suppressor, BRMS1, encoded at chromosome 11q13. Cancer Res, 2000; 60:2764-2769.
26. Shevde L. A., Samant R S., Goldberg S. F., Sikaneta T., Alessandrini A., Donahue H. J., Mauger D. T. and Welch D. R. Suppression of human melanoma metastasis by the metastasis suppressor gene, BRMS1. Exp Cell Res, 2002; 273:229-239.
27. Samant R. S., Debies M. T., Shevde L. A., Verderame M. F. and Welch D. R. Identification and characterization of murine ortholog (Brms1) of breast cancer metastasis suppressor 1 (BRMS1). Int J Cancer, 2002; 97:15-20.
28. Samant R. S., Seraj M. J., Saunders M. M., Sakamaki T., Shevde L. A., Harms J. L., Leonard T. O., Goldberg S. F., Budgeon L. R., Meehan W. J., Winter C. R., Christensen

N. D., Verderame M. F., Donahue H. J. and Welch D. R. Analysis of mechanisms underlying BRMS1 suppression of metastasis Clin Exp Metas, 2001; 18:683-693.

29. Saunders M. M., Seraj M. J., Li Z. Y., Zhou Z. Y., Winter C. R., Welch D. R. and Donahue H. J. Breast cancer metastatic potential correlates with a breakdown in homospecific and heterospecific gap junctional intercellular communication. Cancer Res, 2001; 61: 1765-1767.
30. Meehan W. J., Samant R. S., Hopper J. E., Carozza M. J., Shevde L. A., Workman J. L., Eckert K. E., Welch D. R. Interaction of BRMS1, a metastasis suppressor with RBP1 and the mSin3 histone deacetylase complex. J Biol Chem, 2004; 279:1562-1569.
31. Cheung S. T. et al. Identify Metastasis-associated Genes in Hepatocellular Carcinoma through Clonality Delineation for Multinodular Tumor. Cancer Res, 2002; 62:4711–4721,
32. Kapoor P., Saunders M.M., Li Z., Zhou Z., Schaeffer N., Kunze E.L., Samant R.S., Welch D.R. and Donahue H.J. Breast cancer metastatic potential: Correlation with increased heterotypic gap junctional intercellular communication between breast cancer cells and osteoblastic cells. Int J Cancer, 2004; 111:693-7
33. Cicek M., Fukuyama R., Welch D. R., Sizemore N., Casey G. Breast cancer suppressor gene 1 (brms1) inhibits urokinase plasminogen (upa) gene expression by reducing nfkb activity in human breast and melanoma cell lines. 10th International congress of the Metastasis Research Society; 2004 September 17th-20th; Genoa, Italy. Abstract #5
34. DeWald D. B., Torabinejad J., Samant R. S., Erin N., Johnston D., Welch D. R. Breast cancer metastasis suppressor 1 (brms1) expressing mda-mb-435 cells have significantly reduced levels of phosphatidylinositol 4,5 bisphosphate (ptdins(4,5)p2) . 10th International congress of the Metastasis Research Society; 2004 September 17th-20th; Genoa, Italy. Abstract #65
35. Cicek M., Saman, R. S., Kinter M., Welch D.R. and Casey G. Identification of metastasis associated proteins through protein analysis of metastatic MDA-MB-435 and Metastasis Suppressed BRMS1 transfected MDA-MB-435 cells. Clin Exp Metastasis, 2004; 21:149-157
36. Fleischer T. C., Yun U. J., Ayer D. E. Identification and characterization of three new components of the mSin3A corepressor complex. Mol Cell Biol, 2003; 23:3456-67.
37. Nikolaev A. Y., Papanikolaou N. A., Li M., Qin J., Gu W. Identification of a novel BRMS1-homologue protein p40 as a component of the mSin3A/p33(ING1b)/HDAC1 deacetylase complex. Biochem Biophys Res Commun, 2004; 29; 323:1216-22.
38. Li Z., David G., Hung K. W, Depinho RA, Fu A. K, Ip N. Y. Cdk5/p35 phosphorylates mSds3 and regulates mSds3-mediated repression of transcription. JBC , 2004; 279: 54438-54444.
39. Alland L., David G., Shen-Li H., Potes J., Muhle R., Lee H. C., Hou H. Jr, Chen K., DePinho R. A. Identification of mammalian Sds3 as an integral component of the Sin3/histone deacetylase corepressor complex. Mol Cell Biol, 2002; 22:2743-50.
40. David G., Turner G. M., Yao Y., Protopopov A., DePinho R. A. mSin3-associated protein, mSds3, is essential for pericentric heterochromatin formation and chromosome segregation in mammalian cells. Genes Dev, 2003; 17:2396-405.
41. Kelly W. K., Richon V. M., O'Connor O., Curley T., MacGregor-Curtelli B., Tong W., Klang M., Schwartz L., Richardson S., Rosa .E, Drobnjak M., Cordon-Cordo C., Chiao J. H., Rifkind R., Marks P. A., Scher H. Phase I clinical trial of histone deacetylase inhibitor: suberoylanilide hydroxamic acid administered intravenously. Clin Cancer Res, 2003; 9:3578-88.
42. Kim D. H., Kim M., Kwon H. J. Histone deacetylase in carcinogenesis and its inhibitors as anti-cancer agents. J Biochem Mol Biol, 2003; 36:110-9.

43. Vigushin D. M., Coombes R. C. Histone deacetylase inhibitors in cancer treatment. Anticancer Drugs, 2002; 13:1-13.
44. Sandor V., Bakke S., Robey R. W., Kang M. H., Blagosklonny M. V., Bender J., Brooks R., Piekarz R. L., Tucker E., Figg W. D., Chan K. K., Goldspiel B., Fojo A. T., Balcerzak S. P., Bates S. E. Phase I trial of the histone deacetylase inhibitor, depsipeptide (FR901228, NSC 630176), in patients with refractory neoplasms. Clin Cancer Res, 2002; 8:718-28.
45. Kwon H. J., Kim M. S., Kim M. J., Nakajima H., Kim K. W. Histone deacetylase inhibitor FK228 inhibits tumor angiogenesis. Int J Cancer, 2002; 97:290-6.
46. Richon V. M, O'Brien J. P. Histone deacetylase inhibitors: a new class of potential therapeutic agents for cancer treatment. Clin Cancer Res, 2002; 8:662-4.

Chapter 11

MECHANISMS OF DNA DEMETHYLATING DRUGS AGAINST CANCER PROGRESSION

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Abstract: DNA methylation has been shown over the last several decades to play a critical role in tumorigenesis for almost all cancers. In this chapter, we review the underlying changes in methylation that are found in cancer cells, namely genomic hypomethylation and CpG (or regional) hypermethylation (relative to normal cells). CpG hypermethylation is associated with gene silencing, and a major area of research over the last decade has been the identification of numerous genes that are methylated in sporadic cancers. Many, if not most, tumor suppressor genes that have been identified on the basis of their relationship with familial, inherited cancer syndromes, have been shown to undergo CpG hypermethylation in sporadic, non-inherited cancers. We then discuss the association of CpG hypermethylation with transcriptional silencing and review the mechanism(s) through which methylation regulates transcriptional activity. One under-appreciated (but likely relevant) mechanism of transcriptional control through hypermethylation of promoter elements is the steric hindrance that methyl groups may place upon transcription factor binding. Interestingly, this steric hindrance may occur with transcription factor binding sites (ones that contain the CG dinucleotide), but may also be affected by cytosine methylation adjacent to the putative binding site. One of the critical insights over the last 5-6 years has been the understanding of the inter-relatedness of DNA methylation and chromatin structure, especially as affected by post-translational core histone modifications. This is discussed (along with the potential strategy of combining DNA methylation inhibition with histone deacetylase inhibitors). There are several distinct types of methylation inhibitors including nucleoside inhibitors (such as 5-aza-2'-deoxycytidine, 5-azacytidine or zebularine), non-nucleoside inhibitors (such as procainamide and hydralazine) and antisense oligonucleotides directed against one of the three known DNA methyltransferases (DNMT1, DNMT3a and DNMT3b). Of these three types

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of DNA methylation inhibitors, most clinical experience has been generated with the nucleoside analogues. These require intracellular phosphorylation and incorporation into the host cell DNA in order to function. The mechanisms of action include trapping of DNA methyltransferases onto the DNA (this is only operant with the nucleoside analogues), re-expression of silenced genes that have growth regulatory or pro-apoptotic effects, and DNA damage (again, probably restricted to nucleoside analogues). Interestingly, there is abundant evidence that methylation alone does not determine which genes are expressed after exposure to methylation inhibitors. While enthusiasm for the clinical strategies investigating the incorporation of these agents into our oncologic armamentarium seems warranted, the final portion of this review expresses an element of caution. There are a number of scenarios (which have an experimental basis) in which DNA hypomethylation may lead to adverse outcomes. First, hypomethylation may lead to enhanced mutation (as is seen in mouse cells engineered to lack one of the DNMT enzymes). Second, DNA hypomethylation could conceivably lead to the re-expression of positive growth regulatory genes (such as matrix metalloproteinases which might enhance the invasiveness and metastatic potential of cancers). Third, a mouse expressing a hypomorphic allele of DNMT1 developed aggressive lymphoid tumors (suggesting that in these cells, DNMT1 may function as a tumor suppressor gene). Fourth, DNA damage (which has been demonstrated with the nucleoside inhibitors of DNA methyltransferases) almost invariably is correlated with enhanced mutagenesis and would conceivably lead to an increased risk of tumorigenesis. Finally, unexpected and unusual toxicities might be expected by the use of these agents such as drug induced lupus seen with procainamide and hydralazine, which is related to DNA hypomethylation in T lymphocytes.

Key words: DNA methylation, histones, nucleoside inhibitors, antisense oligonucleotides, transcriptional silencing, gene re-expression, DNA damage

1. INTRODUCTION

Cancer is a genetic disease, characterized by somatic mutations, genomic instability, non-random chromosomal alterations (translocations, deletions, amplifications, amongst others), mutational activation of growth promoting genes ("oncogenes") and mutational inactivation of growth inhibiting ("tumor suppressor") genes. In addition to genetic inactivation of tumor suppressor genes, epigenetic changes, such as DNA methylation and post-translational histone modifications, appear to play crucial roles in tumorigenesis [1, 2]. The rapidly evolving field of epigenetics studies the heritable modifications that regulate gene expression without altering the DNA sequence [3]. Tremendous progress has been made to understand the significance of epigenetic regulation of gene expression in the last decade. It

has been estimated that silencing of tumor suppressor genes by aberrant DNA promoter methylation is at least as common as genetic mutations. In addition, approximately 50% of tumor suppressor genes related to familial cancers have been shown to be silenced by methylation in sporadic cancers [1]. Another epigenetic change, the status of post-translational histone modifications (including acetylation, deacetylation, methylation, phosphorylation and ubiquitination) affects chromatin structure and plays a key role in the regulation of gene expression [4]. Recent evidence has connected these two epigenetic processes in the regulation of gene expression [5, 6].

DNA methylation refers to the addition of a methyl (CH_3) group to the carbon-5 position of the cytosine pyrimidine ring in the 5'-CpG-3' dinucleotide. Non-CpG methylation exists at very low frequency. DNA methylation is maintained by DNA methyltransferase 1 (*DNMT*1) after DNA replication [7]. *DNMT*1 has *de novo* methyltransferase function, but the activity is much higher on hemi-methylated DNA, such that the principle function of *DNMT*1 is to maintain DNA methylation patterns [8]. The other two DNA methyltransferases, *DNMT*3A and *DNMT*3B, are *de novo* methyltransferases and highly expressed in embryonic stem cells [8]. DNA methylation is an epigenetic change, since it is heritable and affects chromatin organization and gene expression without changing of the genetic code [9]. DNA methylation in mammals is involved in several normal processes including genomic imprinting [10, 11], X chromosome inactivation [12] and aging [13].

CpG dinucleotides are not evenly distributed in the human genome. On average, CpGs only present once per 80 dinucleotides for 98% of the genome. This is far below the predicted frequency of one in 16, presumably because 5-methylcytosine is relatively unstable and can undergo spontaneous deamination leading to a base change from cytosine to uracil. Genome-wide evaluation in normal cells shows that the majority (70-80%) of single CpG dinucleotides are methylated. These methylated regions occur mainly in intronic DNA, in the bodies of many genes and in repetitive sequences [14-16]. In contrast, CpG dinucleotides that exist in CpG islands are usually unmethylated in normal tissue. CpG islands are defined as a stretches of DNA which include a G+C content of greater than 50% with a ratio of observed to expected CpG (number of CpG × total number of nucleotides / number of C × number of G) of at least 0.6 [17]. CpG islands tend to be found in the promoter elements of genes and range in size from 0.2 kilobases (kb) to 4 - 5 kb. There are approximately 29,000 CpG islands in the human genome based on human draft sequences [18], and approximately 50 to 60% of genes are associated with CpG islands.

Methylation of CpG islands in gene promoter regions usually is associated with gene silencing [14, 19].

The mechanism (or mechanisms) of transcriptional repression by DNA methylation is not entirely clear. One possibility is that DNA methylation interferes with the binding of transcription factors, such as (AP-2, NFκB) that contain CpGs in their transcription factor binding sites [20-22]. Interestingly, data has also shown that methylation adjacent to transcription factor binding sites (specifically Sp1 sites in the p21 promoter) can also affect transcription factor binding [23]. In a second, alternative mechanism, transcriptional repressors, such as methyl-CpG-binding domain protein 2 (MBD2) and methyl-CpG-binding protein 2 (MeCP2) are able to bind methylated CpGs and recruit histone deacetylase (HDAC) [24, 25]. For example, MBD2 is associated with HDAC1 in the MeCP1 repressor complex (22), while MeCP2 recruits the Sin3-HDAC co-repressor complex [24]. The recruitment of HDACs to the local sites of promoter hypermethylation results in a hypoacetylation of histone H3 and H4 tails. This results in the chromatin structure assuming a condensed form that is non-permissive for transcription [24]. Therefore, methylation of the gene promoter region causes gene inactivation through a number of distinct mechanisms [1, 26].

2. DNA METHYLATION CHANGE IN CANCER

2.1 Genome-wide Hypomethylation

It has been recognized for approximately 20 years that epigenetic changes in neoplasia include overall genomic hypomethylation, with concurrent hypermethylation in CpG islands that are unmethylated in normal tissues [27, 28]. DNA hypomethylation has been observed in every tumor type that has been studied, using distinct techniques such as methylation sensitive restriction endonucleases [29] or high-pressure liquid chromatography (HPLC) to measure the global 5-methylcytosine content [30]. Hypomethylation refers to both decrease of methylation of the entire genome (global hypomethylation) and the localized specific genomic regions, such as the promoter regions of oncogenes. Global hypomethylation represents the decrease of methylation in CpG dinucleotides in both repetitive elements and within the bodies of the genes [31]. The degree of genome-wide hypomethylation parallels the progression of the malignancy [26, 30]. It has been reported that the extent of hypomethylation in breast cancer is correlated significantly with disease stage, tumor size, and degree

of malignancy [32]. Therefore, DNA hypomethylation might serve as prognostic biomarker.

Although the cause of DNA hypomethylation in cancer is not clear [27, 28], multiple mechanisms have been proposed for its contribution to tumor progression [27, 31]. It is conceivable that growth promoting genes may be normally silenced in quiescent cells, and during tumorigenesis, such genes could be activated by demethylation, such as CMYC [33]. Second, hypomethylation has been linked to chromosomal instability in cancer. For example, in breast adenocarcinoma, ovarian epithelial tumors, and sporadic Wilms tumors, pericentromeric heterochromatin of chromosome 1 and 16 are hypomethylated and unstable, which leads to unbalanced chromosomal translocation and chromosomal arm loss [8, 34]. Third, activation of the multiple drug resistance gene MDR1 by hypomethylation might lead to drug resistance of acute myeloid leukemias [35]. Fourth, hypomethylation may promote tumor metastasis [28]. The role of hypomethylation in cancer development has been reviewed in more detail [27, 28, 31].

2.2 CpG island Hypermethylation

Jones and Baylin reviewed evidence that demonstrates the silencing of tumor suppressor genes by promoter methylation plays an important role in tumor development [1]. First, transcriptional silencing of cancer related genes by aberrant CpG island methylation is observed in every type of cancer (with the exception of seminomas [36]). The list of candidate tumor suppressor genes silenced by promoter methylation continues to grow; see also [26]. Second, nearly 50% of the tumor suppressor genes associated with familial cancers where they are inactivated by mutations can be silenced by promoter methylation in sporadic tumors. Examples of such genes include *BRCA1*, *VHL*, *MLH1*, *APC*, *p16*, and *RB*. Third, promoter methylation is regarded as the one “hit” or both “hits” needed to inactivate suppress tumor suppressor genes in Knudson’s two hit model. The methylation can be the second hit when combined with genetic changes, such as LOH or mutation of the other allele. Fourth, cancers with epigenetic inactivation of important genes, such as *MLH1* and *MGMT* DNA repair genes, predispose the genome to genetic changes. Fifth, tumor suppressor genes that are affected by both epigenetic change and/or genetic changes are usually in a region of LOH [1].

3. METHYLATED GENES IN HUMAN CANCER

3.1 Hematopoietic malignancies

As is described in solid tumors, global hypomethylation also can be seen in hematopoietic malignancies, such as acute myeloid leukemia (AML) [37] and chronic lymphocytic leukemia [38]. In addition, hypermethylation of multiple genes in their promoter regions has been reported and summarized [39, 40]. For examples, *p15*, *p16*, Calcitonin, *MYOD1* and Estrogen receptor have been shown to be methylated in many types of leukemia [39, 40].

3.2 In Human Lung Cancer

Similarly, concurrent hypomethylation and CpG island hypermethylation play critical roles in solid tumor development. The role of methylation in oncogenesis has been explained by one or more mechanisms. First, tumor suppressor genes can be inactivated by hypermethylation of CpG islands in promoter regions. Examples include *RB*, *VHL*, *p16*, *p15*, E-cadherin and h*MLH1* [14]. Second, methylated CpG dinucleotides determine hot spots for mutations in the p53 tumor suppressor gene [19, 41]. We will focus on gene promoter hypermethylation using lung cancer as an example/model.

Promoter hypermethylation of one or both alleles is one of the mechanisms leading to aberrant gene function in lung cancer development. More than 30 genes have been reported to show increased promoter methylation in human lung cancer [42]. These genes have been shown to be involved in a variety of cellular functions. Some of the genes that have been demonstrated to be silenced by promoter methylation in lung cancer are listed in Table 1 [26, 42]. Since the genes affected by methylation have such important functions in the regulation of cell cycle control, differentiation, DNA repair, metastasis and cell adhesion, silencing of these genes could play critical roles in lung cancer development and progression. Different strategies have been utilized to detect the methylation, which results in differences in the frequency of methylation in individual genes from different studies [42].

CDKN2A is a well-studied tumor suppressor gene exhibiting promoter methylation in lung cancer [43-46]. *CDKN2A* abnormalities, frequently observed in primary NSCLC and cell lines, include homozygous deletions and promoter methylation of both alleles. These alterations of *CDKN2A* are rare in SCLC. In some studies, p16 promoter methylation shows no difference among histological subtypes or tumor stages [46]. However, in other studies, p16 has been shown to play a very early role in lung cancer

development, and the methylation status increases during tumor progression in both smoking related carcinogen NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone derived from nicotine during smoking, induced lung cancer in rats, and in squamous cell carcinoma of the human lung [47].

4. TRANSCRIPTIONAL REGULATION BY CHROMATIN STRUCTURE

4.1 Histone Acetylation and Histone Methylation

In addition to gene regulation by DNA methylation, alteration of chromatin structure also plays a key role in gene transcriptional regulation. In the nucleus, the DNA double helix wraps around histones and forms the basic nucleosomal structure [48]. The core histone proteins include H2A, H2B, H3 and H4 and form an octamer, around which the DNA is wrapped. The nucleosome units in turn are assembled into a higher order structure, called chromatin [49]. A portion of the nuclear chromatin called heterochromatin is highly condensed throughout the cell cycle and is transcriptionally inactive. In contrast, the remainder of the nuclear chromatin that contains transcriptionally active DNA has a relatively extended conformation and is called euchromatin [50]. The covalent modifications of histones, such as acetylation, phosphorylation and methylation, alter chromatin structure and thereby contribute to this distinction in transcriptional activity. This information was proposed as "histone code" that extends the information from the genetic code [49].

Histone acetylation and deacetylation are well-studied histone modifications. The deacetylated histones have positive charged lysine tails which bind tightly to negatively charged DNA. In this situation, the DNA is not accessible to the transcriptional machinery. However, if the lysine tail is acetylated by histone acetyltransferase (HAT), the bond between the DNA and the lysine residues is weakened, and the DNA can be easily transcribed. The process is reversible, allowing for the acetyl group(s) to be removed by histone deacetylases to silence gene expression [48].

Histone methylation also participates in gene regulation. It has been shown that the methylation of lysine 9 in the tail of histone H3 correlates with transcriptionally repressed chromatin, while methylated lysine 4 on H3 is found in transcriptionally active chromatin flanked by heterochromatin. Recent evidence in *Neurospora crassa* and *Arabidopsis thaliana* shows that Lys9 histone methyltransferase also control DNA methylation [51, 52].

These data suggest the possibility that methylation of H3 Lys9 might be required to determine the sites of DNA methylation [1].

