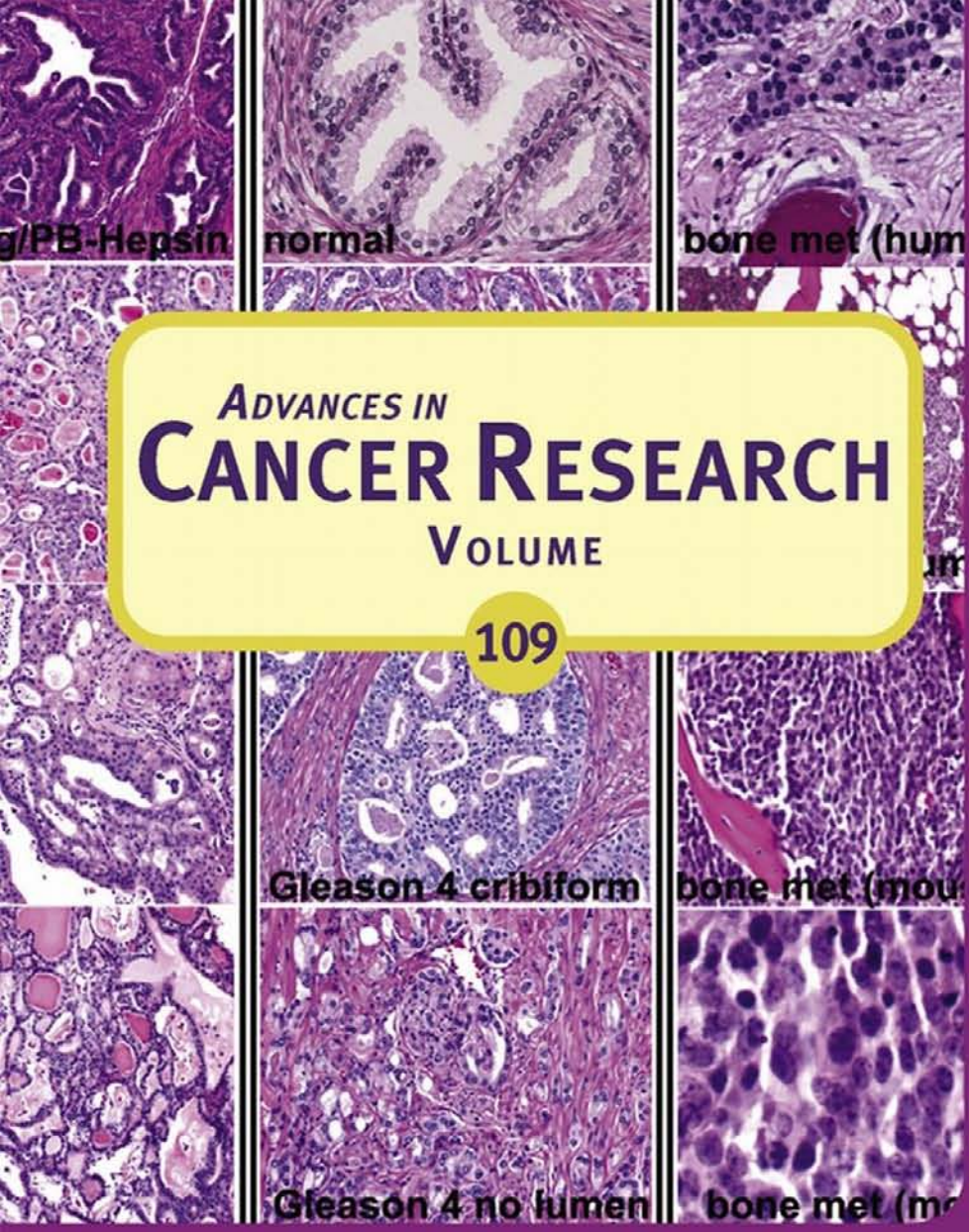

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Mechanisms of Prostate Cancer Initiation and Progression

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Prostate cancer is a major health problem as it continues to be the most frequently diagnosed cancer in men in the Western world. While improved early detection significantly decreased mortality, prostate cancer still remains the second leading cause of cancer-related death in Western men. Understanding the mechanisms of prostate cancer initiation and progression should have a significant impact on development of novel therapeutic approaches that can help to combat this disease. The recent explosion of novel high-throughput genetic technologies together with studies in animal models and human tissues allowed a comprehensive analysis and functional validation of the molecular changes. This chapter will summarize and discuss recently identified critical genetic and epigenetic changes that drive prostate cancer initiation and progression. These discoveries should help concentrate the efforts of drug development on key pathways and molecules, and finally translate the knowledge that is gained from mechanistic studies into effective treatments.

I. EPIDEMIOLOGIC STUDIES IN PROSTATE CANCER

A. Incidence and Prevalence

Prostate cancer is the most common nonskin cancer in men in the Western world. Age is by far the most significant risk factor for development of prostate cancer, with a prevalence of approximately 35% in 60–69-year-old and 46% in 70–81-year-old men (Yin *et al.*, 2008). Incidence of prostate cancer is 1.56 times higher in the Black compared to the Caucasian population. In 2007, there were 2.2 million men alive in the USA who had a diagnosis of prostate cancer (Altekruse *et al.*, 2009). While early detection of prostate cancer significantly improved with the prostate-specific antigen (PSA) screening test and prostate cancer deaths decreased (Etzioni *et al.*, 2008b), the false-positive rate of the test is high and the screening is not cost-effective (Etzioni *et al.*, 2008a). High-grade cancers can cause deceivingly low serum PSA levels and may be missed by the guidelines PSA concentrations that typically prompt prostate biopsies. While in the majority of cases, prostate cancer is treated as a chronic disease, approximately one-third of patients with serological relapse detected by an increase in serum levels of PSA progress to develop symptomatic metastatic disease. While there are several lines of therapy, the available drugs eventually fail and prostate cancer still ranks second among cancer-related causes

of death in U.S. men (Altekruse *et al.*, 2009). Understanding the mechanisms of prostate cancer should help to develop novel targeted approaches to combat this devastating disease.

B. Role of the Environment

The first realization that prostate cancer is triggered by environmental factors occurred when epidemiologic studies demonstrated a vastly lower cancer incidence in Asian countries, compared to the USA and Europe (Zeigler-Johnson *et al.*, 2008). However, Asian-Americans born in the USA experience a much higher prostate cancer prevalence, suggesting that the development of prostate cancer is affected by environmental factors (Gong *et al.*, 2002; Moyad, 1999). Major differences between Asian countries and the USA are diet and lifestyle. Epidemiological studies over many years demonstrated that diets rich in soy and vegetables, in particular, cruciferous vegetables (Kristal and Lampe, 2002), tomatoes (van Breemen and Pajkovic, 2008) and garlic, red wine, and green tea attenuate prostate cancer susceptibility (Thompson, 2007), while meat, fat, and dairy products increase risk (Chan *et al.*, 2005). In addition, obesity and stress increase prostate cancer incidence and grade (Fesinmeyer *et al.*, 2009). To understand the molecular basis of results from epidemiologic studies, various serum and plasma proteins, hormone levels of sex steroids and steroid binding proteins, and genetic polymorphisms have been analyzed in large cohorts of individuals.

The main mechanism by which diet affects prostate cancer development is mediated by a long lasting reduction in androgen stimulation and by a decrease in oxidative stress (Bostwick *et al.*, 2004). The two mechanisms are connected, since oxidative stress through the Nrf2 transcription factor, which binds to antioxidant response elements in the promoter regions of phase-II detoxifying genes, strongly triggers expression of (aldo-ketoreductase family 1 member C1) AKRC1 and AKRC2, the two major dihydrotestosterone (DHT) catabolizing enzymes (Lou *et al.*, 2006; Wang *et al.*, 2006b). Thus, the antioxidant response of cells in the prostate can lead to a decrease in tissue DHT levels and a lower overall activity of the androgen axis. Another bidirectional interaction between oxidative stress and the androgen axis occurs between the regulation of activity of the androgen receptor (AR) and the generation of reactive oxygen species (ROS) (Gong *et al.*, 2009; Mehraein-Ghomi *et al.*, 2010; Pinthus *et al.*, 2007; Sharifi *et al.*, 2008; Shigemura *et al.*, 2007; Veeramani *et al.*, 2008). Thus, a few of the molecular causes for the dietary effects on prostate cancer reduction have been elucidated and many others are being extensively studied.

C. Genetic Predisposition

The inherited genetic background is a significant risk factor for the development of prostate cancer. Inheritance of high-penetrance genetic "risk" alleles in hereditary prostate cancer (HPC) can increase the incidence of prostate cancer by several folds, while more common low-penetrance loci increase the risk only modestly. HPC is characterized by early onset disease (<60 years old) with two or three first-degree relatives affected at a young age and causes a 5- and 11-fold increased risk of prostate cancer. Genes involved in HPC have been identified by positional cloning in affected families.

The HPC gene (*HPC1/RNASEL*) encodes the enzyme ribonuclease L and localizes to chromosome 1q24-25 (Chen *et al*., 2003). RNASEL is involved in the innate immune defense mechanisms and the interferon-mediated signaling, which lowers cell proliferation (Malathi *et al*., 2007). RNASEL reduces antiviral activity and plays an important role in regulation of apoptotic cell death (Zhou *et al*., 1997). The relevance of antiviral defenses to prostate cancer development became more obvious after the recent discovery of a retrovirus in the samples of human prostate tumors in patients with RNASEL mutations (Urisman *et al*., 2006). Sporadic prostate cancers also revealed retroviral infections highlighting the potential role of chronic retroviral infection in inflammation and cancer initiation (Eeles *et al*., 2008; Schlaberg *et al*., 2009). Since retroviral infection is a very exciting potential etiology of prostate cancer, it will be certainly investigated in great details in the near future.