4.2 Connection between DNA Methylation and Histone Modifications

In addition to the previous finding that Lys9 histone methyltransferase controls DNA methylation in some species [51, 52]; DNA methylation has been related to histone acetylation. First, as mentioned above, methyl-CpG-binding proteins, such as MeCP1 and MeCP2 bind to methylated DNA and recruit histone deacetylase to remodel the chromatin structure [1, 26]. Second, a number of investigators have shown that DNA methyltransferases (DNMT1, DNMT3A and DNMT3B) directly associate with histone deacetylase (HDAC). For example, DNMT1 binds to HDAC1 and HDAC2 to recruit histone deacetylase activity [53, 54]. Similarly, DNMT3a can directly recruit HDAC1 [55]. Third, the acetylation of histones was found to be inversely associated with DNA methylation, such as in death-associated protein kinase (*DAPK*) [56]. The acetylation status of histones H3 and H4 related to specific genes can be detected by the chromatin immunoprecipitation assay [56]. Fourth, the DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine, and the histone deacetylase inhibitor, Trichostatin A (TSA), have synergistic effects to induce the re-expression of silenced genes in cancer cells [57]. Therefore, it has been proposed that promoter methylation, together with histone deacetylation, maintains condensed heterochromatin, causing the gene to become inaccessible to the transcriptional machinery, resulting in gene silencing [1].

4.3 Rationale for the Use of DNA Hypomethylating Agents for the Treatment of Human Cancer

DNA methylation plays an essential role in the inactivation of numerous tumor suppressor genes through transcriptional silencing [2], and this modification (unlike other causes of tumor suppressor gene loss such as deletion or mutational inactivation) is reversible Thus, demethylating agents, such as inhibitors of DNA methyltransferase I, has been developed to inhibit DNA methylation for the treatment of cancer patients. Nucleoside analogs, azacytidine (5-azacytidine), decitabine (5-aza-2’-deoxycytidine), fazarabine (1-β-D-arabinofuranosyl-5-azacytosine and DHAC (dihydro-5-azacytidine), have been evaluated in both animal model systems and clinical trials [58]. In addition, non-nucleoside inhibitors including antisense oligonucleotides to *DNMT*1, *DNMT*3a and *DNMT*3b have also been tested in a variety of

preclinical and clinical settings [59]. A final group of non-nucleoside inhibitors that have been described include hydralazine, procainamide and procaine which also induce global hypomethylation and are able inhibit the growth of human cancer cells [59]. In addition, the combination of DNA methyltransferase inhibitors with histone deacetylase inhibitors has shown synergistic effects in the induction of apoptosis in cancer cells and is now being evaluated in clinical trials in a variety of solid and liquid tumors [60].

5. DNA METHYLATION INHIBITORS

There are a number of distinct strategies that can be utilized to inhibit DNA methylation. Three types of inhibitors have been utilized and characterized, including nucleoside analogues (including 5-azacytidine and 5-aza-2'-deoxycytidine), antisense oligonucleotides, and non-nucleoside analogues (such as procainamide).

The nucleoside analogues require incorporation into DNA in order to function, and thereby are phosphorylated to their tri-phosphate form prior to incorporation [61]. Similarly, nucleoside DNMT inhibitors require S-phase in order to function [59], and for this reason, a number of trials utilizing these inhibitors have utilized either continuous infusions, or low dose chronic exposure to the agents for effect [62, 63]. Structurally, nucleoside analogues resemble cytidine (Figure 1A).

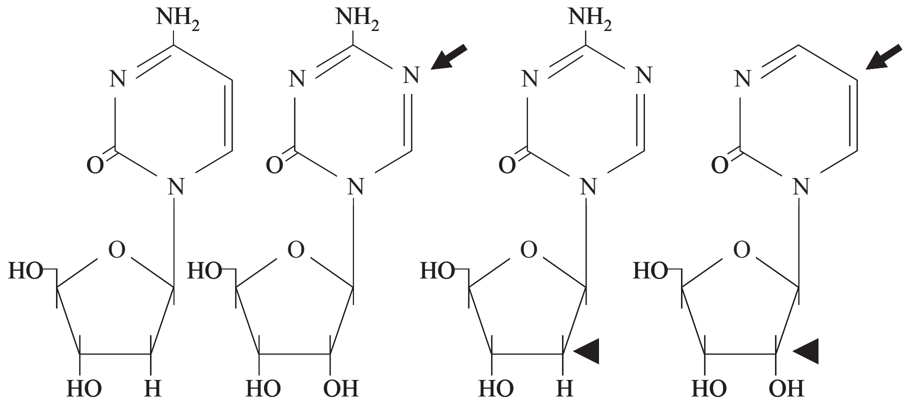

Figure 1A. Structural comparison between 2'-deoxycytidine, 5-azacytidine, 5-aza-2'-deoxycytidine, and zebularine. Arrow shows 5-position on pyrmidine ring that distniguishes "aza" compound, arrowhead shows 2' position on sugar that distinguishes ribose from deoxy-ribose.

5-azacytidine was developed as a cytosine analogue in 1964 [64]. In the mid 1970s, it was found that treatment of uncommitted muscle progenitor cells (murine 10T1/2 cells) with 5-azacytidine led to myotube formation and a more differentiated phenotype [65]. At low levels it interferes with RNA processing and protein production. 5-Azacitidine is degraded by nucleoside deaminase; thus if this enzyme is present at high levels in a cell, they will confer a diminished response to the drug [58]. This drug is most active in cells that are rapidly dividing and are in S phase of the cell cycle. The majority of clinical trials with this drug have been in hematologic malignancies, and the FDA recently approved this agent for the treatment of patients with myelodysplastic syndromes on the basis of a randomized clinical trial (between 5-azacytidine and best supportive care) that demonstrated improved response rate, quality of life and survival, along with decreased transfusion requirements and diminished leukemic transformation [63].

5-aza-2'-deoxycitidine (Decitabine) after being phosphorylated, is preferentially incorporated into DNA [58]. It is entirely inactivated by cytidine deaminase, which is present in high levels in both the liver and the spleen. Thus, *in vivo*, it has a half-life of just 15-20 minutes. The activity and potency of 5-aza-2'-deoxycytidine in the laboratory as well as in the clinical setting appears to be higher than that of 5-azacytidine, though whether this translates into a meaningful benefit for patients is, as yet, unclear.

Jones and colleagues have recently isolated a novel cytidine analogue called zebularine that has a 6 member ring (attached to a ribose sugar), however, lacks the amino group at position 4 of the pyrimidine ring, and also lacks the aza atom at position 5 (unlike the other DNMT inhibitors) [66, 67] (Figure 1A). While the potency of this drug appears less than that of 5-aza-2'deoxycytidine (in animal cells *in vitro*), it has the advantage of being orally available which may make this an attractive drug to eventually apply to cancer therapy (given the option, patients tend to prefer oral rather than intravenous administration of chemotherapy for palliative purposes [68]).

Procainamide, an oral and intravenous type 1 antiarrythmic agent that inhibits sodium dependent depolarization of cardiac muscle, has been found to have demethylating properties on T-cells, which is thought to be part of the mechanism of the unusual toxicity of drug-induced lupus [69, 70]. This agent is not a nucleoside inhibitor (Figure 1B) and is presumed to act on DNA methyltransferases in ways distinct from that of the aza compounds or zebularine.

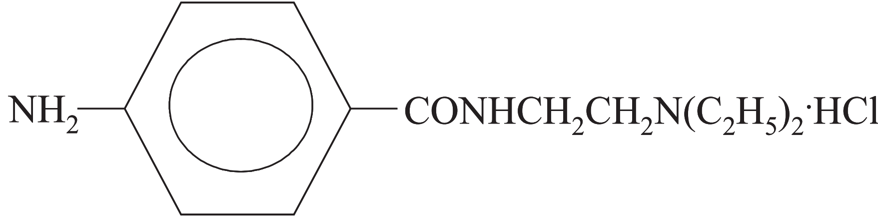

Figure 1B. Structure of Procainamide HCl, a non-nucleoside inhibitor of DNA methyltransferase.

The final class of hypomethylating agents are the antisense oligonucleotides [71]. A variety of inhibitors have been developed against *DNMT*1, *DNMT*3a and *DNMT*3b, but only the *DNMT*1 oligonucleotide has, to date been utilized in human clinical trials [72, 73]. The results of these studies have shown that it is feasible to deliver a 20-mer phosphorothioate antisense to *DNMT*1 and there is preliminary evidence that *DNMT*1 mRNA expression is down regulated in patients treated with this agent by a continuous 7-day infusion [74]. A phase II study in patients with renal cell carcinoma was somewhat disappointing with 7 patients having best response as stable disease with 8 patients having progressive disease [75].

6. MECHANISMS OF DRUG ACTION

The chemical mechanism by which nucleoside inhibitors are able to inhibit DNA methyltransferases appears to be through the incorporation into DNA (after phosphorylation) and subsequent trapping of DNMT onto the DNA. Muller and colleagues have developed an interesting assay that is able to capture the DNMT as it is "trapped" by the aza compounds [76]. Interestingly, this functional trapping of DNMT upon DNA with incorporated 5-aza-2'-deoxycytidine occurs quite quickly. Concurrently with the trapping of DNA methyltransferases onto DNA, "free" DNMT1 (assayed by Western immunoblot) disappears from whole cell protein lysates at the same doses required to cause methylated gene re-expression (such as p16) (Figure 2).

Treatment of transformed cancer cells with DNA methyltransferase inhibitors causes a broad spectrum of changes directly related to the hypomethylating effects of the treatment. This principally is due to the transcriptional de-repression (or relief of transcriptional silencing) that is associated with promoter hypermethylation [77]. It should be noted that genes can also be induced through treatment with DNMT inhibitors that have no promoter hypermethylation [78, 79]. In addition to the effects

caused by the re-expression of growth regulatory genes, there are differentiating effects (that may be related to the re-expression of genes involved in the differentiation pathway) [39, 65], DNMT trapping [80], and DNA damaging effects of the incorporation of nucleoside analogues into DNA [81, 82]. Some of these effects are related to *DNMT* inhibition (and would be seen with nucleoside analogues, antisense oligonucleotides and non-nucleoside inhibitors such as procainamide), while others (DNMT trapping, DNA damaging) are specific to nucleoside inhibitors.

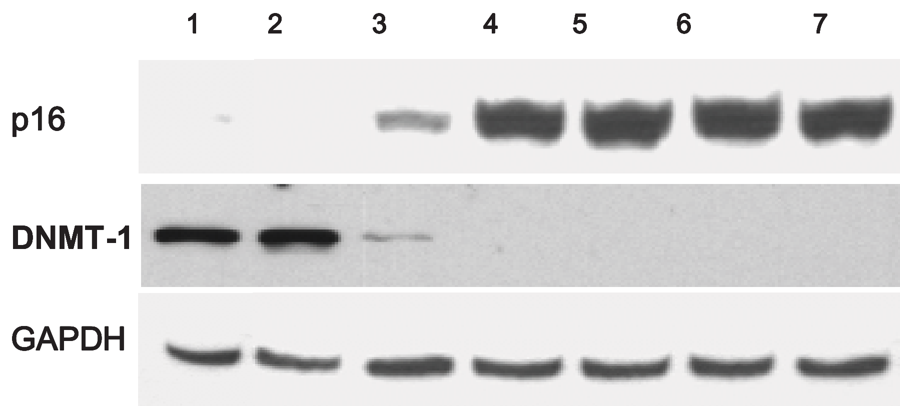

Figure 2. Treatment of lung cancer cells with 5-aza-2'-deoxycytidine at 0, 0.01, 0.1, 0.5, 1, 2, and 5 mM respectively (lanes 1-7) for 72 hours. Western immunblot analysis was used to evaluate p16, DNMT1 and GAPDH expression.

The greatest amount of attention has been paid to the re-expression of genes methylated within the promoter. In part, this is due to the ease of which measurement of re-expression of genes can be evaluated either at the mRNA or at the protein level. All of the genes listed in Table 1 have been shown to be upregulated by treatment with hypomethylating agents (sometimes with synergistic effects seen with the addition of histone deacetylase inhibitors – see below). It should be noted that the genes mentioned in Table 1 represent a small portion of the genes reported in one disease, multiplied by many distinct cancers with many different affected genes. Treatment of cancer cells with varying doses of hypomethylating agents for varying times has confirmed the impression that longer durations of therapy (perhaps to allow for incorporation during S-phase) allows for a greater likelihood (and level) of re-expression [44]. While there has been a number of clinical trials utilizing hypomethylating agents, few of these trials (in cancer patients) have been able to demonstrate gene re-expression, though one phase I study in solid tumor patients did show some evidence of gene hypomethylation in patients treated with 5-aza-2'-deoxycytidine [83].

In contrast, a number of interesting, well designed experiments in baboons and in humans with severe sickle cell anemia utilizing 5-aza-2'-deoxycytidine has been able to demonstrate that continuous, low dose exposure to this agent is capable of inducing fetal hemoglobin expression in patients with improvement in their clinical status [84, 85].

Table 1. Genes with promoter methylation in lung cancer.

Gene	Function
Retinoic acid receptor β (RARb) [109]	Differentiation
RAS association domain family member 1 (RASSF1A) [116, 117]	Signal transduction
Adenomatous polyposis of the colon (APC) [118]	Signal transduction
Tissue inhibitor of metallopreteinase 3 (TIMP3) [109]	Metastasis
p16/*CDKN2A* [44, 109]	Cell cycle
O^6-methylguanine-DNA methyltransferase (*MGMT*) [109]	DNA repair
Retinoblastoma protein interacting zinc finger (RIZ1) [119]	Histone/protein methyltransferase
S100 calcium-binding protein A2 (S100A2) [120]	Candidate tumor suppressor gene
H-cadherin (CDH13) [121]	Cell adhesion
Tumor suppressor in lung cancer 1 (TSLC1) [122, 123]	Cell adhesion
Serum deprivation response factor (SRBC) [124]	Candidate tumor suppressor gene
FHIT1 [125, 126]	Cellular metabolism

These experiments have added impetus to the strategy of utilizing low dose exposures (rather than maximum tolerated doses) in clinical studies with these agents [62]. In our preliminary experience with a phase I study of 5-aza-2'-deoxycytidine in combination with the HDAC inhibitor valproic acid, we have demonstrated doubling of fetal hemoglobin in patients with cancer (but no history of sickle cell disease).

A number of investigators have utilized array technology to evaluate gene expression changes in a more global fashion. The findings have been intriguing for a number of reasons. One aspect that is somewhat surprising is the rather limited number of genes whose expression is significantly altered when analyzed by these approaches [86]. A second intriguing result is that many of the genes whose expression is altered by treatment with

hypomethylating agents are found not to have hypermethylated promoter regions as has been reported for individual genes including *p19* [79, 86, 87]. This second results suggests the possibility either that treatment with hypomethylating agents has secondary effects (other than DNMT inhibition), or alternatively is consistent with either an alteration of a control region that is at some distance from the promoter region of the particular unmethylated gene (such as *p19*), or through alterations of a trans acting factor (such as a transcription factor that may secondarily act on the unmethylated promoter. One group compared the array results seen with treatment of cells (*in vitro*) with the hypomethylating agent 5-aza-2'-deoxycytidine, the HDAC inhibitor Trichostatin A, or through somatic genetic inactivation of *DNMT*1, *DNMT*3b or a double somatic knockout of both *DNMT*1 and *DNMT*3b. The results of this study surprisingly demonstrated more similarity between the expression patterns seen with 5-aza-2'-deoxycytidine and Trichostatin A than that seen with either the single or double somatic knockouts [86]. These results suggest that these agents have effects other than through solely affecting DNMT activity. An interesting comparison would be to evaluate the effects seen with 5-aza-2'-deoxycytidine treatment with that seen after antisense oligonucletide treatment. A somewhat unexpected result seen with array approach is that an almost equal number of genes are found to be down-regulated as are upregulated, a similar result found when HDAC inhibitors are evaluated in these assays [86, 88]. Whether this represents primary or secondary effects is not yet clear and requires further investigation. Finally, the ability of array approaches to demonstrate coordinate regulation of gene families or gene pathways was demonstrated in a number of studies and may help to point the way to novel therapeutic approaches [86, 87].

While most attention regarding the effects of DNMT inhibitors has been focused on the epigenetic regulation of gene expression (with subsequent downstream effects of the affected gene products), there is a growing body of evidence that nucleoside inhibitors of DNA methyltransferases also have a DNA damaging effect. This is demonstrated by the ability of these agents after short term culture, to induce genes that are typically affected by DNA damaging agents [81, 89, 90]. Zhu and colleagues have also obtained direct evidence (through the use of the "Comet" assay) of DNA damage in cells treated with 5-aza-2'-deoxycytidine [81]. Whether this DNA damage phenotype seen by pharmacologic treatment is related the chromosomal instability seen with tumor-linked hypomethylation [34], or the chromosomal instability seen with genetic defects in the methylation machinery (such as that seen in the ICF syndrome associated with *DNMT*3b loss [91-93]), has yet to be demonstrated. Nonetheless, it is likely that this DNA damage pathway will play an important role in the response of tumor cells *in vitro*, and tumors *in vivo* to treatment with DNA methyltransferase

inhibitors. Furthermore, it would also follow that this effect may be an important distinction between the nucleoside and non-nucleoside DNMT inhibitors (antisense and procainamide congeners).

Recent attention has been given to the promise of combining DNA methyltransferase inhibitors with HDAC inhibitors. After it was first reported that there was a synergistic re-activation of epigenetically silenced genes [57], there have been a great number of manuscripts that have evaluated this synergism in a variety of cell systems [60, 94]. It is clear that low doses of these two classes of epigenetically active agents are much more potent in their ability to enhance gene transcription (of tumor suppressor genes such as p16 in lung cancer [60], or tumor differentiation genes such as CD11b or IL3 in acute leukemia cells [94]), and to induce cellular apoptosis and histone acetylation [60]. These *in vitro* findings have led to a number of therapeutic trials in patients with solid and liquid tumors combining agents of these classes. Whether there will be any therapeutic benefit or merely enhanced toxicity has not yet been reported, but clinical investigators anxiously await the results of these trials (both the clinical results as well as the embedded biologic correlates).

7. CAUTION

Although DNA hypomethylating agents hold great promise as a novel cancer treatment strategy, the coexistence of global DNA hypomethylation and CpG island hypermethylation (and their respective contribution to tumorigenesis), has raised some theoretical concerns about using DNA hypomethylating agents for clinical treatment. First, mouse embryonic stem cells engineered to be without the DNA methyltransferase 1 (*DNMT*1) gene showed significantly elevated mutation rates for both the endogenous hypoxanthine phosphoribosyltransferase (*HPRT*) gene and an integrated viral thymidine kinase (*TK*) transgene [95]. Second, DNA hypomethylation leads to genomic instability, a feature of cancer cells, both in human cancer [34] as well as in animal models [96]. Third, mice carrying a hypomorphic *DNMT*1 allele with substantial genome-wide hypomethylation in all tissues developed aggressive T cell lymphomas, which suggests that DNA hypomethylation promotes cancer and conversely that *DNMT*1 may function, in part, as a tumor suppressor gene [97]. Fourth, 5-azacytidine or 5-aza-2'-deoxycytidine induced genomic instability in a human pro-B cell lines. The major change was rearrangements of chromosome 1 involved its pericentromeric region, including both multibranched figures and whole-arm deletions [98]. In addition, 5-aza-2'-deoxycytidine could induce the

expression of matrix metalloproteinases (MMPs) and enhance the invasiveness of pancreatic cancer cells [99].

However, to counter these arguments, investigators have argued that short time treatment using hypomethylating agents, such as 5-aza-2'-deoxycytidine, in clinical treatment induced transient and much less severe demethylation than that has been observed in animal models [100]. Furthermore, no secondary tumor induction has been observed in patients receiving hypomethylating treatment [100]. Therefore, the safety of using demethylating agents should be further evaluated. In the setting of established cancer (which all too often is life limiting), and in which current "established" treatments including cytotoxic chemotherapy and ionizing radiation are well-known carcinogens [101, 102], it may be argued that the risk of DNA damage, and induction of secondary cancers is acceptable. However, in the setting of chemoprevention or even treatment of sickle cell anemia, this would clearly be an unacceptable risk/toxicity. Though not a part of this review, there is significant human and animal model experience suggesting that methylation is an early event in tumorigenesis, and furthermore, that reversal of methylation may be an attractive preventive strategy [47, 103-105]. Further exploration of these potential pitfalls is needed.

Another caution needs to be made against the indiscriminate use of hypomethylating agents for the treatment of cancers. Esteller and colleagues have demonstrated that transcriptional silencing of the DNA-repair gene O^6-methyl-guanine-DNA methyltransferase (*MGMT*) through promoter methylation is associated with an improved survival in patients with gliomas that are treated with alkylating agents. [106] Similarly, methylation of *MGMT* predicts for responsiveness of gliomas to the new cytotoxic agent temozolomide. [107] One could infer that treatment of gliomas with a hypomethylating agent such as 5-aza-2'-deoxycytidine in combination with temozolomide or a nitrosurea would lead to an inferior survival as opposed to treatment with the cytotoxic agent alone. Lest one assume that this is only relevant to incurable neoplasms such as unresectable glioblastoma multiforme, investigators have demonstrated *MGMT* methylation as a predictor of survival for patients with diffuse large B-cell lymphoma (which are also often treated with alkylating agents such as cyclophosphamide. [108] Similarly, though alkylating agents are not used clinically in patients with lung cancer, *MGMT* has been found to be methylated at a high frequency in samples derived from patients with non-small cell lung cancer [109].

It is clear that if one is to develop molecularly targeted agents such as DNA methyltransferase inhibitors (alone and in combination with HDAC inhibitors, conventional cytotoxic chemotherapy or radiation therapy); a

thorough understanding of the molecular phenotype of the treated cancer is an important prerequisite.

A final area of caution is the potential for developing unanticipated side effects from hypomethylating agents. Autoimmune lupus appears to be associated with hypomethylated peripheral blood mononuclear cells. This may be related to lack of methylation of endogenous retroviral elements within the genome which are then recognized as foreign DNA (with subsequent antinuclear antibodies). [69, 110, 111] This involvement of hypomethylated DNA in lupus is presumably the underlying mechanism for the drug-induced lupus related to procainamide and hydralazine use. [112, 113] Interestingly, one of the nucleoside analogues tested by the Cancer and Leukemia Group B (CALGB) for the treatment of malignant mesothelioma was associated by a complication of chest pain (which was thought to be due to serositis/pericarditis). [114, 115] As investigations of DNA methylation inhibitors moves forward, investigators will need to be aware of potentially adverse immune mediated phenomena.

8. CONCLUSION

It is useful at this time to review the mechanisms of epigenetic silencing, agents currently being used to reverse DNA methylation, and potential mechanisms of cancer suppression with this class of agents, particularly as these agents move forward into clinical use in patients with cancer. As noted above, while considerable enthusiasm can be generated with the prospect of epigenetic therapy, it must be recognized that these agents also have potential cost. Clearly two concurrent requirements must be met as investigators move forward: first, a more thorough understanding of the basic biologic and molecular processes that are involved in the epigenetic events that contribute to cancer is necessary, and second, the rational application of epigenetic therapy to patients with malignancy will require careful attention to the details of patient care, biologic correlative studies, and patient safety.

REFERENCES

1. Jones P. A., Baylin S. B. The fundamental role of epigenetic events in cancer. Nat Rev Genet, 2002; 3: 415-28.
2. Jaenisch R., Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet, 2003; 33: 245-54.
3. Wolffe A. P., Matzke M. A. Epigenetics: regulation through repression. Science, 1999; 286: 481-6.

4. Eberharter A., Becker P. B. Histone acetylation: a switch between repressive and permissive chromatin: Second in review series on chromatin dynamics. EMBO Rep, 2002; 3: 224-9.
5. Dobosy J. R., Selker E. U. Emerging connections between DNA methylation and histone acetylation. Cell Mol Life Sci, 2001; 58: 721-7.
6. Nakao, M. Epigenetics: interaction of DNA methylation and chromatin. Gene, 2001; 278: 25-31.
7. Bestor T. H. The DNA methyltransferases of mammals. Hum Mol Genet, 2000; 9: 2395-402.
8. Plass C. Cancer epigenomics. Hum Mol Genet, 2002; 11: 2479-88.
9. Baylin, S. B. Tying it all together: epigenetics, genetics, cell cycle, and cancer [see comments]. Science, 1997; 277: 1948-1949.
10. Plass C., Soloway P. D. DNA methylation, imprinting and cancer. Eur J Hum Genet, 2002; 10: 6-16.
11. Bartolomei M. S., Tilghman S. M. Genomic imprinting in mammals. Annu Rev Genet, 1997; 31: 493-525.
12. Heard E., Clerc P., Avner P. X-chromosome inactivation in mammals. Annu Rev Genet, 1997; 31: 571-610.
13. Ahuja N., Li Q., Mohan A. L. , Baylin S. B., Issa J. P. Aging and DNA methylation in colorectal mucosa and cancer. Cancer Res, 1998; 58: 5489-94.
14. Baylin S. B., Herman J. G., Graff J. R., Vertino P. M., Issa J. P. Alterations in DNA methylation: a fundamental aspect of neoplasia. Adv Cancer Res, 1998; 72: 141-96.
15. Bird A. P. Gene number, noise reduction and biological complexity [see comments]. Trends Genet, 1995, 11, 94-100.
16. Antequera F., Bird A. Number of CpG islands and genes in human and mouse. Proc Natl Acad Sci U S A, 1993; 90: 11995-9.
17. Gardiner-Garden M., Frommer M. CpG islands in vertebrate genomes. J Mol Biol, 1987; 196: 261-82.
18. Venter J. C., Adams M. D., Myers E. W., Li P. W., Mural R. J., Sutton G. G., Smith H. O., Yandell M., Evans C. A., Holt R. A., Gocayne J. D., Amanatides P., Ballew R. M., Huson D. H., Wortman J. R., Zhang Q., Kodira C. D., Zheng X. H., Chen L., Skupski M., Subramanian G., Thomas P. D., Zhang J., Gabor Miklos G. L., Nelson C., Broder S., Clark A. G., Nadeau J., McKusick V. A., Zinder N., Levine A. J., Roberts R. J., Simon M., Slayman C., Hunkapiller M., Bolanos R., Delcher A., Dew I., Fasulo D., Flanigan M., Florea L., Halpern A., Hannenhalli S., Kravitz S., Levy S., Mobarry C., Reinert K., Remington K., Abu-Threideh J., Beasley E., Biddick K., Bonazzi V., Brandon R., Cargill M., Chandramouliswaran I., Charlab R., Chaturvedi K., Deng Z., Francesco V. D., Dunn P., Eilbeck K., Evangelista C., Gabrielian A. E., Gan W., Ge W., Gong F., Gu Z., Guan P., Heiman T. J., Higgins M. E., Ji R. R., Ke Z., Ketchum K. A., Lai Z., Lei Y., Li Z., Li J., Liang Y., Lin X., Lu .F, Merkulov G. V., Milshina N., Moore H. M., Naik A. K., Narayan V. A., Neelam B., Nusskern D., Rusch D. B., Salzberg S., Shao W., Shue B., Sun J., Wang Z. Y., Wang A., Wang X., Wang J., Wei M. H., Wides R. The sequence of the human genome. Science 2001, 5507, 1304-1351.
19. Singal R., Ginder G. D. DNA methylation. Blood, 1999; 93: 4059-70.
20. Bird A. P., Wolffe A. P. Methylation-induced repression--belts, braces, and chromatin. Cell, 1999; 99: 451-4.
21. Robertson K. D., Jones P. A. DNA methylation: past, present and future directions. Carcinogenesis, 2000; 21: 461-7.
22. Tate P. H., Bird A. P. Effects of DNA methylation on DNA-binding proteins and gene expression. Curr Opin Genet Dev, 1993; 3: 226-31.

23. Zhu W. G., Srinivasan K., Dai Z., Duan W., Druhan L. J., Ding H., Yee L., Villalona-Calero M. A., Plass C., Otterson G. A. Methylation of adjacent CpG sites affects Sp1/Sp3 binding and activity in the p21(Cip1) promoter. Mol Cell Biol, 2003; 23: 4056-65.
24. Jones P. L., Veenstra G. J., Wade P. A., Vermaak D., Kass S. U., Landsberger N., Strouboulis J., Wolffe A. P. Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nat Genet, 1998; 19: 187-91.
25. Nan X., Campoy F. J., Bird A. MeCP2 is a transcriptional repressor with abundant binding sites in genomic chromatin. Cell, 1997; 88: 471-81.
26. Costello J. F., Plass C. Methylation matters. J Med Genet, 2001, 38, 285-303.
27. Feinberg A. P., Tycko B. The history of cancer epigenetics. Nat Rev Cancer, 2004; 4: 143-53.
28. Szyf M., Pakneshan P., Rabbani S. A. DNA demethylation and cancer: therapeutic implications. Cancer Lett, 2004; 211: 133-43.
29. Feinberg A. P., Vogelstein B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature, 1983; 301: 89-92.
30. Gama-Sosa M. A., Slagel V. A., Trewyn R. W., Oxenhandler R., Kuo K. C., Gehrke C. W., Ehrlich M. The 5-methylcytosine content of DNA from human tumors. Nucleic Acids Res, 1983; 11: 6883-94.
31. Dunn B. K. Hypomethylation: one side of a larger picture. Ann N Y Acad Sci, 2003; 983: 28-42.
32. Soares J., Pinto A. E., Cunha C. V., Andre S., Barao I., Sousa J. M., Cravo M. Global DNA hypomethylation in breast carcinoma: correlation with prognostic factors and tumor progression. Cancer, 1999; 85: 112-8.
33. Tsujiuchi T., Tsutsumi M., Sasaki Y., Takahama M., Konishi Y. Hypomethylation of CpG sites and c-myc gene overexpression in hepatocellular carcinomas, but not hyperplastic nodules, induced by a choline-deficient L-amino acid-defined diet in rats. Jpn J Cancer Res, 1999; 90: 909-13.
34. Qu G. Z., Grundy P. E., Narayan A., Ehrlich M. Frequent hypomethylation in Wilms tumors of pericentromeric DNA in chromosomes 1 and 16. Cancer Genet Cytogenet, 1999; 109: 34-9.
35. Nakayama M., Wada M., Harada T., Nagayama J., Kusaba H., Ohshima K., Kozuru M., Komatsu H., Ueda R., Kuwano M. Hypomethylation status of CpG sites at the promoter region and overexpression of the human MDR1 gene in acute myeloid leukemias. Blood, 1998; 92: 4296-307.
36. Smiraglia D. J., Szymanska J., Kraggerud S. M., Lothe R. A., Peltomaki P., Plass C. Distinct epigenetic phenotypes in seminomatous and nonseminomatous testicular germ cell tumors. Oncogene 2002; 21: 3909-16.
37. Pfeifer G. P., Steigerwald S., Boehm T. L., Drahovsky D. DNA methylation levels in acute human leukemia. Cancer Lett, 1988; 39: 185-92.
38. Wahlfors J., Hiltunen H., Heinonen K., Hamalainen E., Alhonen L., Janne J. Genomic hypomethylation in human chronic lymphocytic leukemia. Blood, 1992; 80: 2074-80.
39. Claus R., Lubbert M. Epigenetic targets in hematopoietic malignancies. Oncogene, 2003; 22: 6489-96.
40. Santini V., Kantarjian H. M., Issa J. P. Changes in DNA methylation in neoplasia: pathophysiology and therapeutic implications. Ann Intern Med, 2001; 134: 573-86.
41. Denissenko M. F., Chen J. X., Tang M. S., Pfeifer G. P. Cytosine methylation determines hot spots of DNA damage in the human P53 gene. Proc Natl Acad Sci U S A, 1997; 94: 3893-8.

42. Tsou J. A., Hagen J. A., Carpenter C. L., Laird-Offringa I. A. DNA methylation analysis: a powerful new tool for lung cancer diagnosis. Oncogene, 2002; 21: 5450-61.
43. Otterson G. A., Kratzke R. A., Coxon A., Kim Y. W., Kaye F. J. Absence of p16INK4 protein is restricted to the subset of lung cancer lines that retains wildtype RB. Oncogene, 1994; 9: 3375-8.
44. Otterson G. A., Khleif S. N., Chen W., Coxon A. B., Kaye F. J. CDKN2 gene silencing in lung cancer by DNA hypermethylation and kinetics of p16INK4 protein induction by 5-aza 2'deoxycytidine. Oncogene, 1995; 11: 1211-6.
45. Gazzeri S., Gouyer V., Vour'ch C., Brambilla C., Brambilla E. Mechanisms of p16INK4A inactivation in non small-cell lung cancers. Oncogene, 1998; 16: 497-504.
46. Kashiwabara K., Oyama T., Sano T., Fukuda T., Nakajima T. Correlation between methylation status of the p16/CDKN2 gene and the expression of p16 and Rb proteins in primary non-small cell lung cancers. Int J Cancer, 1998; 79: 215-20.
47. Belinsky S. A., Nikula K. J., Palmisano W. A., Michels R., Saccomanno G., Gabrielson E., Baylin S. B., Herman J. G. Aberrant methylation of p16(INK4a) is an early event in lung cancer and a potential biomarker for early diagnosis. Proc Natl Acad Sci U S A, 1998; 95: 11891-6.
48. Wolffe A. P. The cancer-chromatin connection. Science & Medicine July/August 1999; 6: 28-37.
49. Jenuwein T., Allis C. D. Translating the histone code. Science, 2001; 293: 1074-80.
50. Strachan T., Read A. P. Human Molecular Genetics, New York : Wiley-Liss; Oxford, UK: Published in association with BIOS Scientific Publishers, 1996.
51. Jackson, J. P., Lindroth A. M., Cao X., Jacobsen S. E. Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. Nature, 2002; 416: 556-60.
52. Tamaru H., Selker E. U. A histone H3 methyltransferase controls DNA methylation in Neurospora crassa. Nature, 2001; 414: 277-83.
53. Fuks, F, Burgers WA, Brehm A, Hughes-Davies L, Kouzarides T. DNA methyltransferase DNMT1 associates with histone deacetylase activity. Nat Genet, 2000; 24: 88-91.
54. Rountree M. R., Bachman K. E., Baylin S. B. DNMT1 binds HDAC2 and a new co-repressor, DMAP1, to form a complex at replication foci. Nat Genet, 2000; 25: 269-77.
55. Fuks F., Burgers W. A., Godin N., Kasai M., Kouzarides T. DNMT3a binds deacetylases and is recruited by a sequence-specific repressor to silence transcription. Embo J, 2001; 20: 2536-44.
56. Satoh A., Toyota M., Itoh F., Kikuchi T., Obata T., Sasaki Y., Suzuki H., Yawata A., Kusano M., Fujita M., Hosokawa M., Yanagihara K., Tokino T., Imai K. DNA methylation and histone deacetylation associated with silencing DAP kinase gene expression in colorectal and gastric cancers. Br J Cancer, 2002; 86: 1817-23.
57. Cameron E. E, Bachman K. E., Myohanen S., Herman J. G., Baylin S. B. Synergy of demethylation and histone deacetylase inhibition in the re- expression of genes silenced in cancer. Nat Genet, 1999; 21: 103-7.
58. Goffin J., Eisenhauer E. DNA methyltransferase inhibitors-state of the art. Ann Oncol, 2002; 13: 1699-716.
59. Szyf M. DNA methylation and cancer therapy. Drug Resist Updat 2003; 6: 341-53.
60. Zhu W. G., Lakshmanan R. R., Beal M. D., Otterson G. A. DNA methyltransferase inhibition enhances apoptosis induced by histone deacetylase inhibitors. Cancer Res, 2001; 61: 1327-33.

61. Lyons J., Bayar E., Fine G., McCullar M., Rolens R., Rubinfeld J., Rosenfeld C.. Decitabine: development of a DNA methyltransferase inhibitor for hematological malignancies. Curr Opin Investig Drugs, 2003; 4: 1442-50.
62. Issa J. P., Garcia-Manero G., Giles F. J., Mannari R., Thomas D., Faderl S., Bayar E., Lyons J., Rosenfeld C. S., Cortes J., Kantarjian H. M. Phase 1 study of low-dose prolonged exposure schedules of the hypomethylating agent 5-aza-2'-deoxycytidine (decitabine) in hematopoietic malignancies. Blood, 2004; 103: 1635-40. Epub 2003 Nov 06.
63. Silverman L. R., Demakos E. P., Peterson B. L., Kornblith A. B., Holland J. C., Odchimar-Reissig R., Stone R. M., Nelson D., Powell B. L., DeCastro C. M., Ellerton J., Larson R. A., Schiffer C. A., Holland J. F. Randomized controlled trial of azacitidine in patients with the myelodysplastic syndrome: a study of the cancer and leukemia group B. J Clin Oncol, 2002; 20: 2429-40.
64. Sorm F., Piskala A., Cihak A., Vesely J. 5-Azacytidine, a new, highly effective cancerostatic. Experientia, 1964; 20: 202-3.
65. Constantinides P. G., Jones P. A., Gevers W. Functional striated muscle cells from non-myoblast precursors following 5-azacytidine treatment. Nature, 1977; 267: 364-6.
66. Yoo C. B., Cheng J. C., Jones P. A. Zebularine: a new drug for epigenetic therapy. Biochem Soc Trans, 2004; 32: 910-2.
67. Cheng J. C., Matsen C. B., Gonzales F. A., Ye W., Greer S., Marquez V. E., Jones P. A., Selker E. U. Inhibition of DNA methylation and reactivation of silenced genes by zebularine. J Natl Cancer Inst, 2003; 95: 399-409.
68. Liu G., Franssen E., Fitch M. I., Warner E. Patient preferences for oral versus intravenous palliative chemotherapy. J Clin Oncol, 1997; 15: 110-5.
69. Richardson B. DNA methylation and autoimmune disease. Clin Immunol 2003; 109: 72-9.
70. Richardson B., Ray D., Yung R. Murine models of lupus induced by hypomethylated T cells. Methods Mol Med 2004; 102: 285-94.
71. Crooke ST. Potential roles of antisense technology in cancer chemotherapy. Oncogene, 2000; 19: 6651-9.
72. Reid G. K., Besterman J. M., MacLeod A. R. Selective inhibition of DNA methyltransferase enzymes as a novel strategy for cancer treatment. Curr Opin Mol Ther, 2002; 4: 130-7.
73. Ramchandani S., MacLeod A. R., Pinard M., von Hofe E., Szyf M. Inhibition of tumorigenesis by a cytosine-DNA, methyltransferase, antisense oligodeoxynucleotide. Proc Natl Acad Sci U S A, 1997; 94: 684-9.
74. Leslie M., Coulthard S., Plummer E., Judson I., de Bono J., Vidal L., Greystoke A., Lee C., Boddy A., Calvert A. Decreased expression of DNMT1 at the mRNA level following 7 day infusion of the antisense compound MG98 in a phase I study. European Journal of Cancer Supplements, 2004; 2: 132.
75. Winquist E., Knox J., Ayoub J-P., Wood L., Wainman N., Pearce L., Shah A., Eisenhauer E. Phase II study of the DNA methyltransferase 1 (DNMT1) inhibitor MG98 in patients with renal cell carcinoma. A trial of the National Cancer Institute of Canada Clinical Trials Group (NCIC CTG). European Journal of Cancer, 2002; 38: 141.
76. Liu K., Wang Y. F., Cantemir C., Muller M. T. Endogenous assays of DNA methyltransferases: Evidence for differential activities of DNMT1, DNMT2, and DNMT3 in mammalian cells in vivo. Mol Cell Biol, 2003; 23: 2709-19.

77. Bender C. M., Pao M. M., Jones P. A. Inhibition of DNA methylation by 5-aza-2'-deoxycytidine suppresses the growth of human tumor cell lines. Cancer Res, 1998; 58: 95-101.
78. Schmelz K., Sattler N., Wagner M., Lubbert M., Dorken B., Tamm I. Induction of gene expression by 5-Aza-2'-deoxycytidine in acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) but not epithelial cells by DNA-methylation-dependent and -independent mechanisms. Leukemia, 2005; 19: 103-11.
79. Zhu W. G., Dai Z., Ding H., Srinivasan K., Hall J., Duan W., Villalona-Calero M. A., Plass C., Otterson G. A. Increased expression of unmethylated CDKN2D by 5-aza-2'-deoxycytidine in human lung cancer cells. Oncogene, 2001; 20: 7787-96.
80. Juttermann R., Li E., Jaenisch R. Toxicity of 5-aza-2'-deoxycytidine to mammalian cells is mediated primarily by covalent trapping of DNA methyltransferase rather than DNA demethylation. Proc Natl Acad Sci U S A, 1994; 91: 11797-801.
81. Zhu W. G., Hileman T., Ke Y., Wang P., Lu S., Duan W., Dai Z., Tong T., Villalona-Calero M. A., Plass C., Otterson G. A. 5-aza-2'-deoxycytidine activates the p53/p21Waf1/Cip1 pathway to inhibit cell proliferation. J Biol Chem, 2004; 279: 15161-6 Epub 2004 Jan 13.
82. Covey J. M., D'Incalci M., Tilchen E. J., Zaharko D. S., Kohn K. W. Differences in DNA damage produced by incorporation of 5-aza-2'-deoxycytidine or 5,6-dihydro-5-azacytidine into DNA of mammalian cells. Cancer Res, 1986; 46: 5511-7.
83. Aparicio A., Eads C. A., Leong L. A., Laird P. W., Newman E. M., Synold T. W., Baker S. D., Zhao M., Weber J. S. Phase I trial of continuous infusion 5-aza-2'-deoxycytidine. Cancer Chemother Pharmacol, 2003; 51: 231-9. Epub 2003 Feb 27.
84. Saunthararajah Y., Hillery C. A., Lavelle D., Molokie R., Dorn L., Bressler L., Gavazova S., Chen Y. H., Hoffman R., DeSimone J. Effects of 5-aza-2'-deoxycytidine on fetal hemoglobin levels, red cell adhesion, and hematopoietic differentiation in patients with sickle cell disease. Blood, 2003; 102: 3865-70.
85. Koshy M., Dorn L., Bressler L., Molokie R., Lavelle D., Talischy N., Hoffman .R, van Overveld W., DeSimone J. 2-deoxy 5-azacytidine and fetal hemoglobin induction in sickle cell anemia. Blood, 2000; 96: 2379-84.
86. Gius D., Cui H., Bradbury C. M., Cook J., Smart D. K., Zhao S., Young L., Brandenburg S. A., Hu Y., Bisht K. S., Ho A. S., Mattson D., Sun L., Munson P. J., Chuang E. Y., Mitchell J. B., Feinberg A. P. Distinct effects on gene expression of chemical and genetic manipulation of the cancer epigenome revealed by a multimodality approach. Cancer Cell, 2004; 6: 361-71.
87. Suzuki H., Gabrielson E., Chen W., Anbazhagan R., van Engeland M., Weijenberg M. P., Herman J. G., Baylin S. B. A genomic screen for genes upregulated by demethylation and histone deacetylase inhibition in human colorectal cancer. Nat Genet, 2002; 31: 141-9.
88. Glaser K. B., Staver M. J., Waring J. F., Stender J., Ulrich R. G., Davidsen S. K. Gene expression profiling of multiple histone deacetylase (HDAC) inhibitors: defining a common gene set produced by HDAC inhibition in T24 and MDA carcinoma cell lines. Mol Cancer Ther, 2003; 2: 151-63.
89. Qiu W., Zhou B., Zou H., Liu X., Chu P. G., Lopez R., Shih J., Chung C., Yen Y. Hypermethylation of growth arrest DNA damage-inducible gene 45 beta promoter in human hepatocellular carcinoma. Am J Pathol, 2004; 165: 1689-99.
90. Karpf A. R., Moore B. C., Ririe T. O., Jones D. A. Activation of the p53 DNA damage response pathway after inhibition of DNA methyltransferase by 5-aza-2'-deoxycytidine. Mol Pharmacol, 2001; 59: 751-7.