Another HPC gene (*HPC2/ELAC2*) resides on chromosome 17p11 and encodes a protein with poorly understood function (Camp and Tavtigian, 2002). ELAC2 binds SMAD2 and may be involved in prostate cancer via its role in regulation of TGF-beta signaling pathway (Noda *et al*., 2006). *MSR1* (macrophage scavenger receptor 1) is the third identified HPC gene and it resides on chromosome 8p22 (Xu *et al*., 2002). However, the low penetrance of this allele resulted in several studies, which were unable to confirm its association with HPC (Maier *et al*., 2006; Wang *et al*., 2003a).

The X chromosome is believed to play an important role in prostate cancer inheritance, because it contains the AR and because small deletions in hereditary and sporadic prostate cancer in the Xq26.3–q27.3 region were noted (Xu *et al*., 1998). This region contains the genetic locus known as *HPCX*, which explains 16% of HPC cases (Bergthorsson *et al*., 2000; Schleutker *et al*., 2000).

An alternative approach to positional cloning for gene discovery of HPC has been the analysis of known cancer susceptibility genes, such as *BRCA1*, *BRCA2*, and several DNA mismatch repair genes. A subset of HPC was found to occur in individuals with *BRCA1* and *BRCA2* mutations.

In *BRCA2* mutation carriers, prostate cancer incidence is increased and cancers with mutant *BRCA1* and *BRCA2* are clinically more aggressive (Gallagher *et al*., 2010). Individuals with mutations in DNA mismatch repair genes (*MLH1*, *MLH2*, *MLH6*, and *PNS2*) have a risk of prostate cancer that is similar to *BRCA2* carriers (Grindedal *et al*., 2009). The significance of BRCA2 signaling in prostate cancer was recently highlighted by the discovery that the BRCA2-interacting protein PALB2 is involved in familial prostate cancer (Erkko *et al*., 2007). More recently, a genome wide SNP (single nucleotide polymorphism) linkage analysis in 301 HPC families revealed multiple novel loci and their significance will be evaluated in the future studies (Stanford *et al*., 2009).

While positional cloning was successful in identifying genetic causes of HPC, it is not useful in sporadic cancers because of the poor penetrance of cancer alleles. To identify low-penetrance genomic modifications that are associated with prostate cancer risk in the general population, SNPs in genes, most of them loosely associated with prostate cancer, were tested for their incidence in men with prostate cancer. In the last few years, several cohorts of thousands of men were assembled to conduct genome wide association studies (GWAS). These studies were designed with sufficient statistical power to identify genetic changes that are responsible for the development of sporadic prostate cancers and for gene–environment interactions in cancer development. When combined with family history, the predictive strength of cancer risk-associated SNPs increases substantially (Xu *et al*., 2009; Zheng *et al*., 2008). GWAS and other SNP studies resulted in an accumulation of massive amounts of data linking prostate cancer risk to genes and pathways.

The androgen pathway is one of the most important signaling mechanisms involved in prostate cancer. There is a significant association between prostate cancer risk and SNPs in the genes encoding enzymes involved in the synthesis of testosterone and DHT: hydroxysteroid (17-beta) dehydrogenase-1, hydroxy-delta-5-steroid dehydrogenase (Chang *et al*., 2002; Kraft *et al*., 2005; Margiotti *et al*., 2002; Suzuki *et al*., 2005), 5α-reductase-1 (Setlur *et al*., 2010) and -2 (Chang *et al*., 2003; Lindstrom *et al*., 2006; Loukola *et al*., 2004), and CYP17, CYP3A4, CYP19A1 (Sarma *et al*., 2008). There is also association between prostate cancer and the variants of androgen responsive genes—kallikrein family, hK2 and PSA (Cramer *et al*., 2008; Klein *et al*., 2010; Nam *et al*., 2006), and microseminoprotein (Beuten *et al*., 2009; Chang *et al*., 2009; Lou *et al*., 2009), as well as genes involved in estrogen receptor signaling—estrogen receptors α (Hernandez *et al*., 2006) and β (Chen *et al*., 2007; Thellenberg-Karlsson *et al*., 2006). While in most cases, the exact functional role of identified SNP variants is not known, it is thought that many of them are associated with differences in the levels of gene expression or function, which may influence prostate cancer incidence.

There is a strong link between prostate cancer and inflammation. Chronic inflammation causes proliferative inflammatory atrophy (PIA) (De Marzo *et al.*, 2007), which in 30% of cases progresses to the precursor of prostate cancer known as prostatic intraepithelial neoplasia (PIN) (De Marzo *et al.*, 2003). Therefore, it is perhaps not surprising that SNPs in genes involved in inflammation such as cyclooxygenase *(COX-2)* (Danforth *et al.*, 2008a; Fernandez *et al.*, 2008), *interleukin-1* (*IL-1*) (Zabaleta *et al.*, 2009), *IL-6* (Bao *et al.*, 2008; Pierce *et al.*, 2009), *IL-8* (Zabaleta *et al.*, 2009), and *IL-10* (Wang *et al.*, 2009b; Zabaleta *et al.*, 2009), tumor necrosis *factor-α* (*TNF-α*) (Danforth *et al.*, 2008b), and toll-like *receptor-4* (*TLR4*) (Chen *et al.*, 2005a; Song *et al.*, 2009; Stark *et al.*, 2009; Wang *et al.*, 2009b) demonstrated an association with prostate cancer risk.

SNPs in a number of transcription factors or chromatin remodeling enzymes also display an association with prostate cancer. For example, a SNP in the fragile histidine triad gene (*FHIT*), a tumor-suppressor gene involved in the development of the kidney and pancreas, is associated with prostate cancer (Ding *et al.*, 2008; Larson *et al.*, 2005). SNPs associated with prostate cancer risk were also detected in the homeobox B gene, *HNF1B* (Sun *et al.*, 2008b); *JAZF1*, a transcriptional repressor (Thomas *et al.*, 2008); *Mel-18/Bmi*, a chromatin modifying gene (Wang *et al.*, 2009d); and in *NR1H2*, which dimerizes with the retinoid acid receptor and is associated with an aggressive type of prostate cancer (Hooker *et al.*, 2008). The vitamin D receptor is a transcription factor, which stimulates differentiation of prostate epithelial cells, and SNPs that diminish its activity were associated with prostate cancer risk (Ahn *et al.*, 2009; Kidd *et al.*, 2005; McKay *et al.*, 2009; Moon *et al.*, 2006).

With the improvement of high-throughput genotyping, several GWAS studies have provided confirmatory results for cancer susceptibility loci in multiple populations and have had an important contribution to prostate cancer risk prediction. Genetic variants on chromosomes 3, 6, 7 (*JAZ1*), 8 (*c-MYC*), 10 (*MSMB*), 11, 17 (*HNF1B*), 19, and X were associated with prostate cancer risk in PRACTICAL, a worldwide consortium of 13 groups evaluating the most significant SNP (Kote-Jarai *et al.*, 2008). The results from this study supported a multiplicative risk model, which in combination with previously reported SNPs on 8q and 17q explained 16% of familial risk in a high-risk population.

Compelling evidence demonstrates chromosome 8q24 as a susceptibility locus for prostate and several other cancer types (Eeles *et al.*, 2008; Gudmundsson *et al.*, 2009; Sun *et al.*, 2008a; Thomas *et al.*, 2008). The SNPs of the 8q24 region were rigorously tested in a case–control study of 1012 cases and controls with advanced prostate cancer. Of 10 8q24 variants, six were associated with the risk of advanced prostate cancer at *p*-values between 0.001 and 0.038. In addition, a meta-analysis of four 8q24 variants

in 10 studies demonstrated strong associations across a wide array of study designs and populations, and provided confirmation that the three 8q24 regions independently influence the risk of prostate cancer and advanced disease (Cheng *et al*., 2008). Because the 8q24 region is a gene desert, the functionality of SNPs remained unclear until recently long-range interactions of enhancers in 8q24 showed effects on *c-MYC* expression and regulation of the activity of the Wnt pathway (Nielsen *et al*., 1991; Tuupanen *et al*., 2009; Wright *et al*., 2010).

Despite the accumulation of massive amounts of data, which were acquired to understand the genetics of prostate cancer risk, the knowledge gained has not informed about the molecular mechanisms that drive development and progression of prostate cancer. In contrast, significant information has been obtained from the analysis of human prostate cancers and through discovery of genomic, transcriptomic, and protein expression changes. We will now review the knowledge about the morphological and molecular changes that take place during prostate cancer initiation and progression.

II. THE ARCHITECTURE OF THE NORMAL PROSTATE GLAND

The prostate, like all other glandular organs, consists of an epithelial and a stromal compartment, which contain multiple cell types (Fig. 1). It is impossible to understand the cellular mechanisms of prostate cancer without knowing the origin and physiological function of these cells or without a clear idea about the coordination of cellular activities that forms and maintains the entire organ. Significant information about the development and homeostasis of the prostate gland was obtained using wild-type and mutant model organisms, especially mice. While there are important similarities between mouse and human prostates, there are also some differences, and understanding these similarities and differences is important for any prostate cancer investigator.