91. Hansen R. S., Wijmenga C., Luo P., Stanek A. M., Canfield T. K, Weemaes CM, Gartler SM. The DNMT3B DNA methyltransferase gene is mutated in the ICF immunodeficiency syndrome. Proc Natl Acad Sci U S A, 1999; 96: 14412-7.
92. Okano M., Bell D. W., Haber D. A., Li E. DNA methyltransferases DNMT3a and DNMT3b are essential for de novo methylation and mammalian development. Cell, 1999; 99: 247-57.
93. Xu G. L., Bestor T. H., Bourc'his D., Hsieh C. L., Tommerup N., Bugge M., Hulten M., Qu X., Russo J. J., Viegas-Pequignot E. Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. Nature, 1999; 402: 187-91.
94. Klisovic M. I., Maghraby E. A., Parthun M. R., Guimond M., Sklenar A. R., Whitman S. P., Chan K. K., Murphy T., Anon J., Archer K. J., Rush L. J., Plass C., Grever M. R., Byrd J. C., Marcucci G. Depsipeptide (FR 901228) promotes histone acetylation, gene transcription, apoptosis and its activity is enhanced by DNA methyltransferase inhibitors in AML1/ETO-positive leukemic cells. Leukemia, 2003; 17: 350-8.
95. Chen R. Z., Pettersson U., Beard C., Jackson-Grusby L., Jaenisch R. DNA hypomethylation leads to elevated mutation rates. Nature, 1998; 395: 89-93.
96. Eden A., Gaudet F., Waghmare A., Jaenisch R. Chromosomal instability and tumors promoted by DNA hypomethylation. Science, 2003; 300: 455.
97. Gaudet F., Hodgson J. G., Eden A., Jackson-Grusby L., Dausman J., Gray J. W., Leonhardt H., Jaenisch R. Induction of tumors in mice by genomic hypomethylation. Science, 2003; 300: 489-92.
98. Hernandez R., Frady A., Zhang X. Y., Varela M., Ehrlich M. Preferential induction of chromosome 1 multibranched figures and whole-arm deletions in a human pro-B cell line treated with 5-azacytidine or 5-azadeoxycytidine. Cytogenet Cell Genet, 1997; 76: 196-201.
99. Sato N., Maehara N., Su G. H., Goggins M. Effects of 5-aza-2'-deoxycytidine on matrix metalloproteinase expression and pancreatic cancer cell invasiveness. J Natl Cancer Inst, 2003; 95: 327-30.
100. Yang A. S., Estecio M. R., Garcia-Manero G., Kantarjian H. M., Issa J. P. Comment on "Chromosomal instability and tumors promoted by DNA hypomethylation" and "Induction of tumors in nice by genomic hypomethylation". Science, 2003; 302: 1153; author reply 1153.
101. Tucker M. A., Coleman C. N., Cox R. S., Varghese A., Rosenberg S. A. Risk of second cancers after treatment for Hodgkin's disease. N Engl J Med 1988; 318: 76-81.
102. Hancock S. L., Tucker M. A., Hoppe R. T. Breast cancer after treatment of Hodgkin's disease. J Natl Cancer Inst, 1993; 85: 25-31.
103. Laird P. W., Jackson-Grusby L., Fazeli A., Dickinson S. L., Jung W. E., Li E., Weinberg R. A., Jaenisch R. Suppression of intestinal neoplasia by DNA hypomethylation. Cell, 1995; 81: 197-205.
104. Lantry L. E., Zhang Z., Crist K. A., Wang Y., Kelloff G. J., Lubet R. A., You M. 5-Aza-2'-deoxycytidine is chemopreventive in a 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone-induced primary mouse lung tumor model. Carcinogenesis, 1999; 20: 343-6.
105. Nuovo G. J., Plaia T. W., Belinsky S. A., Baylin S. B., Herman J. G. In situ detection of the hypermethylation-induced inactivation of the p16 gene as an early event in oncogenesis. Proc Natl Acad Sci U S A, 1999; 96: 12754-9.
106. Esteller M., Garcia-Foncillas J., Andion E., Goodman S. N., Hidalgo O. F., Vanaclocha V., Baylin S. B., Herman J. G. Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. N Engl J Med, 2000; 343: 1350-4.

107. Paz M. F., Yaya-Tur R., Rojas-Marcos I., Reynes G., Pollan M., Aguirre-Cruz L., Garcia-Lopez J. L., Piquer J., Safont M. J., Balana C., Sanchez-Cespedes M., Garcia-Villanueva M., Arribas L., Esteller M. CpG island hypermethylation of the DNA repair enzyme methyltransferase predicts response to temozolomide in primary gliomas. Clin Cancer Res, 2004; 10: 4933-8.
108. Esteller M., Gaidano G., Goodman S. N., Zagonel V., Capello D., Botto B., Rossi D., Gloghini A., Vitolo U., Carbone A., Baylin S. B., Herman J. G. Hypermethylation of the DNA repair gene O(6)-methylguanine DNA methyltransferase and survival of patients with diffuse large B-cell lymphoma. J Natl Cancer Inst, 2002; 94: 26-32.
109. Zochbauer-Muller S., Fong K. M., Virmani A. K., Geradts J., Gazdar A. F., Minna J. D. Aberrant Promoter Methylation of Multiple Genes in Non-Small Cell Lung Cancers. Cancer Res, 2001; 61: 249-255.
110. Yung R. L., Richardson B. C. Role of T cell DNA methylation in lupus syndromes. Lupus, 1994; 3: 487-91.
111. Januchowski R., Prokop J., Jagodzinski P. P. Role of epigenetic DNA alterations in the pathogenesis of systemic lupus erythematosus. J Appl Genet, 2004; 45: 237-48.
112. Deng C., Lu Q., Zhang Z., Rao T., Attwood J., Yung R., Richardson B. Hydralazine may induce autoimmunity by inhibiting extracellular signal-regulated kinase pathway signaling. Arthritis Rheum, 2003; 48: 746-56.
113. Villar-Garea A., Fraga M. F., Espada J., Esteller M. Procaine is a DNA-demethylating agent with growth-inhibitory effects in human cancer cells. Cancer Res, 2003; 63: 4984-9.
114. Samuels B. L., Herndon J. E., 2nd, Harmon D. C., Carey R., Aisner J., Corson J. M., Suzuki Y., Green M. R., Vogelzang N. J. Dihydro-5-azacytidine and cisplatin in the treatment of malignant mesothelioma: a phase II study by the Cancer and Leukemia Group B. Cancer, 1998; 82: 1578-84.
115. Yogelzang N. J., Herndon J. E., 2nd, Cirrincione C., Harmon D. C., Antman K. H., Corson J. M., Suzuki Y., Citron M. L., Green M. R. Dihydro-5-azacytidine in malignant mesothelioma. A phase II trial demonstrating activity accompanied by cardiac toxicity. Cancer and Leukemia Group B. Cancer, 1997; 79: 2237-42.
116. Dammann R., Li C., Yoon J. H., Chin P. L., Bates S., Pfeifer G. P. Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. Nat Genet, 2000; 25: 315-9.
117. Burbee D. G., Forgacs E., Zochbauer-Muller S., Shivakumar L., Fong K., Gao B., Randle D, Kondo M, Virmani A, Bader S, Sekido Y, Latif F, Milchgrub S, Toyooka S, Gazdar A. F., Lerman M. I., Zabarovsky E., White M., Minna J. D. Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. J Natl Cancer Inst, 2001; 93: 691-9.
118. Virmani A. K., Rathi A., Sathyanarayana U. G., Padar A., Huang C. X., Cunnigham H. T., Farinas A. J., Milchgrub S., Euhus D. M., Gilcrease M., Herman J., Minna J. D., Gazdar A. F. Aberrant methylation of the adenomatous polyposis coli (APC) gene promoter 1A in breast and lung carcinomas. Clin Cancer Res, 2001; 7: 1998-2004.
119. Du Y., Carling T., Fang W., Piao Z., Sheu J. C., Huang S. Hypermethylation in human cancers of the RIZ1 tumor suppressor gene, a member of a histone/protein methyltransferase superfamily. Cancer Res, 2001; 61: 8094-9.
120. Feng G., Xu X., Youssef E. M., Lotan R. Diminished expression of S100A2, a putative tumor suppressor, at early stage of human lung carcinogenesis. Cancer Res, 2001; 61: 7999-8004.
121. Sato M., Mori Y., Sakurada A., Fujimura S., Horii A. The H-cadherin (CDH13) gene is inactivated in human lung cancer. Hum Genet, 1998; 103: 96-101.

122. Fukami T., Fukuhara H., Kuramochi M., Maruyama T., Isogai K., Sakamoto M., Takamoto S., Murakami Y. Promoter methylation of the TSLC1 gene in advanced lung tumors and various cancer cell lines. Int J Cancer, 2003; 107: 53-9.
123. Kuramochi M., Fukuhara H., Nobukuni T., Kanbe T., Maruyama T., Ghosh H. P., Pletcher M., Isomura M., Onizuka M., Kitamura T., Sekiya T., Reeves R. H., Murakami Y. TSLC1 is a tumor-suppressor gene in human non-small-cell lung cancer. Nat Genet, 2001; 27: 427-30.
124. Xu X. L., Wu L. C., Du F., Davis A., Peyton M., Tomizawa Y., Maitra A., Tomlinson G., Gazdar A. F., Weissman B. E., Bowcock A. M., Baer R., Minna J. D. Inactivation of human SRBC, located within the 11p15.5-p15.4 tumor suppressor region, in breast and lung cancers. Cancer Res, 2001; 61: 7943-9.
125. Kim J. S., Kim H., Shim Y. M., Han J., Park J., Kim D. H. Aberrant methylation of the FHIT gene in chronic smokers with early stage squamous cell carcinoma of the lung. Carcinogenesis, 2004; 25: 2165-71. Epub 2004 Jul 01.
126. Zochbauer-Muller S., Fong K. M., Maitra A., Lam S., Geradts J., Ashfaq R., Virmani A. K., Milchgrub S., Gazdar A. F., Minna J. D. 5' CpG island methylation of the FHIT gene is correlated with loss of gene expression in lung and breast cancer. Cancer Res, 2001; 61: 3581-5.

Chapter 12

HISTONE DEACETYLASE INHIBITORS: NOVEL TARGETED ANTI-CANCER AGENTS

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Abstract: The base sequence of DNA provides the genetic code for proteins. In any given cell, only a proportion of genes are expressed. The regulation of expression of genes is determined, in large part, by the structure of the chromatin proteins around which the DNA is wrapped – referred to as epigenetic gene regulation. Post-translational modifications of the histones of chromatin have been established as important factors in regulating gene expression. Among the most extensively studied of these epigenetic modifications are those which involve the acetylation and deacetylation of the lysines in the tails of the core histones. The acetylation/deacetylation of these lysines is controlled by the action of two families of enzymes, histone deacetylases (HDACs) and histone acetyltransferases (HATs). A balance between histone acetylation and deacetylation is essential for the normal growth of cells. This review will focus on HDAC and HDAC inhibitors. HDAC inhibitors represent a relatively new group of targeted anti-cancer compounds which are showing significant promise as agents which have activity against a broad spectrum of both hematologic and solid tumors at doses that are well tolerated by the patients. The HDAC inhibitors are a structurally diverse group of molecules that can induce growth arrest, differentiation and cell death of cancer cells in both *in vitro* as well as *in vivo* in tumor bearing animal models. Several of these agents are currently in clinical trials. Over the past 2-3 years a number of general reviews of areas related to the present review have been published (1-16). This review will primarily cover the literature of the past 3 years as of August 31, 2004.

Key words: Histone deacetylases, suberoylanilde hydroxamic acid, p21, apoptosis, reactive oxygen species, thioredoxin, solid tumors and hematopoietic malignancies

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1. CHROMATIN STRUCTURE AND CHROMATIN REMODELING

Chromatin is structurally complex, consisting of DNA, histones and nonhistone proteins (17-22). The basic repeating unit of chromatin is the nucleosome, composed of approximately 146 bp of DNA wrapped around the histone octamer composed of two copies of each of four histones, H2A, H2B, H3 and H4. The 146 base pair of DNA make two superhelical turns wrapped around the octamer core pairs of histones (23, 24).

The remodeling of the chromatin proteins around which the DNA is wrapped is a fundamental epigenetic mechanism regulating gene expression in vertebrates. It depends largely on the reversible post-translational modification of histone amino acid tails by acetylation of lysines, methylation of lysines and arginines, phosphorylation of serines and ubiquination of lysines (17-23, 25, 26). Two groups of enzymes, histone deacetylases (HDACs) and histone acetyltransferases (HATs), determine the pattern of histone acetylation. Histone acetylation is a dynamic aspect of chromatin structure with half lives of acetylated histone lysines being a few minutes.

It has been a hypothesized that histone modifications acting alone, sequentially or in combination represent a "code" that can be recognized by nonhistone proteins forming complexes involved in the regulation of gene expression (16-22). Recently, this hypothesis has been extended (25). The concept of "modification cassettes" and localized "binary switches" that is, modifications of adjacent sites within neighboring specific locations of histone tails involving, for example, acetylation or methylation of lysine residues and phosphorylation of serines residues which are adjacent or closely adjacent and act as information "units" mediating different signals.

In general, it has been suggested that condensation of chromatin structure primarily through methylation of lysines of the tails of histones, is associated with suppression of gene expression while neutralization of the positive charge of histones, primarily by acetylation of the lysines of the histone tails, leads to a more open structure of chromatin providing access to promoter regions of complexes which are involved in inducing expression of genes. This model is probably an over simplification, since HDAC inhibitors cause about as many genes to be increased in expression, as are suppressed in their transcription (see below) (4, 9).

2. HISTONE DEACETYLASES AND HISTONE ACETYL TRANSFERASES

There are three classes of human HDAC enzymes (9, 27). Class I includes HDAC 1, 2, 3 and 8 which are related to yeast RPD3 deacetylase, have molecular weights of 22-55 kDa and share hemology in their catalytic sites (Table 1). Class II deacetylases includes HDACs 4, 5, 6, and 9, which are larger molecules with molecular weights between 120-135 kDa and are related to yeast HDA1 deacetylase. A subclass of HDACs may be represented by HDAC 6 and 10, which contain two catalytic sites. HDAC 11 has conserved residues in the catalytic core region that are shared by both class I and class II enzymes (28) (Table 1). A third class of HDACs has been identified that have an absolute requirement for NAD, the so called Sir2 family of deacetylases and are not inhibited by compounds that inhibit class I and II HDACs (29). The Sir classes of histone deacetylases appear to not have histones as their primary substrates (27, 29-31).

Recent phylogenetic analysis of bacterial HDACs suggest that all three HDAC classes preceded the evolution of histone proteins. This raises the possibility that the primary activity of some histone deacetylation enzymes is directed against non-histone substrates (32). A recurring theme that emerged from these phylogenetic studies was the common nature of association between HDAC molecules (27, 30, 32). It is well established that class I and class II HDACs are often found together as components of larger protein complexes. There are also examples of direct self-association of HDACs. The functional significance of association between HDAC molecules is not clear. Further analysis of the self associating properties of HDAC proteins may give insight into the function of different HDACs and the selectivity of HDAC inhibitors in altering gene transcription. Contrary to what might be expected from the widespread distribution of HDAC within the chromatin, inhibition of HDACs activity by molecules such as Trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA) and related agents is associated with the alteration in transcription of relatively few genes (9, 33, 34). It is possible that a particular association of HDACs within a transcription factor complex may contribute to the selectivity of the inhibitory effect of various HDAC inhibitors (35). This is an interesting hypothesis to consider in view of the light of recent studies on alterations induced by the HDAC inhibitor, SAHA, on proteins associated with the proximal promoter region of p21 gene. Both HDAC 1 and HDAC 2 were identified to be associated in this complex, but exposure to SAHA caused dissociation of HDAC1 but not HDAC 2 from the proximal promotor associated proteins (35).

Table 1. Human Histone Deacetylases.

Class (homology)	Enzyme	Size (Amino Acids)	Cell Localization	Chromosome Location
1 (Rpd3-like)	HDAC 1	483	Nuclear	1p34
	HDAC2	488	Nuclear	6q21
	HDAC3	428	Nuclear	5q31
	HDAC 8	377	Nuclear	xq13
	HDAC11*	347	Nuclear	3p25.2
II (Had 1-like)	HDAC 4	1084	Nuclear cytoplasmic	q37.2
	HDAC 5	1122	Nuclear cytoplasmic	17q21
	HDAC 7	855	Nuclear cytoplasmic	12q13.1
	HDAC 9	1011	Nuclear cytoplasmic	p21-p15
II B ++	HDAC 6	1215	Nuclear cytoplasmic	xp11.22-23
	HDAC 10	669	Nuclear cytoplasmic	22q13.31

*HDAC II has homology to both Class I and II HDACs

++HDAC 6 and HDAC 10 have two catalytic sites

HDAC 6 contains two catalytic domains (36-38). HDAC 9 gene encodes multiple protein isoforms, some of which display distinct cellular localization patterns (39, 40). HDAC 6 interacts with tubulin and microtubules *in vivo* (38). HDAC10 is a novel class II HDAC which has two C-terminal retinoblastoma protein binding domains and a nuclear receptor binding motif (41, 42). HDAC 10 interacts with HDAC 3 but not HDAC 4 or 6. Class I and class II HDACs each map to a different chromosomal sites (30).

There is now abundant evidence that HDACs are not redundant in their biological function (9, 15, 43, 44). Class I HDACs, such as HDAC 1 and HDAC 3 appear to be important in the regulation of proliferation of cancer cells (44). Parenthetically, this suggests that the development of class I selective HDAC inhibitors may lead to more effective anti-cancer agents than the pan-HDAC inhibitors such as SAHA (see below). Class I HDACs are found almost exclusively in the cell nucleus, while class II shuttle between the nucleus and cytoplasm on certain cellular signals (4, 9, 45) (Table 1). HDAC 1 complexed with myoD serves as a repressor of proliferating myoblasts (46). Another indication that the different HDACs have different functions is the finding that the expression of different HDACs through embryonic development changes with different stages of embryogenesis (47) Targeted disruption of HDAC1 results in embryonic lethality despite increased expression for HDAC2 and 3 (48). HDAC3 has been found to modulate the functions of transcription factors such as TFII-1 (49). Class 2 HDACs block myocyte enhancer factor 2 (MEF2) activation of cardiac hypertrophy (50-52). HDAC 3 is critical for repression of multiple nuclear receptors (53). HDAC 5 has been shown to interact with Ca(2+) calmodulin to inhibit MEF2a (54). HDAC 7 has a role in regulating T-cell differentiation in the thymus which is not shared by other HDACs (55). It is clear from these data that HDACs have many nonhistone protein targets in which they play a role in determining the acetylations status and presumably alter the structure and possible function of these targets (Figure 1).

HDACs and HATs do not bind directly to DNA but are recruited to DNA by transcription factor to complexes that differ in their subunit composition (1, 4, 9, 15, 30, 56, 57). HDACs 1 and 2 are frequently found in complex with Sir3, NURD (nucleosome remodeling and deacetylation), N-Cor (Nuclear receptor co-repressor), mSiN3A, Ni-2/NRD and/or CoREST.

1. ALTERING GENE TRANSCRIPTION

    a. Target: Histone Proteins – accumulation of acetylated histones

    b. Target: Proteins in Transcription factor complexes altered by acetylation

2. ALTERING NON-TRANSCRIPTION PROTEINS

    a. Target: Acetylation of proteins involved in mitosis and cytokinesis

    b. Target: Acetylation of proteins regulation cell cycle progression, a apoptosis , etc.

Figure 1. Model of Mechanism of Action of HDAC Inhibitors

3. HISTONE ACETYLTRANSFERASES, HISTONE DEACETYLASES AND IN HUMAN CANCERS

In general, it is a curious fact that while alterations in the both HATs and HDACs are commonly found in many human cancers, the type of alterations for each family of enzymes is different. Thus, structural alterations in HDACs associated with cancer appear to be rare. HDACs are involved in mediating the function of oncogenic translocation products in specific forms of leukemia and lymphoma (1, 3, 5, 9, 58-65). For example, the oncoprotein that is encoded by one of the translocation-generated fusion genes in acute promyelocytic leukemia (APL), PML-RARα, represses transcription by associating with a corepressor complex that contains HDAC activity (59). In non-Hodgkin's lymphoma, the transcriptional repressor LAZ3/BCL6 (lymphoma-associated zinc finger-3/B cell lymphoma) is inappropriately overexpressed and associated with aberrant transcriptional repression through recruitment of HDAC, leading to lymphoid oncogenic transformation. Acute myeloid leukemia m2 subtype is associated with the t(8;21) chromosomal translation, which produces an AML1-ETO fusion

protein – a potent dominant transcription repressor – though its recruitment of HDAC activity (60).

HDAC 1 affects breast cancer progression, promoting cell proliferation by interacting with estrogen receptor alpha causing a loss in its expression (61). The retinoblastoma tumor suppressor protein recruits HDAC 1 (62, 63). Transcriptional repression by p53 protein utilizes HDACs, the complex formation being mediated by SIN-3 HDAC (64), BRAC1 mutation increases risk for breast and ovarian cancers BRAC1 protein associates with HDAC 1 and 2 (65). Further, altered HDAC or HAT activity may disrupt normal mitosis and cytokinesis. There are other examples of transcriptional repression and altered activity of proteins involved in regulation of cell cycle progression which seem to be mediated by the recruitment of HDACs and provide a further mechanistic rational for the treatment of these neoplasms with inhibitors of HDAC activity.

HDAC inhibitors have also been shown to have activity as immunosuppressive agents (66). HDAC inhibitors block activation-induced CD25 and CE124 expression on CD4 T cells. These observations suggest that HDAC inhibitors may have therapeutic activity in various autoimmune disorders.

Translocations of CBP and p300, resulting in in-frame fusion with a number of genes, have been identified in several hematological malignancies (59, 60, 67-69). MOZ (monocyte – leukemia zinc-finger protein) has been found fused to TIF1 (transcriptional mediator/intermediary factor 2) in a leukemia-associated chromosome 9 inversion [inv(8)(p11;q13)], and to transcripts of CBP in a subtype of acute myeloid leukemia (AML) (60). Translocations of CBP and p300 have also been described in treatment-related leukemias and myelodysplastic syndromes.

Structural disruption of HATs has been found in many human cancers (3, 5, 57-61, 67-74). Genes that encode HAT enzymes are translocated, amplified, overexpressed and/or mutated in various cancers – both hematological and epithelial. Two closely related HATs, CBP and p300, are altered in some tumors by either mutation or translocation (66, 74, 75). Missense mutations in p300 and mutations associated with truncated p300, have been identified in colorectal and gastric primary tumors and in other epithelial cancers. In these cases, the second allele was frequently deleted. Individuals with the Rubinstein–Taybi syndrome – a developmental disorder – carry a mutation in CBP that inactivates its HAT activity. These individuals have an increased risk of cancer (74). Loss of heterozygosity of p300 has been described in 80% of glioblastomas and loss of heterozygosity around the CBP locus has been observed in hepatocellular carcinomas.

4. HISTONE DEACETYLASE INHIBITORS

HDAC inhibitors reported to date can be divided into several structural class including hydroximates, cyclic peptides, aliphatic acids and benzamides (8, 43, 76-78). TSA (79) was the first natural product hydroximate discovered to inhibit HDACs. SAHA is structurally less complex than TSA and is a nanomolar inhibitor of a partially purified HDAC class I and II but not class III (Table 2) (80, 81). *M-carboxycinnamic* acid bishydroxamide (CBHA) is another potent HDAC inhibitor (81) which has been the structural basis for several derivatives including LAQ824 (82, 83) and a sulfonamide derivative, PXD-101, both of which inhibit class one and class II HDACs in nanomolar concentrations. Oxamflatin and scriptaid are hydroximates that are somewhat less potent inhibitors of HDACs (84, 85). Hydroximate replacement with alpha ketoamide moiety provided a potent HDAC inhibitor with an IC50 millimolar activity in cell cultures and in tumor-bearing animals (7).

The cyclic peptide class is one of the most structurally complex group of HDAC inhibitors, which includes the natural product depsipeptide (FK228) and apicidin and the Chaps group of molecules, all active in nanomolar in concentrations (86-91). Depsipeptide has a disulfide bridge and is a prodrug of an active agent.

The groups of allopathic acids, which tend to be relatively weak inhibitors of HDACs being active at micromolar concentrations, include phenylbutyrates and its derivatives and valproic acid (92-96). Both valproic acid and phenylbutyrate are relatively old drugs that have been on the market for non oncological uses, and recently shown to have activity as HDAC inhibitors.

The benzamide class of HDAC inhibitors includes MS-275 and CI994, both of which are active of micromolar concentrations (97, 98). Newer benzamides are being developed which shown to have activity both *in vitro* and in tumor bearing animals models (99). In addition, various trifluoroethyl ketones and alpha ketoamides have been developed which are inhibitors of HDAC activity at micromolar concentrations (7, 100). Another new benzamide, 3-(4-substituted-pheno)-hydroxy-2-troperamide, has been reported to have HDAC inhibitory activity *in vitro* and in tumor bearing animals (101). There are recent reports of thiol compounds modeled after SAHA, one of which, at least, is reported to be as potent as SAHA as an inhibitor of HDAC activity (102). A novel histone deacetylase inhibitor, a SAHA based non-hydroximate semi-carbazide and bromo-acetamides derivatives were reported to be potent HDAC inhibitors (103).

Table 2. Histone Deacetylase Inhibitors in Development

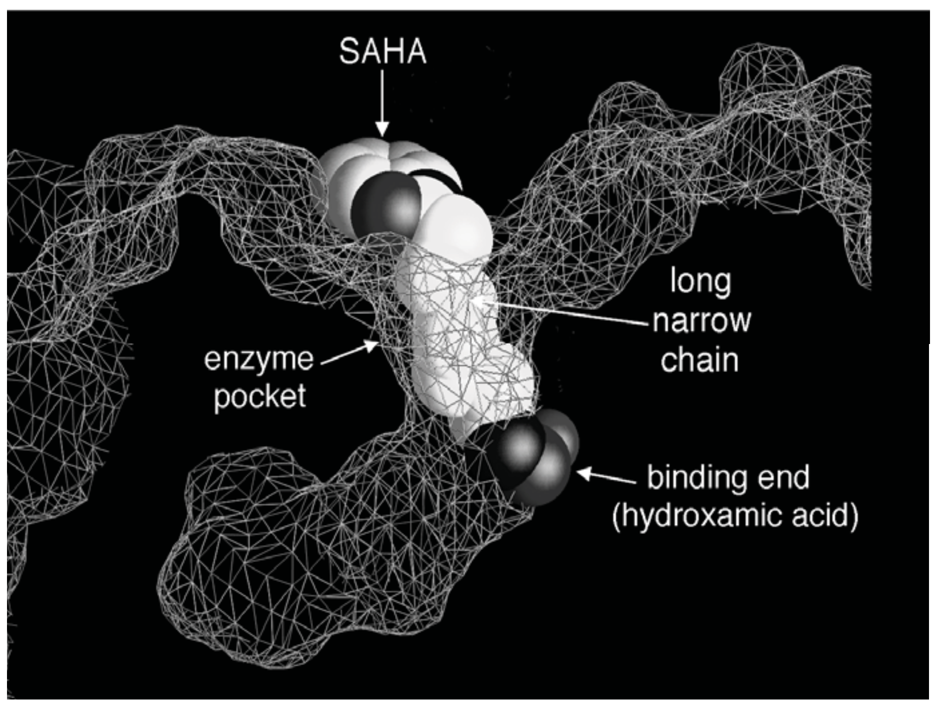
Class	COMPOUND	STRUCTURE	HDAC INHIBITOR ACTIVITY*				
			HDAC*	CELLS	Animal Bearing Tumor	PhI	PhII
Hydroxamate	**Trichostatin A (TSA)**		nM	µM	X	NA	NA
	Suberoyl anilide hydroxamic acid (SAHA)		nM	µM	X	X	X
	CBHA		nM	µM	X	NA	NA
	LAQ-824		nM	µM	X	X	NA
	PXD-101		nM	µM	X	X	NA
Cyclic peptide	**Depsipeptide (FK-228)**		nM	µM	X	X	X

Class	Compound	Structure	HDAC Inhibitor Activity*				
			HDAC*	**CELLS**	**Animal Bearing Tumor**	**PhI**	**PhII**
Cyclic peptide	**Apidicin**		µM	µM	X	NA	NA
Aliphatic Acid	**Valproic Acid**		µM	mM	X	X	X
	Phenyl Butyrate		µM	mM	X	X	X
Benzamide	**MS-275**		µM	µM	X	X	NA
	CI-994		µM	µM	X	X	X

* Concentration s indicated for HDAC and Cells are range of HDACi activity. The X indicates the HDACi has been tested in tumor bearing animals (in vivo), in clinical trials, PhI and PhII. NA – HDACi has not been tested in this stage of development

Evidence has been developed to indicate that different HDAC inhibitors may selectively inhibit different HDACs. For example, TSA was found to be a potent inhibitor of HDACs 1, 3 and 8 while MS-275 (2-Aminophenyl) 4-[(N-pyrydin-3-Metyloxycarbonyl)-(Aminomethyl)-(benzamide)] preferentially inhibited HDAC1 with an IC50 at 0.3 μM compared to HDAC3 with a IC50 of about 8 μM and no inhibitory effect against HDAC8 (104).

The structural details of the HDAC inhibitor enzyme interactions have been elucidated by Finnin, *et al*., and x-ray crystallographic acid studies which they used a homolog of HDAC (HTLP) and resolved its structure with the HDAC inhibitors TSA and SAHA (105) (Figure 2). Structure activity relationship of HDAC inhibitor classes are consistent with the features described in the x-ray crystal structure. There is a direct interaction of the inhibitor with the active zinc site. This interaction appears to be a prerequisite for optimal HDAC inhibitory activity. Because of the evidence that the different HDAC enzymes have different biological activities and in particular class I HDACs 1 and possibly 2 or 3 are important in transformed cell proliferation, considerable efforts are going forward to develop structures that are selective inhibitors of one or another of the HDACs enzymes. Progress has been made in developing high throughput assays which are suitable for screening selective HDAC inhibitors (106).



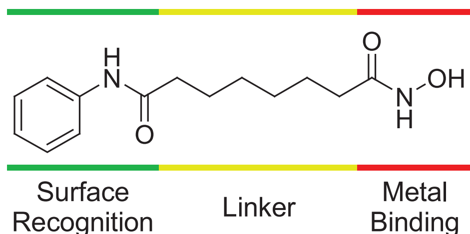


Figure 2. **Upper Panel**: Schematic representation of the crystal structure of the histone deacetylase-like protein with SAHA that inserts into the pocket-like catalytic site of the enzyme. At the base of the catalytic pocket is a zinc molecule with which the hydroxamic moiety of SAHA binds (105). **Lower Panel**: Structural features of hydroxamic acid based inhibitors of HDACs.

5. EFFECTS OF HDAC INHIBITORS

5.1 Effects of HDAC Inhibitors on Gene Expression

The mechanism of the anti-proliferative effects of HDAC inhibitors involves, at least in part, altering the expression of genes either by directly affecting chromatin structure by inducing an accumulation of acetylated histones, or by affecting the activity of transcription factors by altering the structure of component proteins by acetylation (Figure 1).

HDAC inhibitors cause both increased and decreased expression of a finite number of genes (2-10% of the expressed genes) (34,109-112) (Table 3). Microarray analysis of the effects of HDAC inhibitors on gene expression in different cancer cell lines has shown that the patterns of alterations of gene expression may be similar for different cell types and different HDAC inhibitors (109).

One of the most commonly induced genes by HDAC inhibitors is the cell cycle kinase inhibitor $p21^{WAF1}$ (110). The increase in the level of p21 protein can lead to arrest of cells in G1. It has been shown that the HDAC inhibitor induced expression of $p21^{WAF1}$ correlates with an increase in the acetylation of histone associated with the p21 promoter region (35, 110). This suggests that p21 gene promoter is a direct target for HDAC inhibitors. Recently, it was shown that the HDAC inhibitor, SAHA, caused specific modifications in the pattern of acetylation and methylation of lysines in histones H3 and H4 associated with the p21 promoter (35). These changes did not occur in the promoter region of the $p27^{KIP1}$ or of the epsilon globin gene related histones. The $p27^{KIP1}$ gene is expressed in the transformed cells (ARP-1) and the epsilon globin gene is not expressed in these cells and neither gene is altered in its transcription by SAHA. The HDAC inhibitor caused a marked decrease in HDAC1 and Myc and an increase in RNA polymerase II in protein complex bound to the p21 promoter. These findings are consistent with HDAC inhibitor selective increase in transcription of the p21 gene.

In addition to $p21^{WAF1}$, a limited number of genes have been shown to be induced in a variety of cell types in response to HDAC inhibition (34, 44, 46, 108,111 and see reviews 1-9). These genes include several proteins that may play an important role in the antiproliferative effects of the inhibitors (Table 3).

SAHA is a potent inducer of apoptosis of human multiple myeloma cells. Microarray analysis of gene expression in these cells revealed a constellation of anti-proliferative and/or pro-apoptotic genes was altered within 6 hrs of culture with the HDAC inhibitors including down regulation of transcripts

for member of the insulin-like growth factor IGF/IGF-1 receptor and IL6 receptor signaling cascades and anti-apoptotic genes such as caspase inhibitors, oncogenic kinases, DNA synthesis repair enzymes and transcription factors such as E2F-1 (112). SAHA suppressed the activity of the proteasome and expression of its subunits and enhanced multiple myeloma cell sensitivity to proteasome inhibition and other pro-apoptotic agents.

For example, as indicated above, non-histone proteins are substrates for HDACs. Acetylation of the transcriptional activator, p53, leads to increased DNA binding and an increase in the rate of transcription of p53 regulated genes. BCL6 is a transcriptional repressor whose activity is commonly increased in lymphomas. Acetylation of BCL6 leads to an inhibition of transcriptional repression activity (6).

A number of genes are repressed in response to HDAC inhibition. The commonly repressed genes include cyclin D1, erb-B_2 and thymidylate synthase (Table 3). HDAC inhibitor induced transcriptional repression may result from either effects on histone acetylation or alternately, from the increase in acetylation of transcription factors or components of the transcriptional machinery.

HDAC activity is required for transcriptional activation mediated by STAT5 (signal transducer and activator or transcription 5) (113). Inhibiting HDAC activity can prevent expression of genes for which STAT5 is required. This will have an effect of repressing transcription associated with deacetylation. However, the basis of this activation is not well understood since the substrate of the HDAC activity associated with STAT5 does not appear to be STAT5 or histones.

Table 3. Partial List of Genes Transcriptionally Altered by HDAC Inhibitors*

Induced Genes	***Repressed Genes***
$p21^{WAF1}$	Cyclin D1
Gelsolin	ErbB2
Metallothionein 1L	Thymidylate Synthase
Histone H2B	Importin β
Cyclin E	Cyclin A
	VEGF

*See text for discussion of effects of HDAC inhibitors on gene expression in different transformed cells.

5.2 Effects of HDAC inhibitors on Non-transcriptional Proteins

In addition to the effects on gene expression, HDAC inhibitor-induced accumulation of acetylated histones may effect cell cycle progression by

altering the ability of tumor cells to undergo mitosis (22, 114). The acetylation state of histones is important for their proper deposition during DNA synthesis and chromosome segregation during mitosis. An increase in acetylated histones during the S phase (DNA synthesis) and G2 (pre-mitosis) phases of the cell cycle can activate a G2 checkpoint which leads to arrest of cells in the G2 phase. Loss of the G2 checkpoint is a frequent event in cancer cells and may account, in part, for the increase sensitivity of cancer cells compared to normal cells to the pro-apoptotic effects of HDAC inhibitors (115). HDACs and HATs, as indicated above, act on many proteins which are subject to be reversible acetylation on lysine residues (22, 116) (Table 4). The acetylation state of several of these proteins is increased following exposure to Class I and Class II HDAC inhibitors, including alpha-tubulin and Hsp90 (heat shock protein 90). Acetylation of certain of these proteins leads to their activation and anti-proliferative effects.

Table 4. Non-transcriptional Proteins Acetylated by HDAC Inhibitors*

Histone H2A, H2B, H3 and H4	***HMG protein***
P53	MyoD
GATA-1	Hsp90
E2F-1	βCatenin
pRB	+EKLF
c-Jun	NF=-kappaB
α-Tubulin	TFIIE$^{\beta}$
Importin α	TFIIF
Smad7	+TCF
Androgen receptor	+HMGI (Y)
Bc16	+ACTR
	+YY-1

*Partial List

+YY-1	Transcription factor : Yin – Yang 1
EKLF	Kruppel-like factor (erythroid)
TCF	Transcription factor 1 (T-cell specific, HMG – Box)
HMG1 (Y)	High-mobility group at-hook (mus musculos)
ACTR :	NCoA3 (Nuclear Receptor Co-activator 3)

A small molecule, tubacin has been discovered to be a selective inhibitor of HDAC 6 activity and causes an accumulation of acetylated alpha-tubulin, but does not affect acetylation of histones and does not inhibit cell cycle progression (117). This suggests a subset of acetylated non-histone proteins play a role in the anti-proliferative effects of HDAC inhibitors. HDAC inhibitor induced acetylation of alpha-tubulin did decrease cell migration and therefore, while not inhibiting proliferation, may play a role in determining the metastatic capability of cancer cells.

HDAC inhibitors may block the activity of proteins that regulate cell signal transduction pathways by increasing non-histone acetylation. For example, HDAC inhibitors can cause an increase in acetylation of the chaperone protein Hsp90 (118). Acetylation of Hsp90 inhibits the binding of the chaperone protein to client proteins such as AKT resulting in the degradation of the client protein.

Further studies are needed to determine the relative role that altered gene transcription and altered activity of protein regulating cell signaling pathways and cell cycle progression play in HDAC inhibition induced cancer cell death. In certain cases, it may be a combination of both transcriptional and post-translational effects that lead to cancer cell death.

5.3 Activity of HDAC Inhibitors with other Agents

There is an extensive literature reporting on the use of HDAC inhibitors in combination with a number of different anti-cancer agents. Thus, HDAC inhibitors have been reported to be synergistic or additive with radiation therapy (119-121) anthracyclines (122), fludarabine (123, 124), flavopiridol (125), imatinib (126-128), proteasome inhibitor, bortezomib (129, 130), anti-angiogenic agents and nuclear receptor ligands such as all trans-retinoic acid, APO-2l/TRAIL (131-135).

For example, it has been reported that the sequential exposure of bortezomib in conjunction with HDAC inhibitors potently induces mitochondrial disfunction and apoptosis in human multiple myeloma cells through a ROS-dependent mechanism (129). Synergistic induction of apoptosis in breast cancer cells has been reported using butyrate, TNF-alpha, TRAIL or anti-FAS agonist (135). Coadministration of the HSP-90-antagonist, 17-allyalamino-17-demethoxygeldanamycin (17-AAG) with SAHA or sodium butyrate was reported to synergistically act to induce apoptosis in human leukemia cells (136).

HDAC inhibitors act synergistically or additively with DNA damaging agents such as radiation and anthracyclines probably by altering the conformation of chromatin resulting in a more open structure allowing access to DNA and increased ability to cause damage to DNA by these agents. While the induction of p21 may play an important role in the inhibition of cell cycle progression caused by HDAC inhibitors, several studies demonstrate that p21 induction blocks apoptosis in certain cell contexts (137). In fact, blocking SAHA induced p21 expression by combination treatment with flavopiridol leads to increased apoptosis. HDAC inhibitors may alter expression of genes such as BCR-ABL, thymidylate synthase and ERBB2 that cause cells to become more sensitive to various anti-cancer agents such as imatinib and 5-fluorouracial. The

elucidation of the down stream pathways of HDAC inhibition should provide further mechanistic rationale for therapies to be administered in combination with HDAC inhibitors.

5.4 HDAC Inhibitor Induced Cell Death

HDAC inhibitors have been shown to induce growth arrest and death of cancer cells *in vitro* and in *in vivo* in tumor bearing animal models with little or no toxicity (reviewed 1-9). Further, in phase I and II clinical trials HDAC inhibitors, such as SAHA and depsipeptide have shown significant anti-tumor activity against various cancers at doses that are well tolerated by patients (9, 138-142). The results of these studies demonstrated that HDAC inhibitors can induce death of cancer cells, while normal cells appear to be relatively resistance to HDAC inhibitor induced cell death.

One of the major questions with regard to HDAC inhibitor induced transformed cell death is the basis for the relative resistance of normal cells to these agents. HDAC inhibitors, SAHA and MS-275, arrest the growth of both normal human cells and transformed cells but induced rapid cell death of only the transformed cells. Both SAHA and MS-275 caused an accumulation of reactive oxygen species (ROS) and caspase activation in transformed but not normal cells. The increase in caspase activity could be completely blocked by the pan-caspase inhibitor, Z-VAD-fmk, without inhibiting HDAC inhibitor induced death of the transformed cells. The level of thioredoxin protein in normal cells cultured with SAHA or MS-275 was consistently found to be higher than in transformed cells. Thioredoxin is a major reducing protein which has many targets including ribonucleotide reductase which is required for DNA synthesis. Thioredoxin in an active scavenger of ROS. Transfection of the transformed cells, with thioredoxin siRNA decreased proliferation of cells and increased the sensitivity of the transformed cells to SAHA induced death (143). These studies indicated that thioredoxin may play an important role in determining sensitivity or resistance of normal and transformed cells to HDAC inhibitors.

A number of other studies with HDAC inhibitors have addressed the effects of these agents on inducing apoptosis and suppressing cell proliferation of various transformed cells. The HDAC inhibitor depsipeptide has been reported to induce apoptosis and suppress cell proliferation of human glioblastoma cells *in vitro* and *in vivo* (140). This effect of depsipeptide is associated with a decrease in the anti-apoptotic protein BcL-xl and increased expression of BAD a pro-apoptotic factor. LAQ824 significantly inhibits the proliferation of leukemic lymphoblastic cells, and induces apoptosis which was reported to be partially independent of caspase activation (144). MS-275 has been reported to induce caspase dependent

apoptosis in T cell chronic lymphatic leukemia cells (145) but induces caspase independent transformed cell death of other transformed cells (143). Depsipeptide has also been reported to induce a caspase independent cell death of T cell lymphoma cell lines (142).

The HDAC inhibitors SAHA, oxamflatin, and depsipeptide were shown to induce cell death which is mediated by the intrinsic apoptotic pathway. The HDAC induced apoptosis was inhibited by over expression of BcL2 but not by the polycaspase inhibitor, Z-VAD-fmk (146). CBHA induced apoptosis in human neuroblastoma was found to be caspase dependent (147). Taken together, these studies suggest that there are differences in the mechanism of cell death induced by different HDAC inhibitors in different transformed cells.