A. Comparison of Mouse and Human Prostates

In both humans and mice, the prostate is a glandular organ situated at the neck of the bladder. As all exocrine glands, the prostate produces secretions, which are generated in acinar cells and are channeled through the tree-like system of ducts to the urethra. During ejaculation, smooth muscle cell contraction squeezes prostatic secretions into the urethra, where they mix

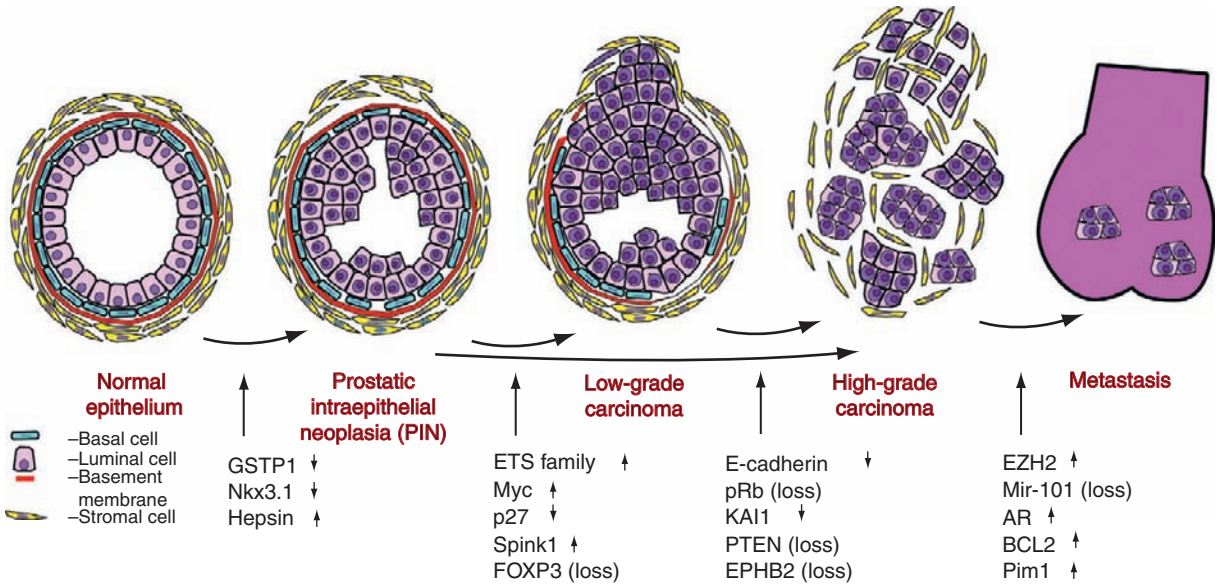

Fig. 1 Model of prostate cancer progression. Histological changes and concomitant genetic and epigenetic events during prostate cancer initiation and progression. The deletion or inactivating mutation in tumor-suppressor genes are denoted as (loss). Overexpression of a gene is shown with an arrow pointing up, while downregulation of expression is shown with an arrow pointing down. Only initial changes in the expression levels are shown. The up- or downregulation of expression may persist at more advanced stages.

with semen from the seminal vesicles. Prostate secretions liquefy the ejaculate and increase the survival of the sperm.

The rodent prostate contains four distinct lobes (dorsal, ventral, lateral, and anterior lobes), which encircle the urethra and are named according to their anatomical location (Hayashi *et al*., 1991; Sugimura *et al*., 1986a) (Fig. 2A). Unlike the rodent prostate, the human prostate gland does not have an obvious lobular organization; however, it does have a distinct zone-specific architecture and compartmentalization, with prostate tumors usually originating in the peripheral zone. The relationships between different murine lobes and human prostate remain unclear. While some investigators believe that the dorsal lobe of the mouse prostate is most similar to the human prostate, the glandular architecture in the murine dorsal lobe is most histologically distinct from the human prostate as epithelial cells often fill and obliterate the lumen of prostate acini.

Both human and rodent prostates consist of two tissue compartments: epithelial and stromal (Fig. 1). Stromal and epithelial compartments in the prostate gland are separated by the basement membrane, a packed structure of collagen fibers containing various extracellular matrix proteins produced by both epithelial (laminins) and stromal cells (collagens) (Bonkhoff *et al*., 1991a, 1992). The epithelium consists of two cell layers. The luminal cell layer is formed by polarized columnar luminal epithelial cells, which produce prostatic secretions. A distinct basal epithelial cell layer separates the luminal cells from the stroma (Brawer *et al*., 1985; Liu *et al*., 1997; Nagle *et al*., 1987; van Leenders and Schalken, 2003). Basal and luminal cells also express different cell-type-specific proteins, which are often used to analyze the gland and to detect glandular pathologies. For example, luminal cells express high levels of AR, low molecular weight cytokeratins 8/18, CD57, and the homeobox domain transcription factor Nkx3.1. In contrast, basal cells express high molecular weight cytokeratins 5/14, p63, CD44, GSTP1, and much lower levels of AR. While in the human prostate, a cellular basal cell layer completely separates the luminal cells from the stroma, this cell layer is much more sparsely populated in rodents with flattened basal cells that extend a thin layer of cytoplasm between the basement membrane and the luminal cell layer. Because the thin layer of cytoplasm is difficult to detect by immunofluorescent staining, it is predominantly thought that the basal cell layer is discontinuous in the rodent prostate (El-Alfy *et al*., 2000). In contrast to mouse, human basal cells become hyperplastic under conditions of androgen suppression, estrogen access, and stromal fibrosis. In addition to luminal and basal cell layers, the human prostate epithelium contains a compartment of transiently amplifying, "intermediate cells," which express markers of both basal and luminal epithelial cells (Verhagen *et al*., 1988). These cells are enriched in PIA, express the c-MET receptor, low AR, and may represent the daughters of the basal cells, which initiate a

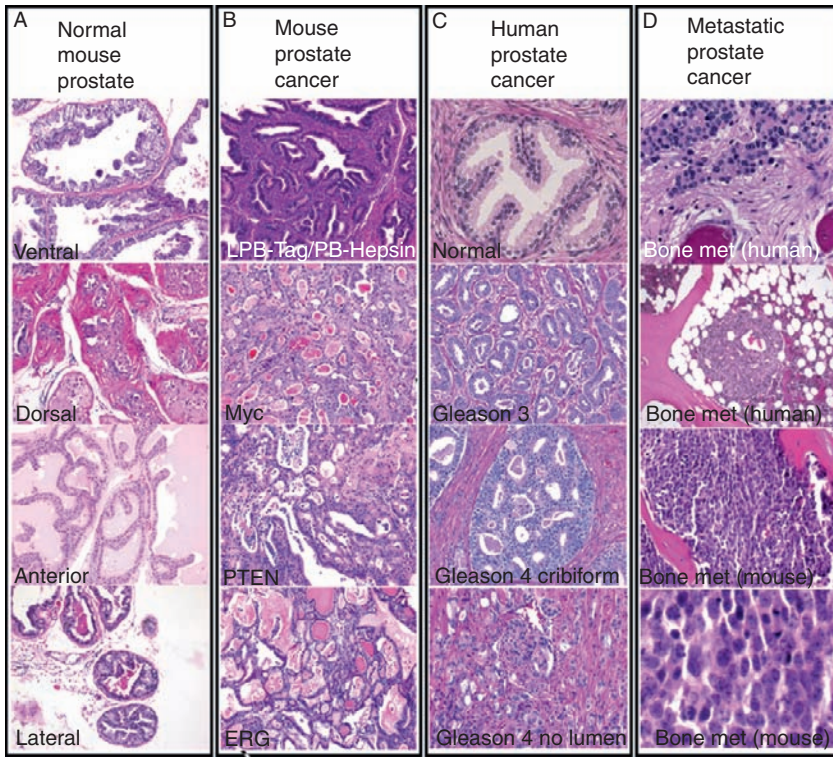

Fig. 2 Histology of human and mouse prostate cancer. (A) Ventral, dorsal, anterior, and lateral lobes of adult mouse prostate. (B) Distinct histologic appearances of primary prostate tumors from different mouse models of prostate cancer. LPB-Tag/PB-Hepsin, transgenic mice expressing *SV-40 T antigen* and *Hepsin* in prostate epithelium (Klezovitch *et al*., 2004). Myc, transgenic mice expressing *c-Myc* in prostate epithelium (Ellwood-Yen *et al*., 2003). PTEN, conditional mutant mice with prostate epithelium-specific deletion of *PTEN* (Wang *et al*., 2003b). ERG, transgenic mice expressing ERG in prostate epithelium (Klezovitch *et al*., 2004). (C) Histology of human prostate. Normal prostate, Gleason Grade 3, Gleason Grade 4 showing a cribiform pattern, Gleason Grade 4 showing glands with a limited ability to form a lumen and invasion into the prostate stroma. (D) Histology of prostate cancer bone metastasis from human and mouse (LPB-Tag/PB-Hepsin; Klezovitch *et al*., 2004) prostate cancer. H&E stainings were performed by Liem Nguyen and the images were taken by Aviva Ventura.

differentiation program and are in the process of becoming luminal cells (van Leenders *et al*., 2000).

In addition, to epithelial cells, the epithelial cell layer also contains rare postmitotic neuroendocrine cells, which produce and secrete various neuropeptides and growth factors necessary for growth of luminal cells (Bonkhoff *et al*., 1991b, 1994, 1995). Synaptophysin and chromogranin A are two widely used markers of prostatic neuroendocrine cells.

The stromal cell compartment of the prostate gland contains several different cell types. Smooth muscle cells represent the most abundant cell type in the stroma (Bartsch *et al*., 1979). In addition, the stromal layer contains myofibroblasts, which form a circumferential cell layer around glands. Interspersed between the mesenchymal cells are endothelial cells of blood vessels and lymphatics, cells of the nerve sheet, and inflammatory cells. As in all glandular organs, the epithelial cells of the prostate require close proximity to the stromal cells and blood vessels for their survival. Approximately 50% of stromal cells as well as prostate endothelial cells and neuronal cells express ARs. In response to androgen, stromal cells secrete "andromedins," which are factors that promote the proliferation, survival, and differentiation of epithelial cells. Androgen suppression or inflammation results in stromal fibrosis, which leads to the atrophy of epithelial cells (Cunha, 1973). There is a significant difference in the architecture and abundance of stroma in human and mouse prostates. The human prostate has a much higher stromal-to-epithelial ratio. In the rodent prostate, stromal cells form a thin layer around branching epithelial tubes. This results in a distinct gland morphology, where the prostate gland contains a loosely assembled collection of glandular ducts and each duct contains its own epithelial and stromal cell compartment (Sugimura *et al*., 1986a).