In studies with transformed cells in culture, tumor cell lines were 10-fold more sensitive to HDAC inhibitors than normal fibroblasts (115). The selective inhibitory effects on transformed cells compared to normal cells do not appear to be due to a difference in the ability to inhibit HDAC activity. Accumulation of acetylated histones occurs in both normal and transformed cells (80, 148).

HDAC inhibitors fall into a class of agents that target an activity (reversible protein acetylation) that occurs in all cells. As reviewed above, the favorable therapeutic index observed in tumor bearing animal studies and in clinical trials appears to result from the differential response of the cancer cells and normal cells to inhibition of HDAC activity.

5.5 HDAC Inhibitor Effects in Tumor Bearing Animals

Several HDAC inhibitors – including TSA, CHAP1 and CHAP31, SAHA, pyroxamide, CBHA, oxamflatin, MS-275, PXD101, and FK-228 inhibit tumor growth in animal models bearing both solid tumors and hematological malignancies with little toxicity (1-15). The solid tumors models include human breast, prostate, lung and stomach cancers, neuroblastoma, medulloblastoma, multiple myeloma and leukemia. HDAC inhibitors cause an accumulation of acetylated histones in tumor and normal tissues [spleen, liver and peripheral mononuclear (PMN) cells] (148). Increased accumulation of acetylated histones is a useful marker of HDAC inhibitor biological activity and has been used to monitor dosing in clinical trials with cancer patient.

TSA, SAHA, valproic acid and depsipeptide are reported to block angiogenesis *in vivo* (149-153). These HDAC inhibitors block hypoxia-induced angiogenesis in different carcinoma models. Hypoxia induces HDAC 1 and angiogenesis, so inhibition of HDACs might have a role in blocking new tumor blood-vessel formation. HDAC inhibitors may inhibit

tumor growth both directly by causing growth arrest, terminal differentiation and/or death of cancer cells, and indirectly, by inhibiting neovascularization of tumors.

6. CLINICAL TRIALS WITH HISTONE DEACETYLASE INHIBITORS

6.1 Aliphatic Acids

Over the last several years an increasing number of histone deacetylase inhibitors have undergone pre-clinical evaluation and have made there way into clinical trials. The first generation agents were the short chain fatty acids, phenyl acetate and phenylbutyrate. Phenylacetate was initially evaluated and approved for the use in children with urea cycle disorders, portal encephalopathy and chemotherapy-induced hyperammonemia. Initial trials of phenylacetate in patients with malignant tumor noted reversible lethargy and confusion at higher doses with modest palliative benefit from the drug (154-158) . Phenylbutyrate (PB), a precursor of phenylacetate after β-oxidization in the liver and kidney, showed more promising results. Carducci and colleagues administered a 5 day continuous infusion of phenylbutyrate to patents with refractory solid tumors. At the highest doses, somnolence and confusion associated with hypokalemia, hyponatremia and hyperuricemia was encountered but these adverse effects were reversible within 12 hours after discontinuation of the drug (157). Pharmacokinetic studies showed that plasma clearance increased continuously beginning 24 hours after start of the constant infusion and that plasma concentrations were able to be maintained above the targeted therapeutic threshold of 500 μmol/liter required for *in vitro* activity. While no objective responses were seen, stabilization of disease, improvement in pain with decrease in analgesic requirements and post-therapy decline in tumor markers were documented (157). In another trial of PB in patients with myelodysplasia and acute myeloid leukemia, a 7 day continuous infusion every 28 days was used (158). Dose limiting toxicities were similar to other trials but the drug was well tolerated at the maximal tolerated dose. In this trial three out eleven patients with myelodysplastic syndrome had hematologic improvement, and one of sixteen patients with AML that was previously dependent on platelet transfusions had recovery of platelets and did not require platelet transfusions (158). An additional four patients with AML also had a decrease in the number of malignant cells circulating in the blood. These

changes were associated with an increase in both the colony-forming units-granulocyte- macrophage and leukemic colony-forming units. A concern about this trial was that the steady state concentration of phenylbutyrate was less than the ED_{50} for differentiation and cytosis *in vitro.* Since higher concentrations were not tolerated, Gore *et al.* suggested that prolonged infusions of PB may increase the cell differentiation and cytosis. These investigators subsequently explored a fixed dose of PB administered as continuous infusion using an ambulatory pump for 7 consecutive days out of 14 days or 21 consecutive days out of 28 days (159). The infusions were tolerated well with mild neurocortical toxicity however an increased number of patients developed infectious complications from the prolonged intravenous access. Few objective responses were seen but hematologic improvements in blood counts were documented in several patients.

An oral preparation of PB has been investigated in 28 patients with refractory solid tumor malignancies (160). These patients were given 9 to 45 grams of oral PB daily. Overall there was good compliance with the medication and oral bioavailability was predicted to be 78%. At the higher doses of oral PB, nausea/vomiting, fatigues and edema became the dose limiting toxicities. In contrast to the intravenous trials with PB, neurocortical symptoms were mild and were not does limiting. The PB pharmacokinetics was linear within the dose ranges studied and the pharmacokinetics parameters paralleled the reports from the intravenous studies. The plasma concentrations of PB remained above the *in vitro* differentiation threshold of 0.5mM at all doses and the duration above this threshold increased from 1.1 hours to 4.1 hours as the dose was escalated from 9 to 45 grams per day (160). There were 23 patients that were evaluable for clinical response to the therapy. While there were no complete or partial tumor regressions reported, seven patients that had progressive disease before entering the study had stable disease for > 6 months during the administration of oral PB (160). These studies with PB suggest that these drugs, as predicted, have a cytostatic effect in tumors, and combining PB with other agents such as Retinoic Acid may be more effective. Warrell and colleagues combined all–trans retinoic acid with oral PB and showed a complete hematologic remission in a patient with retinoic acid refractory acute promyelocytic leukemia (161). It was postulated that phenylbutyrate inhibited the co-repressor complex that contains HDAC for the oncoprotein that is encoded by one of the translocation-generated fusion genes in APL, *PML-RARα* and restored the sensitivity to all-trans retinoic acid (161).

A histone deacetylase inhibitor analog of butyric acid, AN-9 or Pivanex, has been shown to causes apoptosis of cancer cells through signaling cellular differentiation and has entered clinical trials (162). Preclinical trials suggested that Pivanex was more potent than butyric acid for inducing tumor

cell differentiation and altering gene expression. This difference is thought to be related to an increase in the cellular uptake of Pivanex. Phase I studies showed that the drug was well tolerated when given as intravenous infusion for 5 consecutive days every 21 days. An objective response was seen in one patient with squamous cell carcinoma of the lung which prompted a phase II trial in patients with advanced non-small cell carcinoma of the lung. In this multi-center study, forty-seven patients with heavily pre-treated non-small cell carcinoma were treated with Pivanex as a 6 hour continuos intravenous infusion for three consecutive days every 21 days (162). Fatigue, nausea and dysgeusia were the most common adverse effects that were reversible. Three patients had documented partial tumor regression (6%) and 14 out of the 47 (30%) patients had stable disease for 12 weeks or greater (163). Further studies in combination with chemotherapy are planned in this population of patients.

Valproic acid, a common well tolerated anti-epileptic medication, is another short fatty acid that that has recently been shown to be an inhibitor of HDACs (92). A dose escalation study in patients with advanced cancer found that 60 mg/kg of valproic acid administered daily for 5 consecutive days every 21 days was the maximum tolerated dose (164). Neurologic toxicity without hematologic suppression was the dose limiting toxicity encountered. An increase in acetylated histones in peripheral mononuclear cells was also documented. Further phase II studies are ongoing to further characterize valproic acid as an anti-tumor agent in patients.

6.2 Cyclic Peptides

Depsipeptide is a cyclic peptide that has completed phase I evaluation and phase II studies alone or in combination are ongoing to determine the clinical efficacy in a wide range of solid and hematological malignancies. The phase I studies in patients with refractory solid tumors evaluated a 4 hour intravenous infusion administered on days 1 and 5 of a 21 day cycle or weekly on days 1, 8, 15 every 28 days (165, 166). On the 21 day schedule, the dose limiting toxicities were fatigue, nausea\vomiting, thrombocytopenia and cardiac arrhythmias. While the maximal tolerated dose for the 28 day schedule was lower than obtained in the 21 days schedule (13.3 mg/m^2 vs. 17.8 mg/m^2, respectively), the dose limiting toxicities of thrombocytopenia and fatigue were similar. Cardiac abnormalities that included transient EKG changes and CPK elevations were seen in the 21 day schedule but was less evident when the drug was given every 28 days. These phase I studies showed that depsipeptide could be administered safely and treatment with depsipeptide could result in an accumulation of acetylated histones in peripheral mononuclear cells. More importantly, significant tumor regression

was observed in patients with renal cell carcinoma and in patients with cutaneous T-cell lymphomas (141). Eight additional patients (22%) were reported to have stable disease in one study. Preliminary results of a phase II study in cutaneous T-cell and relapsed peripheral T-cell lymphoma has been reported. Seven out of 14 patients (50%) with cutaneous T-cell lymphoma that have failed 2 or less chemotherapies achieved an objective response, three with an overall complete response (166). The duration of these responses ranged from 6 to 34 months. In patients with peripheral T-cell lymphoma 4 out of 17 patients had a partial response (166). Therapy was well tolerated and no cumulative toxicity was seen.

Depsipeptide has been investigated in patients with Chronic Lymphocytic Leukemia (CLL) and Acute Myeloid Leukemia (AML). Ten patients with CLL and AML were administered 13 mg/m^2 of depsipeptide on days 1, 8 and 15 every 28 days (141). While no patients with CLL had a partial response, all patients had an improvement of the peripheral leukocyte counts (mean decrease of 58%) and one patient had a 46% reduction in the lymphadenopathy observed (168). Only 3 of the 10 patients tolerated more than two cycles of therapy and all had progressive symptoms of fatigue, nausea and vomiting. Similar results were also seen in the 10 patients with ALL. No complete or partial responses were seen but hematological improvement was observed with one patient developing a tumor lysis syndrome from the therapy. While this dosing schedule was poorly tolerated in this patient population, the study uniquely demonstrated that a 100% increase in H4 acetylation can be achieved in CLL cells with depsipeptide treatment. For the first time in patients, these investigators were able to show that an increase in p21 protein expression occurred concurrently with H4 acetylation of the promoter (166). Other phase II trials in solid and hematological tumors are continuing to explore the spectrum of clinical activity of depsipeptide.

6.3 Hydroximates

SAHA, LAQ824, LBH589A and PXD-101 are the HDAC inhibitors in the class that have moved forward in clinical trials. Initially, phase I trials in patients with refractory hematologic and solid tumors with SAHA administered intravenously over 2 hours for 5 consecutives days given for 3 weeks out of 4 weeks was well tolerated with thrombocytopenia and neutropenia being dose limiting toxicities in hematologic patients (167). No dose limiting toxicities were encountered in the solid tumor patients. This study demonstrated that SAHA could be administered safely and plasma concentration exceeded the therapeutic threshold where antiproliferative activities in cell culture were seen at all dose levels (167). In patients,

intravenous SAHA was shown to inhibit histone deacetylase in normal and malignant cells with a broad range of anti-tumor effects seen in solid and hematological tumors. The prolonged intravenous administration of SAHA established the "proof of principal" but was burdensome and was associated with catheter related complications. Subsequently an oral preparation was developed that showed good oral bioavailability, favorable pharmacokinetic profile and three dosing schedules were defined that include a 400 mg daily, 200 mg po twice daily and 300 mg po twice daily for three consecutive days every week (168). Peak plasma concentration were substantially lower than observed with the intravenous formulation however the anti-tumor activity in solid tumors and hematological malignancies was continued to be observed. Patients with renal cell carcinoma, squamous cell carcinoma of the head and neck, papillary thyroid cancer, mesothelioma, B and T cell lymphomas have shown objective tumor regression (168). Fatigue, dehydration and anorexia were the dose limiting toxicities that were rapidly reversible on discontinuation of SAHA. Patients were able to tolerate prolonged administration of the drug for some cases for over 2 years with minimal or no cumulative toxicity. Preliminary results of a phase II study with oral SAHA in patients with refractory cutaneous T-cell lymphoma (CTCL) showed that five out the 13 (38%, 95% CI 14-68%) patients treated had partial objective response to therapy with 5 additional patients having stable disease (139). The symptomatic relief of pruritus associated with the cutaneous lymphoma also improved quickly in the majority of patients and the median time to objective response was 11 weeks. In patients with myelodysplastic syndrome or advanced leukemias, 250 mg po TID for 14 days every 21 days was well tolerated (167). As in the other studies with SAHA, the most common non-hematologic adverse events were nausea, vomiting, diarrhea, fatigue and dyspepsia. Out of the 15 patients treated in this phase I study, one patient with AML achieved a complete hematologic remission with one additional patient with AML observed to have hematologic improvement (167). Two other patients with CLL had reduction in the size the lymphadenopathy. In a preliminary report from a phase II study using 400 mg of oral SAHA in patients with advanced squamous cell head and neck cancer, the therapy was well tolerated but no complete or partial responses were seen (169). The clinical results of SAHA have been encouraging and currently multiple phase II studies with SAHA, as a single agent and in combination with other biologic or chemotherapeutic drugs are ongoing.

LAQ824 is a very potent histone deacetylase inhibitor that is completing phase I clinical trial in solid and hematologic malignancies. Patients were given a 3 hour infusion on days 1-3 every 21 days. In patients with refractory solid tumors transient transaminitis, fatigue, atrial fibrillation without

changes in QTc and elevated creatinine were the dose limiting events. Both studies showed an increase in acetylated histone in peripheral mononuclear cells after therapy at the higher dose levels that lasted 24 to 48 hours after last dose was administered (170, 171). The mean terminal half life is 8 – 18 hours and there was noted to 1.5 fold drug accumulation over the three days period. Three out 28 solid tumor patients had stable disease and one patient with AML treated out 21 patients with hematologic malignancies achieved a complete response in these phase I studies (170, 171). LAQ824 is a novel HDAC inhibitor that is well tolerated, showed a dose dependent pharmadynamic effect on the peripheral mononuclear cells and has clinical activity. Further studies are ongoing. LBH589A is another HDAC inhibitor that can inhibit tumor cell line growth at nanomolar levels. Phase I studies are ongoing but this drug has shown biologic activity in peripheral mononuclear cells and preliminary pharmakinetics have shown prolonged half-life of 15-20 hours (172). Phase I studies with PXD101 are ongoing.

6.4 Benzamides

Phase I studies with MS-275 in patients with solid tumors and hematologic malignancies evaluated an oral daily dosing schedules for 28 days. This was poorly tolerated; however 14 day schedule safely administered to patients with mild to moderate fatigue, nausea, hypoalbuminemia, headache, anxiety, dyspepsia, vomiting, dysgeusia, anemia, fever and hyponatremia observed (173). Peak plasma concentrations were observed at 6-24 hours after oral administration and therapeutic plasma concentrations were obtained in patients. In all patients on the study, an accumulation of acetylated histone H3 in peripheral blood mononuclear cells was also documented post-therapy. Further studies are ongoing to optimize the oral treatment schedule.

CI-994 (N-Acetylamide) is an orally bioavailable compound that has been shown to cause phosphorylation and degradation of nuclear proteins with subsequent accumulation of acetylated histones in malignant cell lines. CI-994 is a weak inhibitor of histone deacetylase however it has shown cytostatic effects in transformed tumor cell lines (174-176). Major adverse events seen with CI-994 are nauseas, vomiting, diarrhea, fatigue, neutropenia and anemia. Two out of 32 (7%) patients with non-small cell lung cancer treated with CI-994 had a partial response with quarter of the patients having stable disease for minimum of 8 weeks (177). In a phase II study in patients with metastatic renal cell carcinoma, stable disease for greater than 8 weeks was also observed in over half of the patients. No complete or partial tumor regression was documented in the renal cell carcinoma trial (174). In a trial that combined CI-994 with capecitabine, thrombocytopenia and hand–foot

syndrome complicated therapy although a partial response in one patient with colorectal cancer was seen (170). The addition of CI-994 to chemotherapy in two randomized trials showed that the CI-994 provided no survival benefits to patients with non-small lung cancer (176,177). Further clinical development of CI-994 has not been further pursued.

7. CONCLUSIONS AND PERSPECTIVES

HDAC inhibitors are promising new targeted anti-cancer agents. HDAC inhibitors cause cancer cell growth arrest, differentiation, apoptosis and cell death of a broad spectrum of malignant cells, both solid tumors and hematologic malignancies. Normal cells are much less sensitive to HDAC inhibitors than transformed cells.

Some understanding is emerging as to the basis of the selectivity of HDAC inhibitors in altering transcription of genes; why normal cells are so relatively resistant to inhibitors compared to transformed cells; the non-histone targets of HDAC inhibitors and what role they play in arresting or regulating growth and inducing death of cancer cells?

It remains an important question as to whether HDAC isotype specific inhibitors can be developed and what effect inhibiting specific HDACs will have on cell growth and development.

Further clinical studies are needed to define the optional program for administration of these agents both as monotherapy and in combination with other agents that inhibit cancer cell growth.

There has been a marked increase in interest in research on HDACs, HATs and HDAC inhibitors which undoubtedly will lead to answering these and other questions related to the development of these agents as effective therapeutics.

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founder of Aton and both institutions and the founder had an equity position in Aton Pharma, Inc. PAM is a scientific consultant to Merck.

REFERENCES

1. Cress W. D. and Seto E. Histone deacetylases, transcriptional control, and cancer. J Cell Physiol, 2000; 184: 1-16.
2. Kelly W. K., O'Connor O. A., and Marks P. A. Histone deacetylase inhibitors: from target to clinical trials. Expert Opin Investig Drugs, 2002;11: 1695-1713.
3. Marks P., Rifkind R. A., Richon V. M., Breslow R., Miller T. and Kelly W. K. Histone deacetylases and cancer: causes and therapies. Nat Rev Cancer, 2001; 1: 194-202.
4. de Ruijter A. J., van Gennip A. H., Caron H. N., Kemp S. and van Kuilenburg A. B. Histone deacetylases (HDACs): characterization of the classical HDAC family. Biochem J, 2003; 370: 737-749.
5. Jones P. A. and Baylin S. B. The fundamental role of epigenetic events in cancer. Nat Rev Genet, 2002; 3: 415-428.
6. Johnstone R. W. and Licht J. D. Histone deacetylase inhibitors in cancer therapy: is transcription the primary target? Cancer Cell, 2003; 4: 13-18.
7. Curtin M. and Glaser K. Histone deacetylase inhibitors: the abbott experience. Curr Med Chem, 2003; 10: 2373-2392.
8. Miller T. A., Witter D. J. and Belvedere S. Histone deacetylase inhibitors. J Med Chem, 2003; 46: 5097-5116.
9. Marks P. A., Richon V. M., Miller T. and Kelly W. K. Histone deacetylase inhibitors. Adv Cancer Res, 2004; 91: 137-168.
10. Rosato R. R. and Grant S. Histone deacetylase inhibitors in clinical development. Expert Opin Investig Drugs, 2004; 13: 21-38.
11. Vigushin D. M. and Coombes R. C. Targeted histone deacetylase inhibition for cancer therapy. Curr Cancer Drug Targets, 2004; 4: 205-218.
12. Somech R., Izraeli S. and J. Simon A. Histone deacetylase inhibitors--a new tool to treat cancer. Cancer Treat Rev, 2004; 30: 461-472.
13. Lindemann R. K., Gabrielli B. and Johnstone R. W. Histone-deacetylase inhibitors for the treatment of cancer. Cell Cycle, 2004; 3: 779-788.
14. Rosato R. R. and Grant S. Histone deacetylase inhibitors in cancer therapy. Cancer Biol Ther, 2003; 2: 30-37.
15. Secrist J. P., Zhou X., and Richon V. M. HDAC inhibitors for the treatment of cancer. Curr Opin Investig Drugs, 2003; 4: 1422-1427.
16. Jenuwein T. and Allis C. D. Translating the histone code. Science, 2001; 293: 1074-1080.
17. Spotswood H. T. and Turner B. M. An increasingly complex code. J Clin Invest, 2002; 110: 577-582.
18. Turner B. M. Histone acetylation and an epigenetic code. Bioessays, 2000; 22: 836-845.
19. Zhang Y. and Reinberg D. Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. Genes Dev, 2001; 15: 2343-2360.

20. Agalioti T., Chen G. and Thanos D. Deciphering the transcriptional histone acetylation code for a human gene. Cell, 2002; 111: 381-392.
21. Richards E. J. and Elgin S. C. Epigenetic codes for heterochromatin formation and silencing: rounding up the usual suspects. Cell, 2002; 108: 489-500.
22. Kouzarides T. Acetylation: a regulatory modification to rival phosphorylation? Embo J, 2000; 19: 1176-1179.
23. Wolffe A. P. and Pruss D. Deviant nucleosomes: the functional specialization of chromatin. Trends Genet, 1996; 12: 58-62.
24. Luger K., Mader A. W., Richmond R. K., Sargent D. F. and Richmond T. J. Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature, 1997; 389: 251-260.
25. Fischle W., Wang Y. and Allis C. D. Binary switches and modification cassettes in histone biology and beyond. Nature, 2003; 425: 475-479.
26. Trojer P., Brandtner E. M., Brosch G., Loidl P., Galehr J., Linzmaier R., Haas H., Mair K., Tribus M. and Graessle S. Histone deacetylases in fungi: novel members, new facts. Nucleic Acids Res, 2003; 31: 3971-3981.
27. Khochbin S., Verdel A., Lemercier C. and Seigneurin-Berny D. Functional significance of histone deacetylase diversity. Curr Opin Genet Dev, 2001; 11: 162-166.
28. Gao L., Cueto M. A., Asselbergs F. and Atadja P. Cloning and functional characterization of HDAC11, a novel member of the human histone deacetylase family. J Biol Chem, 2002; 277: 25748-25755.
29. Kyrylenko S., Kyrylenko O., Suuronen T. and Salminen A. Differential regulation of the Sir2 histone deacetylase gene family by inhibitors of class I and II histone deacetylases. Cell Mol Life Sci, 2003; 60: 1990-1997.
30. Gray S. G. and Ekstrom T. J. The human histone deacetylase family. Exp Cell Res, 2001; 262: 75-83.
31. Grozinge, C. M., Hassig C. A. and Schreiber S. L. Three proteins define a class of human histone deacetylases related to yeast Hda1p. Proc Natl Acad Sci U S A, 1999; 96: 4868-4873.
32. Gregoretti I. V., Lee Y. M. and Goodson H. V. Molecular evolution of the histone deacetylase family: functional implications of phylogenetic analysis. J Mol Biol, 2004; 338: 17-31.
33. Van Lint C., Emiliani S. and Verdin E. The expression of a small fraction of cellular gene is changed in response to histone hyperacetylation. Gene Expr, 1996; 5: 245-254.
34. Verdin E., Dequiedt F. and Kasler H. G. Class II histone deacetylases: versatile regulators. Trends Genet, 2003; 19: 286-293.
35. Gui C. Y., Ngo L., Xu W. S., Richon V. M. and Marks P. A. Histone deacetylase (HDAC) inhibitor activation of p21WAF1 involves changes in promoter-associated proteins, including HDAC1. Proc Natl Acad Sci U S A, 2004; 101: 1241-1246.
36. Hubbert C., Guardiola A., Shao R., Kawaguchi Y., Ito A., Nixon A., Yoshida M., Wang X. F. and Yao T. P. HDAC6 is a microtubule-associated deacetylase. Nature, 2002; 417: 455-458.
37. Voelter-Mahlknecht S. and Mahlknecht U. Cloning and structural characterization of the human histone deacetylase 6 gene. Int J Mol Med, 2003; 12: 87-93.
38. Zhang Y., Li N., Caron C., Matthias G., Hess D., Khochbin S. and Matthias P. HDAC-6 interacts with and deacetylates tubulin and microtubules in vivo. EMBO J, 2003; 22: 1168-1179.
39. Zhou X., Richon V. M., Wang A. H., Yang X. J., Rifkind R. A. and Marks P. A. Histone deacetylase 4 associates with extracellular signal-regulated kinases 1 and 2,

and its cellular localization is regulated by oncogenic Ras. Proc Natl Acad Sci U S A, 2000; 97: 14329-14333.

40. Petrie K., Guidez F., Howell, L., Healy L., Waxman S., Greaves M. and Zelent A. The histone deacetylase 9 gene encodes multiple protein isoforms. J Biol Chem, 2003; 278: 16059-16072.
41. Fischer D. D., Cai R., Bhatia U., Asselbergs F. A., Song C., Terry R., Trogani N., Widmer R., Atadja P. and Cohen D. Isolation and characterization of a novel class II histone deacetylase, HDAC10. J Biol Chem, 2002; 277: 6656-6666.
42. Tong J. J., Liu J., Bertos N. R. and Yang X. J. Identification of HDAC10, a novel class II human histone deacetylase containing a leucine-rich domain. Nucleic Acids Res, 2002; 30: 1114-1123.
43. Arts J., de Scheppe, S. and Van Emelen K. Histone deacetylase inhibitors: from chromatin remodeling to experimental cancer therapeutics. Curr Med Chem, 2003; 10: 2343-2350.
44. Glaser K. B., Li J., Staver M. J., Wei R. Q., Albert D. H. and Davidsen S. K. Role of class I and class II histone deacetylases in carcinoma cells using siRNA. Biochem Biophys Res Commun, 2003; 310: 529-536.
45. Wang A. H. and Yang X. J. Histone deacetylase 4 possesses intrinsic nuclear import and export signals. Mol Cell Biol, 2001; 21: 5992-6005.
46. Mal A. and Harter M. L. MyoD is functionally linked to the silencing of a muscle-specific regulatory gene prior to skeletal myogenesis. Proc Natl Acad Sci U S A, 2003;100: 1735-1739.
47. McGraw S., Robert C., Massicotte L. and Sirard M. A. Quantification of histone acetyltransferase and histone deacetylase transcripts during early bovine embryo development. Biol Reprod, 2003; 68: 383-389.
48. Lagger G., O'Carroll D., Rembold M., Khier H., Tischler J., Weitzer G., Schuettengruber B., Hauser C., Brunmeir R., Jenuwein T. and Seiser C. Essential function of histone deacetylase 1 in proliferation control and CDK inhibitor repression. The EMBO Journal, 2002; 21: 2672-2681.
49. Wen Y. D., Cress W. D., Roy A. L. and Seto E. Histone deacetylase 3 binds to and regulates the multifunctional transcription factor TFII-I. J Biol Chem, 2003; 278: 1841-1847.
50. McKinsey T. A., Zhang C. L. and Olson E. N. Control of muscle development by dueling HATs and HDACs. Curr Opin Genet Dev, 2001; 11: 497-504.
51. Czubryt M. P., McAnally J., Fishman G. I. and Olson E. N. Regulation of peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1 alpha) and mitochondrial function by MEF2 and HDAC5. Proc Natl Acad Sci U S A, 2003; 100: 1711-1716.
52. McKinsey T. A., Zhang C. L. and Olson E. N. Signaling chromatin to make muscle. Curr Opin Cell Biol, 2002; 14: 763-772.
53. Ishizuka T. and Laza, M. A. The N-CoR/histone deacetylase 3 complex is required for repression by thyroid hormone receptor. Mol Cell Biol, 2003; 23: 5122-5131.
54. Berger I., Bieniossek C., Schaffitzel C., Hassle, M., Santelli E. and Richmond T. J. Direct interaction of Ca2+/calmodulin inhibits histone deacetylase 5 repressor core binding to myocyte enhancer factor 2. J Biol Chem, 2003; 278: 17625-17635.
55. Dequiedt F., Kasler H., Fischle W., Kiermer V., Weinstein M., Herndier B. G. and Verdin E. HDAC7, a thymus-specific class II histone deacetylase, regulates Nur77 transcription and TCR-mediated apoptosis. Immunity, 2003; 18: 687-698.
56. Urnov F. D., Yee J., Sachs L., Collingwood T. N., Bauer A., Beug H., Shi Y. B. and Wolffe A. P. Targeting of N-CoR and histone deacetylase 3 by the oncoprotein v-

erbA yields a chromatin infrastructure-dependent transcriptional repression pathway. EMBO J, 2000; 19: 4074-4090.

57. Yoon H. G., Chan D. W., Huang Z. Q., Li J., Fondell J. D., Qin J. and Wong J. Purification and functional characterization of the human N-CoR complex: the roles of HDAC3, TBL1 and TBLR1. EMBO J, 2003; 22: 1336-1346.
58. Timmermann S., Lehrmann H., Polesskaya A. and Harel-Bellan A. Histone acetylation and disease. Cell Mol Life Sci, 2001; 58: 728-736.
59. He L.-Z., Tolentino T., Grayson P., Zhong S., Warrell R. P., Jr., Rifkind R. A., Marks P. A., Richon V. M. and Pandolfi P. P. Histone deacetylase inhibitors induce remission in transgenic models of therapy-resistant acute promyelocytic leukemia. J. Clin Invest, 2001; 108: 1321-1330.
60. Wang J., Hoshino T., Redner R. L., Kajigaya S. and Liu J. M. ETO, fusion partner in t(8;21) acute myeloid leukemia, represses transcription by interaction with the human N-CoR/mSin3/HDAC1 complex. Proc Natl Acad Sci U S A, 1998; 95: 10860-10865.
61. Kawai H., Li H., Avraham S., Jiang S. and Avraham H. K. Overexpression of histone deacetylase HDAC1 modulates breast cancer progression by negative regulation of estrogen receptor alpha. Int J Cancer, 2003; 107: 353-358.
62. Chan H. M., Krstic-Demonacos M., Smith L., Demonacos C. and La Thangue N. B. Acetylation control of the retinoblastoma tumour-suppressor protein. Nat Cell Biol, 2001; 3: 667-674.
63. Magnaghi-Jaulin L., Groisman R., Naguibneva I., Robin P., Lorain S., Le Villain J. P., Troalen F., Trouche D. and Harel-Bellan A. Retinoblastoma protein represses transcription by recruiting a histone deacetylase. Nature, 1998; 391: 601-605.
64. Murphy M., Ahn J., Walker K. K., Hoffman W. H., Evans R. M., Levine A. J. and George D. L. Transcriptional repression by wild-type p53 utilizes histone deacetylases, mediated by interaction with mSin3a. Genes Dev, 1999; 13: 2490-2501.
65. Yarden R. I. and Brody L. C. BRCA1 interacts with components of the histone deacetylase complex. Proc Natl Acad Sci U S A, 1999; 96: 4983-4988.
66. Skov S., Rieneck K., Bovin L. F., Skak K., Tomra S., Michelsen B. K. and Odum N. Histone deacetylase inhibitors: a new class of immunosuppressors targeting a novel signal-pathway essential for CD154 expression. Blood, 2003; 101: 1430-1438.
67. Gayther S. A., Batley S. J., Linger L., Bannister A., Thorpe K., Chin S. F., Daigo Y., Russell P., Wilson A., Sowter H. M., Delhanty J. D., Ponder B. A., Kouzarides T. and Caldas C. Mutations truncating the EP300 acetylase in human cancers. Nat Genet, 2000; 24: 300-303.
68. Fenrick R. and Hiebert S. W. Role of histone deacetylases in acute leukemia. J Cell Biochem Suppl, 1998; 30-31: 194-202.
69. Smirnov D. A., Hou S. and Ricciardi R. P. Association of histone deacetylase with COUP-TF in tumorigenic Ad12-transformed cells and its potential role in shut-off of MHC class I transcription. Virology, 2000; 268: 319-328.
70. Choi J. H., Kwon H. J., Yoon B. I., Kim J. H., Han S. U., Joo H. J. and Kim D. Y. Expression profile of histone deacetylase 1 in gastric cancer tissues. Jpn J Cancer Res, 2001; 92: 1300-1304.
71. Toh Y., Yamamoto M., Endo K., Ikeda Y., Baba H., Kohnoe S., Yonemasu H., Hachitanda Y., Okamura T. and Sugimachi K. Histone H4 acetylation and histone deacetylase 1 expression in esophageal squamous cell carcinoma. Oncol Rep, 2003; 10: 333-338.
72. Neumeister P., Albanese C., Balent B., Greally J. and Pestell R. Senescence and epigenetic dysregulation in cancer. Int J Biochem Cell Biol, 2002; 34: 1475.

73. Lehrmann H., Pritchard L. L. and Harel-Bellan A. Histone acetyltransferases and deacetylases in the control of cell proliferation and differentiation. Adv Cancer Res, 2002; 86: 41-65.
74. Murata T., Kurokawa R., Krones A., Tatsumi K., Ishii M., Taki T., Masuno M., Ohashi H., Yanagisawa M., Rosenfeld M. G., Glass C. K. and Hayashi Y. Defect of histone acetyltransferase activity of the nuclear transcriptional coactivator CBP in Rubinstein-Taybi syndrome. Hum Mol Genet, 2001; 10: 1071-1076.
75. Giles R. H., Peters D. J. and Breuning M. H. Conjunction dysfunction: CBP/p300 in human disease. Trends Genet, 1998; 14: 178-183.
76. Yoshida M., Matsuyama A., Komatsu Y. and Nishino N. From discovery to the coming generation of histone deacetylase inhibitors. Curr Med Chem, 2003; 10: 2351-2358.
77. Marchion D. C., Bicaku E., Daud A. I., Richon V., Sullivan D. M. and Munster P. N. Sequence-specific potentiation of topoisomerase II inhibitors by the histone deacetylase inhibitor suberoylanilide hydroxamic acid. J Cell Biochem, 2004; 92: 223-237.
78. Sabet, R. S., Marcotte, P., Glaser, K., Burns, D. J., Warrior, U., and Groebe, D. R. Screening for inhibitors of histone deacetylase by incorporating a spraying method to micro-arrayed compound screening. Comb Chem High Throughput Screen, 2004; 7: 93-100.
79. Yoshida M., Kijima M., Akita M. and Beppu T. Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. J Biol Chem, 1990; 265: 17174-17179.
80. Richon V. M., Emiliani S., Verdin E., Webb Y., Breslow R., Rifkind R. A. and Marks P. A. A class of hybrid polar inducers of transformed cell differentiation inhibits histone deacetylases. Proc Natl Acad Sci U S A, 1998; 95: 3003-3007.
81. Richon V. M., Webb Y., Merger R., Sheppard T., Jursic B., Ngo L., Civoli F., Breslow R., Rifkind R. A. and Marks P. A. Second generation hybrid polar compounds are potent inducers of transformed cell differentiation. Proc Natl Acad Sci U S A, 1996; 93: 5705-5708.
82. Remiszewski S. W. The discovery of NVP-LAQ824: from concept to clinic. Curr Med Chem, 2003; 10: 2393-2402.
83. Atadja P., Gao L., Kwon P., Trogani N., Walker H., Hsu M., Yeleswarapu L., Chandramouli N., Perez L., Versace R., Wu A., Sambucetti L., Lassota P., Cohen D., Bair K., Wood A. and Remiszewski S. Selective growth inhibition of tumor cells by a novel histone deacetylase inhibitor, NVP-LAQ824. Cancer Res, 2004; 64: 689-695.
84. Su G. H., Sohn T. A., Ryu B. and Kern S. E. A novel histone deacetylase inhibitor identified by high-throughput transcriptional screening of a compound library. Cancer Res, 2000; 60: 3137-3142.
85. Kim Y. B., Lee K. H., Sugita K., Yoshida M. and Horinouchi S. Oxamflatin is a novel antitumor compound that inhibits mammalian histone deacetylase. Oncogene, 1999; 18: 2461-2470.
86. Furumai R., Matsuyama A., Kobashi N., Lee K. H., Nishiyama M., Nakajima H., Tanaka A., Komatsu Y., Nishino N., Yoshida M. and Horinouchi S. FK228 (depsipeptide) as a natural prodrug that inhibits class I histone deacetylases. Cancer Res, 2002; 62: 4916-4921.
87. Furumai R., Komatsu, Y., Nishino, N., Khochbin, S., Yoshida, M., and Horinouchi, S. Potent histone deacetylase inhibitors built from trichostatin A and cyclic tetrapeptide antibiotics including trapoxin. Proc Natl Acad Sci U S A, 2001; 98: 87-92.

88. Colletti S. L., Myers R. W., Darkin-Rattray S. J., Gurnett A. M., Dulski P. M., Galuska S., Allocco J. J., Ayer M. B., Li C., Lim J., Crumley T. M., Cannova C., Schmatz D. M., Wyvratt M. J., Fisher M. H. and Meinke P. T. Broad spectrum antiprotozoal agents that inhibit histone deacetylase: structure-activity relationships of apicidin. Part 1. Bioorg Med Chem Lett, 2001; 11: 107-111.
89. Colletti S. L., Myers R. W., Darkin-Rattray S. J., Gurnett A. M., Dulski P. M., Galuska S., Allocco J. J., Ayer M. B., Li C., Lim J., Crumley T. M., Cannova C., Schmatz D. M., Wyvratt M. J., Fisher M. H. and Meinke P. T. Broad spectrum antiprotozoal agents that inhibit histone deacetylase: structure-activity relationships of apicidin. Part 2. Bioorg Med Chem Lett, 2001; 11: 113-117.
90. Singh S. B., Zink D. L., Liesch J. M., Mosley R. T., Dombrowski A. W., Bills G. F., Darkin-Rattray S. J., Schmatz D. M. and Goetz M. A. Structure and chemistry of apicidins, a class of novel cyclic tetrapeptides without a terminal alpha-keto epoxide as inhibitors of histone deacetylase with potent antiprotozoal activities. J Org Chem, 2002; 67: 815-825.
91. Komatsu Y., Tomizaki K. Y., Tsukamoto M., Kato T., Nishino N., Sato S., Yamori T., Tsuruo T., Furumai R., Yoshida M., Horinouchi S. and Hayashi H. Cyclic hydroxamic-acid-containing peptide 31, a potent synthetic histone deacetylase inhibitor with antitumor activity. Cancer Res, 2001; 61: 4459-4466.
92. Phiel C. J., Zhang F., Huang E. Y., Guenther M. G., Lazar M. A. and Klein P. S. Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. J Biol Chem, 2001; 276: 36734-36741.
93. Boivin A. J., Momparler L. F., Hurtubise A, and Momparler R. L. Antineoplastic action of 5-aza-2'-deoxycytidine and phenylbutyrate on human lung carcinoma cells. Anticancer Drugs, 2002; 13: 869-874.
94. Gurvich N., Tsygankova O. M., Meinkoth J. L. and Klein P. S. Histone deacetylase is a target of valproic acid-mediated cellular differentiation. Cancer Res, 2004; 64: 1079-1086.
95. Eyal S., Yagen B., Sobol E., Altschuler Y., Shmuel M. and Bialer M. The activity of antiepileptic drugs as histone deacetylase inhibitors. Epilepsia, 45: 737-744, 2004.
96. Gottlicher M. Valproic acid: an old drug newly discovered as inhibitor of histone deacetylases. Ann Hematol, 2004; 83 Suppl 1: S91-92.
97. Saito A., Yamashita T., Mariko Y., Nosaka Y., Tsuchiya K., Ando T., Suzuki T., Tsuruo T. and Nakanishi O. A synthetic inhibitor of histone deacetylase, MS-27-275, with marked in vivo antitumor activity against human tumors. Proc Natl Acad Sci U S A, 1999; 96: 4592-4597.
98. Prakash S., Foster B. J., Meyer M., Wozniak A., Heilbrun L. K., Flaherty L., Zalupski M., Radulovic L., Valdivieso M. and LoRusso P. M. Chronic oral administration of CI-994: a phase 1 study. Invest New Drugs, 2001; 19: 1-11.
99. Maeda T., Nagaoka Y., Kuwajima H., Seno C., Maruyama S., Kurotaki M. and Uesato S. Potent histone deacetylase inhibitors: N-hydroxybenzamides with antitumor activities. Bioorg Med Chem, 2004; 12: 4351-4360.
100. Vasudevan A., Ji Z., Frey R. R., Wada C. K., Steinman D., Heyman H. R., Guo Y., Curtin M. L., Guo J., Li J., Pease L., Glaser K. B., Marcotte P. A., Bouska J. J., Davidsen S. K. and Michaelides M. R. Heterocyclic ketones as inhibitors of histone deacetylase. Bioorg Med Chem Lett, 2003; 13: 3909-3913.
101. Kim D. K., Lee J. Y., Kim J. S., Ryu J. H., Choi J. Y., Lee J. W., Im G. J., Kim T. K., Seo J. W., Park H. J., Yoo J., Park J. H., Kim T. Y. and Bang, Y. J. Synthesis and biological evaluation of 3-(4-substituted-phenyl)-N-hydroxy-2-propenamides, a new class of histone deacetylase inhibitors. J Med Chem, 2003; 46: 5745-5751.

102. Suzuki T., Kouketsu A., Matsuura A., Kohara A., Ninomiya S., Kohda K. and Miyata, N. Thiol-based SAHA analogues as potent histone deacetylase inhibitors. Bioorg Med Chem Lett, 2004; 14: 3313-3317.
103. Suzuki T., Nagano Y., Matsuura A., Kohara A., Ninomiya S., Kohda K. and Miyata N. Novel histone deacetylase inhibitors: design, synthesis, enzyme inhibition, and binding mode study of SAHA-based non-hydroxamates. Bioorg Med Chem Lett, 2003; 13: 4321-4326.
104. Hu E., Dul E., Sung C. M., Chen Z., Kirkpatrick R., Zhang G. F., Johanson K., Liu R., Lago A., Hofmann G., Macarron R., de los Frailes M., Perez P., Krawiec J., Winkler J. and Jaye M. Identification of novel isoform-selective inhibitors within class I histone deacetylases. J Pharmacol Exp Ther, 2003; 307: 720-728.
105. Finnin M. S., Donigian J. R., Cohen A., Richon V. M., Rifkind R. A., Marks P. A., Breslow R. and Pavletich N. P. Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. Nature, 1999; 401: 188-193.
106. Wegener D., Hildmann C., and Schwienhorst A. Recent progress in the development of assays suited for histone deacetylase inhibitor screening. Mol Genet Metab, 2003; 80: 138-147.
107. Matsuyama A., Shimazu T., Sumida Y., Saito A., Yoshimatsu Y., Seigneurin-Berny D., Osada H., Komatsu Y., Nishino N., Khochbin S., Horinouchi S. and Yoshida M. In vivo destabilization of dynamic microtubules by HDAC6-mediated deacetylation. Embo J, 2002; 21: 6820-6831.
108. Butler L. M., Zhou X., Xu W.-S., Scher H. I., Rifkind R. A., Marks P. A. and Richon V. M. The histone deacetylase inhibitor SAHA arrests cancer cell growth, up-regulates thioredoxin-binding protein-2, and down-regulates thioredoxin. PNAS, 2002; 99: 11700-11705.
109. Gray S. G., Qian C. N., Furge K., Guo X. and Teh B. T. Microarray profiling of the effects of histone deacetylase inhibitors on gene expression in cancer cell lines. Int J Oncol, 2004; 24: 773-795.
110. Richon V. M., Sandhoff T. W., Rifkind R. A. and Marks P. A. Histone deacetylase inhibitor selectively induces p21WAF1 expression and gene-associated histone acetylation. Proc Natl Acad Sci U S A, 2000; 97: 10014-10019.
111. Chambers A. E., Banerjee S., Chaplin T., Dunne J., Debernardi S., Joel S. P. and Young B. D. Histone acetylation-mediated regulation of genes in leukaemic cells. Eur J Cancer, 2003; 39: 1165-1175.
112. Mitsiades C. S., Mitsiades N. S., McMullan C. J., Poulaki V., Shringarpure R., Hideshima T., Akiyama M., Chauhan D., Munshi N., Gu X., Bailey C., Joseph M., Libermann T. A., Richon V. M., Marks P. A. and Anderson K. C. Transcriptional signature of histone deacetylase inhibition in multiple myeloma: biological and clinical implications. Proc Natl Acad Sci U S A, 2004; 101: 540-545.
113. Rascle A., Johnston J. A. and Amati B. Deacetylase activity is required for recruitment of the basal transcription machinery and transactivation by STAT5. Mol Cell Biol, 2003; 23: 4162-4173.
114. Warrener R., Beamish H., Burgess A., Waterhouse N. J., Giles N., Fairlie D. and Gabrielli B. Tumor cell-selective cytotoxicity by targeting cell cycle checkpoints. Faseb J, 2003; 17: 1550-1552.
115. Qiu L., Kelso M. J., Hansen C., West M. L., Fairlie D. P. and Parsons P. G. Anti-tumour activity in vitro and in vivo of selective differentiating agents containing hydroxamate. Br J Cancer, 1999; 80: 1252-1258.
116. Polevoda B. and Sherman F. The diversity of acetylated proteins. Genome Biol, 2002; 3: reviews 0006.