III. DEVELOPMENT AND MAINTENANCE OF THE PROSTATE GLAND

The prostate starts to develop during late embryogenesis (embryonic day 17.5 in mice) as an outgrowth of epithelial buds in the urogenital sinus (Prins and Putz, 2008). Under the influence of androgen, these buds display rapid extension and branching morphogenesis resulting in the formation of typical glandular organ architecture. The initial organ outgrowth continues vigorously after birth and it is largely completed before reaching sexual maturity (8–9 weeks after birth in mice). The developing prostate is a highly proliferative tissue, with the majority of cellular divisions appearing at the tips of growing prostatic ducts. In contrast, in the adult, the gland becomes largely quiescent, with very few cells showing either mitotic cell divisions or apoptotic cell death (Bhatia-Gaur *et al*., 1999).

The male hormone, testosterone, plays a critical role during prostate initiation, morphogenetic development, and maintenance (Cunha, 1973; Lasnitzki and Mizuno, 1977). AR signaling is active in both epithelial and stromal cell compartments. During early prostate morphogenesis, AR signaling in the mesenchyme is especially critical. This finding was made using tissue recombination experiments in which purified epithelial and

mesenchymal cells from the embryonic urogenital sinus were mixed and transplanted under the capsule of a recipient adult nude mice, where they formed prostate tissue (Cunha *et al*., 1987, 1995; Hayward *et al*., 1997). Tissue recombination experiments with mesenchymal cells lacking the AR and wild-type epithelial cells failed to produce prostate tissue (Cunha *et al*., 1995). Androgen signaling in epithelial cells is also important because reciprocal tissue recombination experiment of wild-type mesenchymal cells and AR-mutant epithelial cells resulted in formation of prostate tissue; however, the epithelial cells failed to differentiate (Cunha and Young, 1991; Donjacour and Cunha, 1993).

In adult prostate, the loss of androgen signaling upon castration results in rapid and dramatic death of the majority of luminal epithelial cells, which express high levels of AR and require androgen signaling for their survival (English *et al*., 1987; Evans and Chandler, 1987). Subsequently, the prostate gland regresses with only a fraction of cells at the proximal region of the prostate surviving the testosterone depletion. Remarkably, readministration of testosterone to these castrated animals via implantation of hormone pellets results in the rapid regrowth of the prostate gland. The entire process of hormone-mediated regression and regrowth can be repeated for more than 15 cycles (Sugimura *et al*., 1986b,c; Tsujimura *et al*., 2002). This androgen-regulated cycles of regression and regrowth of the prostate gland were used to investigate the stem cell population of the mouse prostate, which is believed to be castration resistant and residing close to the urethra (Tsujimura *et al*., 2002). Historically, it was believed that stem cells reside among the basal prostate epithelial cell population, which are insensitive to androgen deprivation, and this was reinforced recently by the discovery that a single basal cell expressing c-kit (CD117), CD44, CD133, and Sca1 and negative for Lin generated an entire prostate ductal system after transplantation *in vivo* (Leong *et al*., 2008). However, the view that stem cells only reside in the basal compartment was dramatically challenged by identification of stem cells among the castration-resistant Nkx3.1-positive (CARN) luminal cells (Wang *et al*., 2009e). It is possible that there are independent stem cell populations within both the luminal and basal cell populations (Shen *et al*., 2008). Alternatively, the CARN stem cells might be "transient-amplifying" cells that can acquire stem cell characteristics through dedifferentiation (Shen *et al*., 2008).

Identifying the stem cell population(s) in the prostate gland is a very exciting task and may provide important insights concerning the origin of prostate cancer and, especially, castration-resistant prostate cancer, which may be arising from androgen-independent stem cell population. Nevertheless, it is important to remember that under normal conditions, the process of stem cell self-renewal and differentiation plays only a minor role in gland homeostasis, as the majority of prostate epithelial cells are long lived and show limited cell proliferation or cell death activities.

IV. MORPHOLOGICAL AND CYTOLOGICAL CHANGES THAT TAKE PLACE DURING PROSTATE CANCER INITIATION AND PROGRESSION

Technically, prostate carcinoma involves an accumulation of cancerous epithelial cells; however, nonepithelial cell types also play a very important role in prostate cancer initiation and progression. In general, significant changes in both epithelial and stromal cell compartments take place during prostate cancer initiation and progression (Fig. 1). While normal adult prostate is largely quiescent, this can change in older individuals, leading to a hyperplastic prostate epithelium. This condition is called benign prostatic hyperplasia (BPH) and is not considered to be the precursor to prostate carcinoma (Kristal *et al.*, 2010). While the number of luminal epithelial cells increases in BPH, the columnar appearance and nuclear cytology remain normal. Up to 25% of men in their 50s and up to 90% in their 80s develop prostate hyperplasia; however, it only becomes symptomatic and requires treatment in 10% of the cases (Bushman, 2009). Drug-attenuating DHT synthesis blocks the enlargement-promoting effects of androgen and reduce the size of the prostate gland. Alpha adrenergic blockers which cause relaxation of smooth muscle are an alternative treatment (Auffenberg *et al.*, 2009).

A. Prostatic Intraepithelial Neoplasia

In contrast to BPH, high-grade PIN, which is the *in situ* carcinoma, is considered to be a precursor to invasive prostate carcinoma. While PIN was initially divided into high- and low-grade lesions, low-grade PIN is not a precursor to carcinoma and therefore receives little attention. PIN usually appears in the peripheral regions of the prostate gland and is characterized by dysplasia of prostate epithelial cells. In PIN, luminal epithelial cells accumulate and show nuclear enlargement with prominent nucleoli (Ayala and Ro, 2007). Even though the integrity of the basal cell layer decreases, basal cells are still present in PIN. As long as basal cells are present, the basement membrane is preserved in PIN lesions and it contains laminin 332 (Laminin-5), which is produced by basal cells. Cancer cells from high-grade PIN lesions can invade and generate frank prostate carcinoma.

B. Prostate Adenocarcinoma

The majority of tumors in the prostate are adenocarcinomas. Histologically, the transition from PIN lesions to adenocarcinomas is characterized by several histological changes in invasive epithelial cells with a cytokeratin

profile characteristic of luminal cells: excessive branching morphogenesis, loss of the basal cell layer, and cytologic atypia with enlargement of nuclei and nucleoli (Figs. 1 and 2). Prostate cancer is suspected based on digital rectal examination, ultrasound, and increased levels of PSA in blood plasma. The tumors are often multifocal and they usually develop in the peripheral zone of the prostate gland.

C. Gleason Grading System

As most epithelial tumors, invasive prostate cancers can be divided into low and high grades, based on their histologic features. The prostate cancer grading scheme was developed by the pathologist Donald F. Gleason and became known as the "Gleason Score" system (Gleason and Mellinger, 1974). This system relies entirely on the architectural pattern of the tumor and the overall score is determined as the sum of two most prevalent growth patterns (Fig. 3). The Gleason Score system has recently been refined to improve the consistency of grading between pathologists. In general, low-grade tumors, for example, Gleason Grade 3 and below, form well-organized ductal structures, which are surrounded by stroma. In contrast, high-grade tumors, Gleason Grades 4 and 5, possess a severely disrupted glandular architecture. High-grade cancers are indistinguishable from low grade based on cytokeratin expression. However, in contrast to low-grade cancers, high-grade cancers are either unable to form a lumen or exhibit gland fusion and cribiform morphology, suggesting that cells have developed autonomy from the stroma. The Gleason Score is the primary predictor of tumor recurrence and progression to metastatic disease. The assignment of a tertiary growth pattern which encompasses a higher grade, in addition to the two most prevalent patterns, has improved prognostic ability (Epstein, 2010) (http://pathology2.jhu.edu/gleason/patterns.cfm). While pure Gleason Score 3 cancers rarely lead to death from prostate cancer, high-grade cancers can be lethal (Miyamoto *et al*., 2009). Approximately 2–3% of patients diagnosed with prostate cancer die of their disease.

D. Multifocal Nature of Prostate Cancer

Multifocal tumor is one of the most typical features of human prostate cancer. High-grade PIN (*in situ* carcinoma) develops concurrently at multiple sites, presumably due to the presence of a field effect, which facilitates cancer initiation. Multiple studies have attempted to identify the molecular causes that underlie the field defect and that may lead to the onset of cancer, because they would provide logical targets for cancer prevention (Troyer

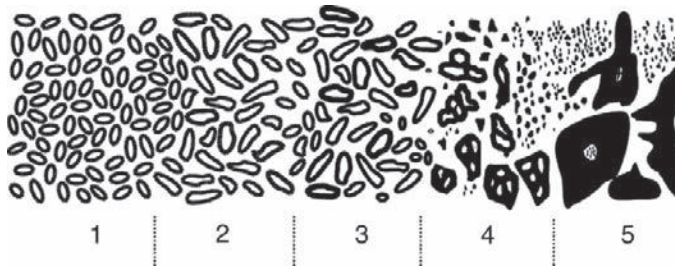

Fig. 3 Schematic diagram of the Gleason Grading system for human prostate cancer. This system is based on the architectural growth pattern and not on cytological features. It takes into account the glandular morphology of the cancer and the interaction with the tumor stroma. The Gleason system takes into account the heterogeneity of prostate tumors and calculates a sum of the two most prevalent patterns, for example, Gleason Score 3 + 4 = 7. Pattern 1—an accumulation of round glands of similar size; most Gleason 1 cancers would today be classified as adenosis and not carcinoma because of the detection of rare basal cells with the help of immunohistochemical staining. Pattern 2—a tumor mass that consists of large cancerous glands of slightly variable diameter with a sharply demarcated edge. Pattern 3—an accumulation of discrete glandular units with lumen formation of variable size and shape, each surrounded by stromal cells, with infiltration between normal glands. Pattern 4—four growth patterns: (1) fused glandular units; (2) glands with ill-defined lumen formation; (4) cribiform; (5) hypernephroid (sheets of cells that resemble renal carcinoma cells). Pattern 5—two growth patterns: (1) sheets of cancer cells, often with central necrosis; (2) single cancer cells in the stroma. Briefly, in low-grade cancers each gland forms a lumen and is surrounded by stroma, while high-grade cancers either lose the ability to form a lumen or become autonomous from the stroma. Areas of necrosis only exist in Gleason Grade 5 cancers. Sarcomatoid morphology, as has been observed in some mouse models, does not exist in human prostate cancer.

et al., 2009). The multifocality of prostate cancer hinders the accurate assessment of the tumor grade (Conti *et al.*, 2009; Dall'Era *et al.*, 2008; Wayenberg *et al.*, 1993), and in approximately 30% of patients the tumor grade is underestimated (Falzarano *et al.*, 2010). The accurate diagnosis of tumor grade is an area of intense investigation because men with truly indolent cancers can choose to delay treatment.

Whether or not prostate cancer can progress from low to high grade is controversial (Albertsen, 2010; Epstein *et al.*, 2001; Turley *et al.*, 2008). Microscopically, regions of Gleason Grades 3 and 4 growth patterns frequently coexist in the same cancer nodule and are intermixed. While it is conceivable that this grade admixture is derived from two cancer initiation events, the histologic appearance suggests that under some conditions, Gleason Grade 3 pattern can progress to Gleason Grade 4. In particular, after refining the Gleason Grading criteria, this type of potential grade progression is more frequently observed (Epstein, 2010), and may have important ramifications in identifying biomarkers of high-grade prostate cancer.

E. Cellular Origin of Prostate Cancer

The newly acquired ability to isolate and analyze different cellular populations originating from prostate cancer tissues has enabled investigators to address critical questions of prostate cancer biology: What is the cell origin of prostate cancer? Is the development of prostate cancer driven by tumor initiating cells, which are sometimes erroneously called cancer stem cells, or does prostate cancer originate in more differentiated luminal cells? What is the precise identity of the cells that can undergo oncogenic transformation? Does the luminal phenotype of prostate cancer cells strictly imply a luminal origin?

One possibility is that transformation of terminally differentiated luminal cells results in partial dedifferentiation and acquisition of immortality. An alternative possibility is that oncogenic transformation takes place in the prostate stem cells, which are believed to be residing among the basal cells. The transformed stem cells then, through partial and incomplete differentiation of their daughters, would generate proliferative cells with a luminal phenotype, constituting the bulk of the tumor. Indeed, recent reconstitution experiments demonstrated that basal but not luminal cells contain a prostate cancer initiating cell population (Lawson *et al.*, 2010). In other solid tumor types, targeting the oncogenic event to the stem cell population results in rapid and vigorous tumor formation, while tumor development is much more attenuated when the same oncogenic event is targeted to more differentiated cell population (Barker *et al.*, 2009).

A recent study challenged the concept that cells in the basal compartment give rise to prostate cancer. In this study, stem cells ("CARNs," castration-resistant Nkx3.1-expressing cells) were identified in the luminal cell compartment. These rare luminal cells express NkX3.1 in the absence of testicular androgens. Furthermore, genetic inactivation of PTEN, specifically in CARN cells in the mouse prostate, resulted in prostate cancer development (Wang *et al.*, 2009e). Since CARN cells are luminal, the CARN-cell origin of prostate cancer is an attractive possibility that can explain luminal phenotype of prostate cancer cells. In humans, the equivalent to CARNs may be the "intermediate cells," which exist in the suprabasal and luminal layers of the prostate epithelium. Additional experiments utilizing a more detailed characterization of the differentiation program in the prostate epithelium and defining CARN cells in human prostate will be necessary for identification of the cellular origin of prostate cancer.

F. Changes in Prostate Stroma During Prostate Cancer Initiation and Progression

While prostate cancer is an epithelial neoplasm, cells in the prostate stroma also play a critical role in cancer initiation and progression (Josson *et al.*, 2010). One of the most significant changes that takes place early in

prostate cancer initiation is the formation of regions of PIA of epithelial cells, which are associated with inflammation in the stromal compartment (De Marzo *et al*., 1999). Interestingly, despite the decrease in the density of epithelial cells, many of these regions show increases in epithelial cell proliferation (Ruska *et al*., 1998; Shah *et al*., 2001). Regions of PIA are often contingent with areas containing high-grade PIN and carcinoma, and therefore might be a precursor to prostate neoplasia (De Marzo *et al*., 2007). Lymphocytic inflammation and other types of infiltrating immune cells are an important part of the tumor microenvironment. For instance, tumor-infiltrating macrophages and lymphocytes activate the inflammation-responsive IkappaB kinase (IKK)-alpha in prostate cancer cells, and IKK-alpha stimulates tumor metastasis by repressing the metastasis suppressor MASPIN (Ammirante *et al*., 2010; Luo *et al*., 2007). Inflammation is also important at the final stages of prostate cancer as it is associated with the appearance of castration-resistant tumors. The initial death of cancer cells during androgen ablation therapy is associated with infiltration of tumor masses by B lymphocytes, which produce lymphotoxin that stimulates activation of IKK-alpha and STAT3 to promote the formation of castration-resistant tumors (Ammirante *et al*., 2010).

Stromal cells also undergo changes during cancer initiation and progression. For instance, fibroblasts in the close proximity to the cancer cells (cancer-associated fibroblasts, CAFs) differ from the normal cells in expression of cell surface and secreted proteins (Dakhova *et al*., 2009; Dean and Nelson, 2008). These cells produce increased levels of various growth factors and cytokines that can influence and perhaps even induce transformation of epithelial cells (Hayward *et al*., 2001; Sung *et al*., 2008).

G. Prostate Cancer in Mouse Models

Several genetic mouse models of prostate cancer have been generated, which differ significantly in regard to the histological appearance of primary prostate tumors (Fig. 2B) (Shappell *et al*., 2004). Models utilizing prostate-specific expression of SV40 large T antigen (TRAMP and LADY (LPB-Tag), and their derivatives) display massive overproliferation of prostate epithelial (and stromal) cells, which results in tremendous increases in the prostate gland size (Gingrich *et al*., 1996; Kasper *et al*., 1998). These models develop metastatic prostate cancer, including bone metastases in LPB-Tag/PB-Hepsin mice; however, metastatic lesions in these animals display prominent neuroendocrine differentiation that resembles human AR- and PSA-negative small cell carcinoma (Kaplan-Lefko *et al*., 2003; Klezovitch *et al*., 2004; Masumori *et al*., 2001). When only a fragment of SV40 large T antigen predicted to inactivate all Rb-family genes was expressed in prostate

epithelium of transgenic mice, resulting model ($TgAPT_{121}$ mice) displayed development of prostate adenocarcinoma with no evidence of neuroendocrine tumors, but these mice also showed no evidence of metastasis (Hill *et al.*, 2005). Stromal cells in the normal mouse prostate form a very thin sheet covering the epithelial ducts, but in some prostate cancer models, the size of the stromal compartment is greatly enlarged and accompanies the expansion of epithelial cells in PIN lesions. Stromal cell compartments between ducts often fuse to generate an overall stromal appearance that is more similar to the human prostate (Fig. 2B).

In addition to models expressing exogenous viral proteins, several genetic models of prostate cancer have been generated, which exclusively utilize genetic changes that are confirmed in human prostate cancer. Conditional deletion of PTEN or overexpression of c-Myc in the prostate epithelium are two models that most faithfully replicate the development of human adenocarcinoma (Ellwood-Yen *et al.*, 2003; Wang *et al.*, 2003b) (Fig. 2B). Prostate cancer metastasis is rarely seen in these animals. In addition to the loss of PTEN and overexpression of Myc, the sustained activation of FGF signaling pathway in mouse prostate epithelial cells can also cause development of prostate cancer (Acevedo *et al.*, 2007). These tumors display prominent epithelial-to-mesenchymal transition (EMT) and form sarcomatoid carcinomas. Rare metastatic lesions contain spindle-shaped cells, which may have had a prostate epithelial origin and lost their epithelial morphology, as they dedifferentiated (Acevedo *et al.*, 2007). Androgen signaling plays an important role in human prostate cancer and AR is mutated and hyperactivated in many metastatic prostate tumors. Overexpression of one of such mutants (AR E231G) in mouse prostate epithelium is sufficient to cause the development of prostate cancer in 50-week-old mice (Han *et al.*, 2005).

While there are multiple similarities between human and mouse prostate cancer, there are also major differences. One of the most significant differences is a universal loss of basal cells in human prostate cancer, but the presence of the basal cell populations in some mouse cancers (Wang *et al.*, 2006a). Another notable difference is the paucity of metastasis in mouse models. In the rare cases that develop metastases, the metastatic lesions are usually small and appear only when the size of the primary tumor is extremely large. While human prostate cancers preferentially metastasize to lymph node and the bone, presently, bone metastasis have consistently been seen in only one genetic model of prostate cancer (LPB-Tag/PB-Hepsin mice; Klezovitch *et al.*, 2004; Fig. 2D). Overall, while tumor pathology and metastatic potential in mouse models significantly differ from human tumors, it remains to be seen whether these differences are due to inherent differences between mice and humans, or due to the failure of mouse models to capture the primary molecular mechanisms responsible for prostate tumor formation in human patients.

V. EARLY GENETIC AND EPIGENETIC EVENTS IN THE TRANSITION FROM NORMAL PROSTATE TO PIN AND INVASIVE CANCER

While environmental factors play an important role in the risk of prostate cancer, as in all other cancer types, the actual development of prostate cancer is primarily a genetic disease, which is caused by changes in gene expression and function (Fig. 1). HPC accounts for only a small fraction of cancers, and the bulk of prostate cancers arises in a sporadic fashion due to cancer initiation by *de novo* genetic changes in the prostate gland throughout the life of an individual. While previously these events were difficult to identify, novel technologies involving analysis of gene, protein, and tissue expression by microarrays, analysis of genomic copy number variation by comparative genomic hybridization (CGH), and, most recently, next-generation high-throughput sequencing have together revolutionized prostate cancer biology. These advances allowed for tremendous increases in the knowledge of the critical genetic and epigenetic events that take place during prostate cancer initiation and progression. The changes identified using these technologies were further analyzed in tissue culture and in live organisms. These functional studies established the causality of genetic and epigenetic events that are involved in prostate cancer initiation and progression. In Section V.A, we will review the most well-characterized genetic and epigenetic changes that are known to play an important role in prostate cancer.