117. Haggarty S. J., Koeller K. M., Wong J. C., Grozinger C. M. and Schreiber S. L. Domain-selective small-molecule inhibitor of histone deacetylase 6 (HDAC6)-mediated tubulin deacetylation. Proc Natl Acad Sci U S A, 2003; 100: 4389-4394.
118. Yu Z., Zhang W. and Kone B. C. Histone deacetylases augment cytokine induction of the iNOS gene. J Am Soc Nephrol, 2002; 13: 2009-2017.
119. Paoluzzi L. and Figg W. D. Histone Deacetylase Inhibitors are Potent Radiation Protectants. Cancer Biol Ther, 2004; 3: 612-613.
120. Kim J. H., Shin J. H. and Kim I. H. Susceptibility and radiosensitization of human glioblastoma cells to trichostatin A, a histone deacetylase inhibitor. Int J Radiat Oncol Biol Phys, 2004; 59: 1174-1180.
121. Zhang Y., Adachi M., Zhao X., Kawamura R. and Imai, K. Histone deacetylase inhibitors FK228, N-(2-aminophenyl)-4-[N-(pyridin-3-yl-methoxycarbonyl)amino-methyl]benzamide and m-carboxycinnamic acid bis-hydroxamide augment radiation-induced cell death in gastrointestinal adenocarcinoma cells. Int J Cancer, 2004;110: 301-308.
122. Kim M. S., Blake M., Baek J. H., Kohlhagen G., Pommier Y. and Carrier F. Inhibition of histone deacetylase increases cytotoxicity to anticancer drugs targeting DNA. Cancer Res, 2003; 63: 7291-7300.
123. Acharya M. R. and Figg W. D. Histone Deacetylase Inhibitor Enhances the Anti-Leukemic Activity of an Established Nucleoside Analogue. Cancer Biol Ther, 2004; 3: 719-720.
124. Maggio S. C., Rosato R. R., Kramer L. B., Dai Y. Rahmani M., Paik D. S., Czarnik A. C., Payne S. G., Spiegel S. and Grant S. The histone deacetylase inhibitor MS-275 interacts synergistically with fludarabine to induce apoptosis in human leukemia cells. Cancer Res, 2004; 64: 2590-2600.
125. Rosato R. R., Almenara J. A., Yu C. and Grant S. Evidence of a functional role for p21WAF1/CIP1 down-regulation in synergistic antileukemic interactions between the histone deacetylase inhibitor sodium butyrate and flavopiridol. Mol Pharmacol, 2004; 65: 571-581.
126. Yoshida C. and Melo J. V. Biology of chronic myeloid leukemia and possible therapeutic approaches to imatinib-resistant disease. Int J Hematol, 2004; 79: 420-433.
127. Brieger A., Boehrer S., Schaaf S., Nowak D., Ruthardt M., Kim S. Z., Atadja P., Hoelzer D., Mitrou P. S., Weidmann E. and Chow K. U. In bcr-abl-positive myeloid cells resistant to conventional chemotherapeutic agents, expression of Par-4 increases sensitivity to imatinib (STI571) and histone deacetylase-inhibitors. Biochem Pharmacol, 2004; 68: 85-93.
128. Kim J. S., Jeung H. K., Cheong J. W., Maeng H., Lee S. T., Hahn J. S., Ko Y. W. and Min Y. H. Apicidin potentiates the imatinib-induced apoptosis of Bcr-Abl-positive human leukaemia cells by enhancing the activation of mitochondria-dependent caspase cascades. Br J Haematol, 2004; 124: 166-178.
129. Pei X. Y., Dai Y. and Grant S. Synergistic induction of oxidative injury and apoptosis in human multiple myeloma cells by the proteasome inhibitor bortezomib and histone deacetylase inhibitors. Clin Cancer Res, 2004; 10: 3839-3852.
130. Adachi M., Zhang, Y., Zhao X., Minami T., Kawamura R., Hinoda Y. and Imai K. Synergistic effect of histone deacetylase inhibitors FK228 and m-carboxycinnamic acid bis-hydroxamide with proteasome inhibitors PSI and PS-341 against gastrointestinal adenocarcinoma cells. Clin Cancer Res, 2004; 10: 3853-3862.
131. Guo F., Sigua C., Tao J., Bali P., George P., Li Y., Wittmann S., Moscinski L., Atadja P. and Bhalla K. Cotreatment with histone deacetylase inhibitor LAQ824 enhances Apo-2L/tumor necrosis factor-related apoptosis inducing ligand-induced death

inducing signaling complex activity and apoptosis of human acute leukemia cells. Cancer Res, 2004; 64: 2580-2589.

132. Neuzil J., Swettenham E. and Gellert N. Sensitization of mesothelioma to TRAIL apoptosis by inhibition of histone deacetylase: role of Bcl-xL down-regulation. Biochem Biophys Res Commun, 2004; 314: 186-191.

133. Zhang X. D., Gillespie S. K., Borrow J. M. and Hersey P. The histone deacetylase inhibitor suberic bishydroxamate: a potential sensitizer of melanoma to TNF-related apoptosis-inducing ligand (TRAIL) induced apoptosis. Biochem Pharmacol, 2003; 66: 1537-1545.

134. Nakata S., Yoshida T., Horinaka M., Shiraishi T., Wakada M. and Sakai T. Histone deacetylase inhibitors upregulate death receptor 5/TRAIL-R2 and sensitize apoptosis induced by TRAIL/APO2-L in human malignant tumor cells. Oncogene, 2004; 23:6261-6271.

135. Chopin V., Slomianny C., Hondermarck H. and Le Bourhis X. Synergistic induction of apoptosis in breast cancer cells by cotreatment with butyrate and TNF-alpha, TRAIL, or anti-Fas agonist antibody involves enhancement of death receptors' signaling and requires P21(waf1). Exp Cell Res, 2004; 298: 560-573.

136. Rahmani M., Yu C., Dai Y., Reese E., Ahmed W., Dent P. and Grant S. Coadministration of the heat shock protein 90 antagonist 17-allylamino- 17-demethoxygeldanamycin with suberoylanilide hydroxamic acid or sodium butyrate synergistically induces apoptosis in human leukemia cells. Cancer Res, 2003; 63: 8420-8427.

137. Vrana J. A., Decker R. H., Johnson C. R., Wang Z., Jarvis W. D., Richon V. M., Ehinger M., Fisher P. B. and Grant S. Induction of apoptosis in U937 human leukemia cells by suberoylanilide hydroxamic acid (SAHA) proceeds through pathways that are regulated by Bcl-2/Bcl-XL, c-Jun, and p21CIP1, but independent of p53. Oncogene, 1999; 18: 7016-7025.

138. Kelly W. K., Richon V. M., O'Connor O., Curley T., MacGregor-Curtelli B., Tong W., Klang M., Schwartz L., Richardson S., Rosa E., Drobnjak M., Cordon-Cordo C., Chiao J. H., Rifkind R., Marks P. A. and Scher H. Phase I Clinical Trial of Histone Deacetylase Inhibitor: Suberoylanilide Hydroxamic Acid Administered Intravenously. Clin Cancer Res, 2003; 9: 3578-3588.

139. Duvic M., Rakhshandra T., Chiao J. and Chiao N. Phase II trial of oral suberoylanilide hydroxamic acid (SAHA) for cutaneous T-cell lymphoma (CTCL) and peripheral T-cell lymphoma (PTCL). Proc Amer Soc of Hem, 2003.

140. Sawa H., Murakami H., Kumagai M., Nakasato M., Yamauchi S., Matsuyama N., Tamura Y., Satone A., Ide W., Hashimoto I. and Kamada H. Histone deacetylase inhibitor, FK228, induces apoptosis and suppresses cell proliferation of human glioblastoma cells in vitro and in vivo. Acta Neuropathol (Berl), 2004; 107: 523-531.

141. Sandor V., Bakke S., Robey R. W., Kang M. H., Blagosklonny M. V., Bender J., Brooks R., Piekarz R. L., Tucker E., Figg W. D., Chan K. K., Goldspiel B., Fojo A. T., Balcerzak S. P. and Bates S. E. Phase I trial of the histone deacetylase inhibitor, depsipeptide (FR901228, NSC 630176), in patients with refractory neoplasms. Clin Cancer Res, 2002; 8: 718-728.

142. Piekarz R. and Bates S. A review of depsipeptide and other histone deacetylase inhibitors in clinical trials. Curr Pharm Des, 2004; 10: 2289-2298.

143. Ungerstedt J., Sowa Y, Xu W-S, Shao Y., Perez G., Ngo L., Holmgren A., Jiang X., Marks P. A. Role of thioredoxin in the response of normal and transformed cells to histone deacetylase inhibitor. Proc. Natl. Acad. Sci. USA, 2005; 102:673-678.

144. Romanski A., Bacic B., Bug G., Pfeifer H., Gul H., Remiszewski S., Hoelzer D., Atadja P., Ruthardt M. and Ottmann O. G. Use of a novel histone deacetylase inhibitor to induce apoptosis in cell lines of acute lymphoblastic leukemia. Haematologica, 2004; 89: 419-426.
145. Lucas D. M., Davis M. E., Parthun M. R., Mone A. P., Kitada S., Cunningham K. D., Flax E. L., Wickham J., Reed J. C., Byrd J. C. and Grever M. R. The histone deacetylase inhibitor MS-275 induces caspase-dependent apoptosis in B-cell chronic lymphocytic leukemia cells. Leukemia, 2004; 18: 1207-1214.
146. Peart M. J., Tainton K. M., Ruefli A. A., Dear A. E., Sedelies K. A., O'Reilly L. A., Waterhouse N. J., Trapani J. A. and Johnstone R. W. Novel mechanisms of apoptosis induced by histone deacetylase inhibitors. Cancer Res, 2003; 63: 4460-4471.
147. Glick R. D., Swendeman S. L., Coffey D. C., Rifkind R. A., Marks P. A., Richon V. M. and La Quaglia M. P. Hybrid Polar Histone Deacetylase Inhibitor Induces Apoptosis and CD95/CD95 Ligand Expression in Human Neuroblastoma. Cancer Res, 1999; 59: 4392-4399.
148. Butler L. M., Agus D. B., Scher H. I., Higgins B., Rose A., Cordon-Cardo C., Thaler H. T., Rifkind R. A., Marks P. A. and Richon V. M. Suberoylanilide hydroxamic acid, an inhibitor of histone deacetylase, suppresses the growth of prostate cancer cells in vitro and in vivo. Cancer Res, 2000; 60: 5165-5170.
149. Kim J. S., Lee S., Lee T., Lee Y. W. and Trepel J. B. Transcriptional activation of p21(WAF1/CIP1) by apicidin, a novel histone deacetylase inhibitor. Biochem Biophys Res Commun, 2001; 281: 866-871.
150. Kwon H. J., Kim M. S., Kim M. J., Nakajima H., and Kim K. W. Histone deacetylase inhibitor FK228 inhibits tumor angiogenesis. Int J Cancer, 2002; 97: 290-296.
151. Deroanne C. F., Bonjean K., Servotte S., Devy L., Colige A., Clausse N., Blacher S., Verdin E., Foidart J. M., Nusgens B. V. and Castronovo V. Histone deacetylases inhibitors as anti-angiogenic agents altering vascular endothelial growth factor signaling. Oncogene, 2002; 21: 427-436.
152. Kim S. H., Ahn S., Han J. W., Lee H. W., Lee H. Y., Lee Y. W., Kim M. R., Kim K. W., Kim W. B. and Hong S. Apicidin is a histone deacetylase inhibitor with anti-invasive and anti-angiogenic potentials. Biochem Biophys Res Commun, 2004; 315: 964-970.
153. Zgouras D., Becker U., Loitsch S. and Stein J. Modulation of angiogenesis-related protein synthesis by valproic acid. Biochem Biophys Res Commun, 2004; 316: 693-697.
154. Thibault A., Cooper M. R., Figg W. D., Venzon D. J., Sartor A. O., Tompkins A. C., Weinberger M. S., Headlee D. J., McCall N. A., Samid D. and et al. A phase I and pharmacokinetic study of intravenous phenylacetate in patients with cancer. Cancer Res, 1994; 54: 1690-1694, 1994.
155. Thibault A., Samid D., Cooper M. R., Figg W. D., Tompkins A. C., Patronas N., Headlee D. J., Kohler D. R., Venzon D. J. and Myers C. E. Phase I study of phenylacetate administered twice daily to patients with cancer. Cancer, 1995; 75: 2932-2938.
156. Chang S. M., Kuhn J. G., Robins H. I., Schold S. C., Spence A. M., Berger M. S., Mehta M. P., Bozik M. E., Pollack I., Schiff D., Gilbert M., Rankin C. and Prados M. D. Phase II study of phenylacetate in patients with recurrent malignant glioma: a North American Brain Tumor Consortium report. J Clin Oncol, 1999; 17: 984-990.
157. Carducci M. A., Gilbert J., Bowling M. K., Noe D., Eisenberger M. A., Sinibaldi V., Zabelina Y., Chen T. L., Grochow L. B. and Donehower R. C. A Phase I clinical and

pharmacological evaluation of sodium phenylbutyrate on an 120-h infusion schedule. Clin Cancer Res, 2001; 7: 3047-3055.

158. Gore S. D., Weng L. J., Zhai S., Figg W. D., Donehower R. C., Dover G. J., Grever M., Griffin C. A., Grochow L. B., Rowinsky E. K., Zabalena Y., Hawkins A. L., Burks K. and Miller C. B. Impact of the putative differentiating agent sodium phenylbutyrate on myelodysplastic syndromes and acute myeloid leukemia. Clin Cancer Res, 2001; 7: 2330-2339.

159. Gore S. D., Weng L. J., Figg W. D., Zhai S., Donehower R. C., Dover G., Grever M. R., Griffin C., Grochow L. B., Hawkins A., Burks K., Zabelena Y. and Miller C. B. Impact of prolonged infusions of the putative differentiating agent sodium phenylbutyrate on myelodysplastic syndromes and acute myeloid leukemia. Clin Cancer Res, 2002; 8: 963-970.

160. Gilbert J., Baker S. D., Bowling M. K., Grochow L., Figg W. D., Zabelina Y., Donehower R. C. and Carducci M. A. A phase I dose escalation and bioavailability study of oral sodium phenylbutyrate in patients with refractory solid tumor malignancies. Clin Cancer Res, 2001; 7: 2292-2300.

161. Warrell R. P., Jr., He L. Z., Richon V., Calleja E. and Pandolfi P. P. Therapeutic targeting of transcription in acute promyelocytic leukemia by use of an inhibitor of histone deacetylase. J Natl Cancer Inst, 1998; 90: 1621-1625.

162. Reid T., Valone F., Lipera W., Irwin D., Paroly W., Natale R., Sreedharan S., Keer H., Lum B., Scappaticci F. and Bhatnagar A. Phase II trial of the histone deacetylase inhibitor pivaloyloxymethyl butyrate (Pivanex, AN-9) in advanced non-small cell lung cancer. Lung Cancer, 2004; 45: 381-386.

163. Atmaca A., Maurer A. B., Heinzel T., Gottlicher M., Neumann A., Al-Bartran S., Martin E., Bartsch I., Knuth A., Jaeger E. and Nordwest K. A dose escalating phase I study with valproic acid (VPA) in patients with advanced cancer. Proc Am Soc Clin Onc, 2004; 22: 3169.

164. Marshall J. L., Rizvi N., Kauh J., Dahut W., Figuera M., Kang M. H., Figg W. D., Wainer I., Chaissang C., Li M. Z. and Hawkins M. J. A phase I trial of depsipeptide (FR901228) in patients with advanced cancer. J Exp Ther Oncol, 2002; 2: 325-332.

165. Piekarz R., Frye R. A., Turner M. L., Wright J., Leonard J., Allen S. and Bates S. Update on the phase II trial and correlative studies of depsipeptide in patients with cutaneous T-cell lymphoma and relapsed peripheral T-cell lymphoma. Proc Am Soc Clin Onc, 2004; 22: 3028.

166. Byrd J., Marcucci G., Parthun M., Xiao J., Klisovic R. B., Moran M., Lin T., Liu S., Sklenar A. R., David M. E., Lucas D. M., Fischer B., Shank R., Tejaswi S. L., Binkley P., Wright J., Chan K. K. and Grever M. A phase I and pharmacodynamic study of depsipeptide (FK228) in chronic lymphocytic leukemia and acute myeloid leukemia. Blood, 2005; 105: 959-967.

167. Kelly W. K., O'Connor O., Richon V., Curley T., Richardson S., Thapi D., Chiao J., Rifkind R., Marks P. and Scher H. Phase I clinical trial of an oral histone deacetylase inhibitor: suberoylanilide hyroxamic acid (SAHA). 15th AACR-NCI-EORTC International Conference: Molecular Targets and Cancer Therapeutics, Boston MA 226, 2003.

168. Garcia-Manero G., Issa J. P., Cortes J., Koller C., O'Brien S., Estey E., Canalli A., Chiao J., Richon V. and Kantarjian H. Phase I study of oral suberoylanilide lydroxamic acid (SAHA) a histone deacetylase inhibitor. Proc Amer Soc Clin Oncol, 2004; 22: 3027.

169. Blumenschein G., Lu C., Kies M., Glisson B., Papadimitrakopoulou V., Zinner R., Kim E., Gillenwater A., Chiao J. and Hong W. Phase II clinical trial of

suberoylanilide hydroxamic acid (SAHA) in patients with recurrent and/or metastatic head and neck cancer. Proc Amer Soc Clin Oncol, 2004; 22: 5578.

170. Ottmann O. G., Deangelo D. J., Stone R., Preifer H., Lowenberg B., Atadja P., Peng B., Scott J., Dugan M., Sonneveld P. and Wolfgang J. A. A phase I pharmacokinetic and pharmacodynamic study of a novel histone deacetylase inhibitor LAQ824 in patients with hematologic malignancies. Proc Amer Soc Clin Oncol, 2004; 22: 3024.
171. Rowinsky E. K., Pacey S., Patnaik A., O'Donnell A., Mita M., Atadja P., Peng B., Dugan M., Scott J. W. and DeBono J. S. A phase I pharmacokinetic and Pharmacodynamic study of a novel histone deacetylase (HDAC) inhibitor LAQ824 in patients with advanced solid tumors. Proc Amer Soc Clin Oncol, 2004; 22: 3022.
172. Beck J., Fischer T., Rowinsky E. K., Huber C., Mita M., Atadja P., Peng B., Kwong C., Dugan M. and Patnaik A. Phase I pharmacokinetic and pharmacodynamic study of LBH589A: A histone deacetylase inhibitor. Proc Amer Soc Clin Oncol, 2004; 22: 3025.
173. Ryan Q. C., Headlee D. J., Sparreboom A., Figg W. D., Zhai S., Trepel J. B., Murgo A., Elsayed Y., Karp J. E. and Sausville E. A. A phase I trial of an oral histone deacetylase inhibitor, MS-275, in advanced solid tumor and lymphoma patients. Proc Amer Soc Clin Oncol, 2003; 22:200.
174. O'Shaughnessy J., Flaherty L., Fiorica J. and Grove W. Phase II trial of CI-994 in patients with metastatic renal cell carcinoma. Proc Amer Soc Clin Oncol, 1999.
175. Kimmel K. A., Kindler H. L., Janisch S., Olson E. N. and Grove W. Phase I dose-finding study of CI-994 in combination with capecitabine in patients with advanced solid tumors. Proc Amer Soc Clin Oncol, 87a, 2001.
176. Ardizzoni A. and Loprevite M. Histone deacetylation inhibitors. Suppl Tumori, 2002; 1: S52-54.
177. Wozniak A., O'Shaughnessy J., Fiorica J. and Grove W. Phase II trial of CI-994 in patients with advanced nonsmall cell lung cancer. Proc Amer Soc Clin Oncol, 1999; 487a.

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