Epigenetic changes resulting in increased methylation and silencing of several critical genes are amongst the earliest events that take place during prostate cancer initiation (Nelson *et al*., 2009; Schulz and Hoffmann, 2009). Several genes including *GSTP1*, *APC*, *RASSF1A*, *RABB2*, and *MDR1* were reported to be consistently hypermethylated, which can robustly distinguish normal prostate from prostate adenocarcinoma (Enokida *et al*., 2005; Florl *et al*., 2004). While the significance of many of these events is not known presently, one of these genes *GSTP1* is heavily implicated in prostate cancer initiation.

A. Decreased Expression of GSTP1 (Glutathione-S-Transferase P1)

GSTP1 is an enzyme that decreases oxidative damage in cells by catalyzing the conjugation of toxic compounds to glutathione. Promoter hypermethylation and silencing of *GSTP1* expression is an early event in prostate cancer

initiation, which is seen in up to 70% of PIN lesions and in 90–95% of prostate carcinomas (Lee *et al*., 1994). Decreased GSTP1 expression, which normally occurs during differentiation of basal to luminal cells, may predispose luminal cells to increased oxidative damage. This will in turn result in accumulation of genetic changes ultimately resulting in cell transformation and cancer.

B. Decreased Expression of NKX3.1

The loss of heterozygocity at chromosome 8p21-22 is an early and frequent event (up to 12% in PIN and up to 85% in carcinoma) in prostate carcinoma (Bethel *et al*., 2006; Vocke *et al*., 1996). One of the most likely candidate genes in this region is a prostate-specific homeobox transcription factor Nkx3.1 (Swalwell *et al*., 2002). Nkx3.1 is prominently expressed in prostate luminal epithelial cells and it is important for normal differentiation of prostate epithelium (Abate-Shen *et al*., 2008). Decreased levels of Nkx3.1 may result in deficient prostate epithelial differentiation and promote a nondifferentiated phenotype that can contribute to neoplastic transformation. Expression of *Nkx3.1* is decreased in human prostate cancer, and deletion of *Nkx3.1* in mice results in development of PIN lesions (Abdulkadir *et al*., 2002; Bhatia-Gaur *et al*., 1999). While PIN lesions in *Nkx3.1/–/–* mice do not progress to prostate cancer, *Nkx3.1* shows prominent cooperation with *PTEN*, as double heterozygous *Nkx3.1* and *PTEN* mice develop prostate adenocarcinomas, which in some cases display metastasis to lymph nodes and distant organs (Abate-Shen *et al*., 2003). Moreover, human germ line mutations in Nkx3.1, which decreases its interaction with DNA, result in genetic predisposition to prostate cancer (Zheng *et al*., 2006). While significant functional analysis in cell lines and genetically engineered mice strongly suggests an important role of *Nkx3.1* haploinsufficiency in prostate cancer, *Nkx3.1* is not a classical tumor-suppressor gene because the second allele of *Nkx3.1* is not lost or mutated in prostate cancer (Ornstein *et al*., 2001; Voeller *et al*., 1997).

C. Increased Lipid Metabolism

Lipid biosynthesis is markedly increased in PIN and invasive cancers. The two enzymes that are responsible for this increase are α-methylacyl-coA-racemase (AMACR) and fatty acid synthase (FASN). AMACR is used clinically in difficult cases to distinguish true prostate cancer from benign histologic mimicry of cancer and has significantly increased the accuracy of diagnosing prostate cancer in prostate needle biopsies (Luo *et al*., 2002).

The overexpression of FASN is an early event in prostate cancer initiation and is observed in PIN. FASN expression was inversely proportional to the apoptotic rate. A recent systematic and extensive analysis of FASN overexpression led to classifying *FASN* as an oncogene (Migita *et al.*, 2009). The study demonstrated that forced expression in immortalized prostate epithelial cells promoted cell proliferation and anchorage independent growth in soft agar. Furthermore, when transplanted into mice, FASN expressing human prostate epithelial cells formed invasive tumors. The transgenic expression of FASN in mouse prostate resulted in PIN, but not in invasive carcinoma (Migita *et al.*, 2009). Given its oncogenic activity, FASN is a treatment target in prostate cancer (Kuhajda *et al.*, 2000).

D. Recurrent Chromosomal Rearrangement and Overexpression of ETS Family Proteins

Previously, it was believed that recurrent chromosomal rearrangements were extremely rare in carcinoma. Dr. Arul Chinnaiyan's group discovered a common and recurrent gene fusion event in human prostate cancer, recurrent fusions may also involve other ETS-family genes, such as ETV4 and ETV5 which results in a fusion of sequences in the androgen-driven *TMPRSS2* promoter with a portion of the coding region of *ERG* or *ETV1* ETS family transcription factors (Tomlins *et al.*, 2005). ETS family transcription factors are homologous to the viral *v-ETS* erythroblastosis oncogene. These initial findings of gene fusions were confirmed and extended by many laboratories (Kumar-Sinha *et al.*, 2008). In addition to ERG or ETV1, recurrent fusions may also involve other ETS-family genes,such as ETV4 and ETV5 (Helgeson *et al.*, 2008; Tomlins *et al.*, 2007). Interestingly, TMPRSS2 and majority of other genes donating their promoter elements to ETS family proteins are positively regulated by androgen signaling (Lin *et al.*, 1999), which is prominently implicated in human prostate cancer (see below). Remarkably, AR signaling is likely to also be the mechanism restricting TMPRSS2–ERG fusions to prostate cancer, because it brings the TMPRSS2 and ERG genes in close proximity to facilitate the subsequent recombination event (Mani *et al.*, 2009). Rearrangement of *ERG* and *TMPRSS2* is by far the most frequent event in human prostate cancer. It is reported to happen early in prostate cancer (up to 20% of high-grade PIN lesions) and it can be detected in up to 50% of all prostate adenocarcinomas (Kumar-Sinha *et al.*, 2008). Rearrangements involving other ETS genes are much less frequent and account for about 10% of prostate carcinomas. Overall, about 60% of all human prostate adenocarcinomas present with gene rearrangement resulting in overexpression of an ETS family transcription factor.

Since the discovery of recurrent gene fusions in prostate cancer, extensive efforts have been made to understand the significance of these events. Analysis of the potential association between *TMPRSS2–ERG* rearrangements and patient prognosis has revealed complex and confusing data. While the majority of studies report association between fusion and poor patient outcome, others report no clinical association or even an association with a better patient prognosis (Kumar-Sinha *et al.*, 2008). It has to be noted that, since recurrent gene fusion is a very frequent event that occurs in majority of prostate tumors, these prognostic association studies cannot suggest the role of ETS proteins in etiology of prostate cancer; however, they can compare the potential aggressiveness of ETS rearrangement positive and negative tumors.

The functional significance of ETS rearrangements in prostate cancer has been analyzed in cell lines and *in vivo*. Gain-of-function and loss-of-function experiments in cell lines have revealed an important role of ETS proteins in cell invasion (Helgeson *et al.*, 2008; Klezovitch *et al.*, 2008; Tomlins *et al.*, 2007). Some reports also found an important positive role of ETS proteins in cancer cell proliferation and tumor progression (Wang *et al.*, 2008). Overexpression of ETS genes in prostate epithelium of transgenic mice results in development of PIN lesions, which do not readily progress to invasive adenocarcinoma in adult mice (Klezovitch *et al.*, 2008; Tomlins *et al.*, 2007, 2008a), but do show development of prostate cancer in older animals (Fig. 2B). However, tissue recombination experiments demonstrated rapid development of prostate cancer in cells overexpressing ERG in combination with AKT pathway activation or AR upregulation (Zong *et al.*, 2009). Overall, these *in vivo* data indicate that while ETS proteins overexpression is observed in cancer, additional genetic or epigenetic events must be cooperating with ETS proteins to drive development of frank carcinoma. Indeed, *ERG* expression prominently cooperates with the loss of *PTEN*. While transgenic animals expressing low levels of ERG protein in prostate epithelium reveal no obvious histological phenotype, crossing these mice with *PTEN*+/− animals reveals development of prostate adenocarcinoma in double mutant *ERG*+/*PTEN*+/− mice (Carver *et al.*, 2009; King *et al.*, 2009). In addition, significant correlation was established between the loss of PTEN and overexpression of ETS family proteins in human carcinomas, indicating that overexpression of ETS cooperates with activation of phosphatidylinositol 3-kinases pathway in development of prostate carcinoma (Carver *et al.*, 2009; Han *et al.*, 2009).

While the significance of recurrent rearrangements of ETS family genes in prostate cancer is beginning to be understood, the molecular mechanisms of ETS function in prostate cancer remain unclear. An integrative analysis recently demonstrated an important role of ETS proteins in the binding and downregulation of AR activity in addition to the direct activation of polycomb group methyltransferase EZH2. Together these events can activate a dedifferentiation program in prostate epithelial cells (Yu *et al.*, 2010).

E. Overexpression of SPINK1

Recurrent rearrangement of ETS genes takes place in about 60% of human prostate tumors (Kumar-Sinha *et al*., 2008), indicating that there may be several additional molecular mechanisms that are responsible for human cancer development. Indeed, specific gene expression changes were identified, which take place only in ETS fusion-negative tumors. One of these is the overexpression of *SPINK1* (Tomlins *et al*., 2008b). *SPINK1* is prominently overexpressed in approximately 10% of prostate tumors, and overexpression strongly correlates with aggressive cancer and reduced progression-free survival (Leinonen *et al*., 2010; Tomlins *et al*., 2008b). Knockdown of *SPINK1* in the human prostate cancer line 22RV1, which overexpresses endogenous *SPINK1*, reduces cell invasion (Tomlins *et al*., 2008b). SPINK1 is a secreted trypsin inhibitor, which, by preventing premature activation of pancreatic proteases, protects from chronic pancreatitis (Witt *et al*., 2000). SPINK1 also binds and regulates the activity of EGFR (Ozaki *et al*., 2009). It is possible that SPINK1 performs similar functions in prostate cancer.

F. Loss of FOXP3

FOXP3 is a forkhead/winged-helix transcription factor localized on the X chromosome (Xp11.23) and involved in X-linked autoimmunity-immunodeficiency syndrome. FOXP3 is somatically deleted or mutated in human prostate cancer (Wang *et al*., 2009a). FOXP3 is a potent transcriptional repressor of c-Myc, which is one of the commonly upregulated genes in human prostate cancer. Importantly, modeling of inactivation of *FOXP3* in prostate epithelia using genetically engineered mice results in upregulation of c-Myc and development of PIN lesions, indicating an important role of *FOXP3* inactivation in prostate cancer (Wang *et al*., 2009a). Interestingly, since males have only one copy of *FOXP3*, a single inactivating hit in *FOXP3* would be sufficient to activate carcinogenesis.

The list of genetic changes that occur during the initiation of prostate cancer is clearly much longer and reflects the magnitude and complexity of mechanisms that are associated with the early development of prostate cancers. The late onset of prostate cancer suggests that genetic changes that lead to cancer development accumulate over many years, are likely to be heterogeneous. It is plausible that there are several different subtypes of human prostate cancer, which are driven by different molecular mechanisms. Perhaps, subclassifying cancers according to genetic criteria will facilitate the discoveries of specific genes and pathways that are responsible for

the development of a particular tumor subtype. For example, exon sequencing of 218 prostate tumors demonstrated frequent deletion of 3p14.1-p13 in cancers with *TMPRSS2–ERG* gene fusion (Taylor *et al.*, 2010). Comprehensive annotation of prostate cancer genomes in this study resulted in identification of six independent cancer subtypes, which were independent of Gleason Grade and demonstrated a risk of tumor progression that was proportional to the extent of genomic alterations.

VI. TRANSITION TO HIGH-GRADE CARCINOMA AND METASTASIS

Prostate tumors with Gleason sums of ≥ 7 are high-grade lesions that are more likely to metastasize and to progress to a lethal form of prostate cancer. A recent study found that prostate cancer cells metastasize early during tumor development. In this study, bone marrow aspirates were obtained in >500 men, who underwent prostate cancer surgery. Many men had low-grade cancers. Surprisingly, in 68% disseminated prostate cancer cells were detected in the bone marrow sample, even in patients with low-grade cancer (Morgan *et al.*, 2009). Since the recurrence rate in this patient population is approximately 15%, only a small number of men with metastatic prostate cancers in the bone marrow will experience a relapse of their cancer. Interestingly, when cancer cells were present in a repeat biopsy, sometimes years after prostate cancer surgery, the amount of persistent cancer cells increased the risk of cancer recurrence. The results from this study have important implications for the pathophysiology of prostate cancer metastasis. It appears that (1) the metastatic process begins in the early stages of cancer development, (2) the majority of metastatic prostate cancer cells remain in a dormant state, and (3) tumor growth in lymph node or bone marrow is the bottleneck of cancer recurrence (Husemann *et al.*, 2008; Klein, 2009; Podsypanina *et al.*, 2008). These concepts are in agreement with the seed-and-soil hypothesis of cancer metastasis, which was proposed 100 years ago and is still debated. In the section below, we will discuss several genetic and epigenetic events that take place at high frequency in high-grade prostate tumors and are likely to be causally involved in the development of high-grade cancer and its progression to metastasis.

A. Loss of PTEN

PTEN is a well-known tumor suppressor involved in variety of human cancer types (Salmena *et al.*, 2008). PTEN is a lipid phosphatase that opposes the enzymatic function of PI3 kinases and activates PI3K/Akt

signaling pathway. PTEN is lost in up to 17% of primary prostate tumors and there is significant correlation between PTEN loss and Gleason Score and clinical stage (Reid *et al*., 2010). Moreover, tumors that maintain PTEN expression are less likely to progress. Up to one-third of all castration-resistant metastatic prostate tumors display loss of PTEN (Shen and Abate-Shen, 2007). The most compelling evidence for the role of PTEN in prostate cancer is from the phenotype of genetically engineered mice. Conditional inactivation of *PTEN* in prostate epithelium results in development of high-grade prostate cancer, which occasionally develops small metastatic lesions in the distant organs (Wang *et al*., 2003b). The development of primary tumors is even more significant in mice with prostate-specific inactivating mutations of both *PTEN* and *p53* tumor-suppressor genes (Chen *et al*., 2005b). Even loss of one allele of *PTEN* is likely to be significant in prostate cancer. Indeed, double heterozygous *Nkx3.1/PTEN* mice develop androgen-independent prostate cancer (Gao *et al*., 2006). Taken together, the data reported from the analysis of human tumors and mouse models of prostate cancer strongly indicate an important role for *PTEN* in the origination of lethal form of prostate cancer.

B. Amplification and Overexpression of C-MYC

C-MYC is a proto-oncogene frequently amplified in variety of human malignancies. C-MYC is a transcription factor involved in activation of the cell-cycle progression and protein biosynthesis. It resides on chromosome 8q24 and this region often shows gain in advanced human prostate cancer. c-MYC is upregulated in 76% of PIN lesions prior to amplification of the chromosomal region that contains the *c-MYC* locus on chromosome 8p, and c-MYC protein expression and genomic amplification do not correlate (Gurel *et al*., 2008). C-*MYC* copy number is modestly increased in approximately 30–40% of primary prostate tumors and 90% of metastatic prostate cancers (Ishkanian *et al*., 2009; Jenkins *et al*., 1997; Sato *et al*., 2006) and is associated with poor patient outcome (Sato *et al*., 2006). Experiments with mouse models of prostate cancer provided strong causal connection between the overexpression of c-MYC and the development of prostate cancer. Transgenic mice overexpressing c-myc in prostate epithelium develop prostate adenocarcinoma, but metastatic lesions are rare in these animals (Ellwood-Yen *et al*., 2003). These functional studies, as well as the overexpression data in human tumors provide strong evidence of an important role of c-MYC signaling in the development and progression of prostate carcinoma.

C. Overexpression of Hepsin

Hepsin is a cell-surface serine protease, which is highly (>10-fold) overexpressed in up to 90% of primary prostate tumors (Dhanasekaran *et al.*, 2001; Landers *et al.*, 2005; Stephan *et al.*, 2004). Hepsin is a promiscuous protease that degrades laminin, and cleaves and activates pro-HGF to stimulate the HGF-Met tumor invasion pathway (Kirchhofer *et al.*, 2005; Tripathi *et al.*, 2008). In addition, it can efficiently cleave and activate pro-urokinase-type plasminogen activator, which is also heavily implicated in tumor invasion and metastasis (Moran *et al.*, 2006). The most compelling evidence for the role of hepsin in prostate cancer came from animal models. Mice overexpressing hepsin in the prostate epithelium do not develop tumors and show mild problems with the integrity of the basement membrane (Klezovitch *et al.*, 2004). However, hepsin overexpression in the nonmetastatic prostate cancer (LPB-Tag line 12T-f) mouse model (Kasper *et al.*, 1998) results in mice displaying prominent metastases (Klezovitch *et al.*, 2004). Similar data concerning the role of Hepsin in prostate tumor progression were also obtained in an orthotopic xenograft model of human prostate cancer (Li *et al.*, 2009). These data indicate that while hepsin is upregulated in prostate cancer as early as PIN lesion formation, functionally, it plays an important role at later stages of tumor invasion and metastatic progression. Interestingly, while bone is the preferred site of human prostate tumor metastasis, bone metastasis is rare in mouse models of prostate cancer. LPB-Tag/PB-Hepsin is the only mouse model of prostate cancer that consistently develops bone metastasis (Klezovitch *et al.*, 2004). Coincidently, this is the only animal model that captures Hepsin overexpression, which is seen so frequently in human tumors. Future research will help to determine whether this is simply a coincidence, or whether Hepsin is involved in the development of prostate cancer bone metastasis.

D. Overexpression of PIM1

PIM1 is serine/threonine kinase involved in positive regulation of cell-cycle progression and inhibition of apoptosis. It is implicated as an oncogene in human lymphomas, and gastric, colorectal, and prostate cancers (Shah *et al.*, 2008). In prostate, PIM1 is overexpressed in about 50% of primary prostate tumors (Dhanasekaran *et al.*, 2001). While the role of PIM1 in prostate cancer has not been analyzed in genetic animal models, PIM1 promotes tumorigenesis and transcriptional activity of c-Myc in prostate cancer cell lines *in vitro* and *in vivo* (Kim *et al.*, 2010).

E. Loss of EPHB2

EPHB2 is a receptor tyrosine kinase for ephrin-B family of ligands. A combination of array-based CGH and nonsense-mediated RNA decay microarray revealed deletion and mutation-mediated loss of *EPHB2* in prostate tumors and metastasis (Huusko *et al*., 2004). In prostate cancer cell lines, reexpression of EPHB2 attenuates clonogenic growth, indicating that the loss of *EPHB2* is likely to be an important part of prostate cancer progression. Interestingly, a variant *EphB2* present at high frequency in African-American population is associated with increased risk for prostate cancer (Kittles *et al*., 2006).

F. Loss of MicroRNA-101 and Overexpression of EZH2

EZH2 is a critical enzymatic component of polycomb repressive complex 2, which methylates histone H3 on dimethyl lysine-27 to generate H3K27-trimethyl and silences gene transcription (Sellers and Loda, 2002). Normally, EZH2 is involved in the dowregulation of expression of genes involved in differentiation; however, *EZH2* is also prominently overexpressed in advanced prostate cancers and overexpression confers a poor prognosis in localized prostate cancers (Varambally *et al*., 2002). *EZH2* is amplified in a subset of advanced prostate tumors (Saramaki *et al*., 2006). While the *in vivo* analysis of the role of EZH2 in animal models of prostate cancer has yet to be reported, silencing of *EZH2* expression in prostate cancer cell lines attenuates their proliferation (Varambally *et al*., 2002). Mechanistically, EZH2 regulates the expression of a variety of genes and some of its known targets, such as E-cadherin and DAB2IP, have been already implicated in prostate cancer (Cao *et al*., 2008; Min *et al*., 2010).

The recently discovered microRNA regulation of signaling pathways has opened a novel dimension in prostate cancer research. For instance, miR-101 is a microRNA frequently deleted in human prostate tumors (Varambally *et al*., 2008). Interestingly, EZH2 is a direct target of mir-101 and loss of mir-101 expression may provide an explanation of one of the mechanisms responsible for upregulation of EZH2, disregulation of epigenetic control of gene expression, and cancer (Varambally *et al*., 2008).

VII. MECHANISMS OF CASTRATION-RESISTANT PROSTATE CANCER

The majority of prostate adenocarcinomas are formed by luminal-type prostate epithelial cells that require activity of AR signaling for survival. Surgical or chemical castration prominently downregulates AR signaling

resulting in the death of the majority of prostate cancer cells (Huggins and Hodges, 1941). Unfortunately, cancer-initiating cells do not die after this treatment and cancers recur as a castration-resistant and potentially lethal disease. The major cause of castration resistance is likely due to the continuous transcriptional activity of the AR (Chen *et al.*, 2008, 2009), despite castrate levels of circulating androgens. Tumor cells can employ several mechanisms that promote survival despite castration, which will be discussed below.

A. Overexpression of AR

The AR is commonly overexpressed in advanced prostate cancer (Setlur and Rubin, 2005). An interesting study compared androgen-sensitive and castration-resistant cancers from same patients. Despite a marked increase in protein expression and in the frequency of AR gene amplification in castration-resistant cancer, there was no correlation between these events (Edwards *et al.*, 2003). It was also clear from this study that not all castration-resistant cancers overexpressed AR, and that additional mechanisms are responsible for castration resistance. Recently, several mechanisms that can lead to the "androgen hypersensitivity syndrome" in castration-resistant prostate cancer have been identified (Vis and Schroder, 2009), and these are reviewed in the sections below.

B. Variant AR Transcripts

Several mutations have been identified in the *AR* gene in castration-resistant tumors. These mutations provide a growth advantage by reducing androgen concentrations needed for activation of AR, or by increasing the affinity of AR to progesterone and glucocorticoid hormones, or by affecting the recruitment of AR corepressors or coactivators. In normal prostate epithelial cells, the inactive AR is complexed with Hsp90 chaperone and resides in the cytoplasm (Georget *et al.*, 2002). Androgen binding to AR induces a conformational change in the receptor, which generates a hydrophobic cleft in the ligand binding domain (LBD) that is then able to bind the unstructured amino-terminus. ARs subsequently homodimerizes, translocate to the nucleus, and bind to the androgen-response elements in the promoters and enhancers of target genes. Coactivators and corepressors are usually recruited to the amino-terminus and thereby regulate the transcriptional activity of the AR (Dehm and Tindall, 2007). There are at least 70 different somatic missense mutations in the *AR* gene in patients with prostate cancer (Gottlieb *et al.*, 2004). Of 44 mutations that were tested, 20 possessed

increased functional activity (Shi *et al*., 2002). The mutations are concentrated in the N-terminus and in the LBD and affect dimerization, binding of AR to transcriptional coactivators and corepressors, and modify the interaction of AR with chromatin (Jaaskelainen *et al*., 2006; Li *et al*., 2006; Welsbie *et al*., 2009). In addition, mutations in the hinge region of AR affect its nuclear translocation (Buchanan *et al*., 2001). Specifically, the S296R and T877A mutations, which were identified in castration-resistant prostate cancer, change hormone specificity from androgen to estrogen and progesterone (Sack *et al*., 2001) (Li *et al*., 2008). Overall, the functional outcome of AR mutations is to circumvent the decrease in the levels of testosterone and continue to activate androgen-mediated signaling pathways.

In addition to mutations in AR, utilization of splice variants of *AR* provides a novel mechanism for the escape from androgen suppression (Dehm *et al*., 2008; Hu *et al*., 2009; Marcias *et al*., 2010). Alternative splicing of *AR* gene transcripts can result in the formation of AR variants with modified functions. Multiple splice variants of AR were identified in prostate cancer cell lines and castration-resistant prostate tumors. These variants often truncate the C-terminal domain of AR making it constitutively active and hormone insensitive (Dehm *et al*., 2008; Marcias *et al*., 2010). Importantly, these splice variants were upregulated during prostate cancer progression and were enriched in prostate cancer xenograft tumors, which were resistant to therapy (Dehm *et al*., 2008; Guo *et al*., 2009). The drugs currently used for chemical castration in prostate cancer decrease testosterone levels in the circulation and inhibit androgen binding to the AR LBD. Since mutations and splice variants reduce the androgen dependence of the AR, a novel class of drugs is necessary to directly target the AR, independent of androgen. EPI-001 is a recently developed novel compound, which binds to the amino-terminus of the AR and blocks its transactivation (Andersen *et al*., 2010). This compound is specific for the AR and inhibits the growth of castration-resistant xenografts without causing toxicity to the animals. Another effective and high affinity AR inhibitor, MDV3100, has recently been tested in a phase I/II clinical trial against treatment-refractory prostate cancer (Scher *et al*., 2010). Another drug inhibits nuclear translocation, DNA binding, and coactivator recruitment of AR, and it will soon be tested in clinical trials (Tran *et al*., 2009).

C. Intracrine Androgen Synthesis

Another mechanism for resistance to castration is the autocrine activation of androgen synthesis by cancer cells. Indeed, some recurrent prostate cancers develop the capacity to synthesize testosterone from adrenal androgens or cholesterol (Titus *et al*., 2005), which leads to persistence of intraprostatic

androgen despite testosterone suppression (Mostaghel *et al*., 2007; Page *et al*., 2006). Under conditions of *de novo* synthesis of androgens by cancer cells, drugs that effectively block hormone synthesis would be most beneficial. For example, the CYP17 inhibitor, abiraterone, which blocks androgen synthesis by cancer cells, demonstrated clinical efficacy in patients who failed prior treatment with ketochonazol, an inhibitor of adrenal androgen synthesis (Ryan *et al*., 2010), or who had previously received docetaxel (Danila *et al*., 2010).

D. Hyperactivity of the AR Through Phosphorylation

The drastic decrease in the levels of circulating androgens in castrated patients can be overcome by hyperactivation of signaling pathways resulting in an increased activity of AR. For instance, phosphorylation of steroid hormone receptors by various kinases in the N-terminal transactivation domain can increase their transcriptional activity. IL-6 is an autocrine cytokine in the prostate epithelium and activation of its receptor significantly lowers the androgen dependence of the AR (Hobisch *et al*., 1998). Mechanistically, IL-6 receptor activation leads to increased AR phosphorylation by MAPK, Etk, and Pim1 kinases (Giri *et al*., 2001; Kim *et al*., 2004). The activation of several tyrosine kinases, such as HER2, IGFR, Ack1, and Src, can also result in AR phosphorylation and in an increase in AR activity (Guo *et al*., 2006; Mahajan *et al*., 2007; Mendoza *et al*., 2002; Qi *et al*., 2009; Sugita *et al*., 2004; Wu *et al*., 2005). In addition to the AR itself, the AR coactivator, SRC3, can also integrate signaling pathways via multiple phosphorylation sites that are necessary to promote the transcriptional activity of the AR (Wu *et al*., 2004). Overall, inhibition of phosphorylation of AR may provide a viable therapeutic intervention for castration-resistant tumors; however, the heterogeneity of kinases that can phosphorylate and transactivate the AR is a significant complication for the development of such treatments.

E. Changes in AR Transcriptional Activity in Castration-Resistant Prostate Cancer

In addition to the increase in AR activity in castration-resistant cancers, recent studies suggest that AR target genes can differ in castration-resistant compared to androgen-sensitive cancers. The difference in transcriptional target genes in androgen-sensitive versus castration-resistant cancer is caused by other transcription factors, which bind to their own DNA-binding

sequences in the proximity of AR-response elements (AREs) and regulate the occupancy of AREs by the AR (Agoulnik *et al*., 2008; Lupien and Brown, 2009). FoxA1, GATA2, PAX2, Oct1, ETS, and Nkx3.1 can affect AR binding to AREs, and their expression levels differ in castration-resistant prostate cancer (Bohm *et al*., 2009; Jia *et al*., 2008; Shyr *et al*., 2010; Simmons and Horowitz, 2006; Wang *et al*., 2007; Yu *et al*., 2010; Zhang *et al*., 2010). The major mechanism that regulates the occupancy of AREs is histone modifications (Chen *et al*., 1999). Gene activation and repression are specifically regulated by histone methylation status at distinct lysine residues. Interestingly, AR target genes display significantly higher levels of histone-activating marks H3K4 and H3K27 methylation and H3-K9/K14 acetylation in castration-independent prostate cancer (Chen *et al*., 1999; Jia *et al*., 2006; Kang *et al*., 2004; Lupien and Brown, 2009; Lupien *et al*., 2008; Metzger *et al*., 2005, 2008; Wang *et al*., 2001; Wissmann *et al*., 2007). The demethylases, JMJD2C, and LSD1, cooperatively promote androgen-dependent transcription by association with AR, thereby removing the repressive histone mark (H3K9) from target genes by demethylation of H3K9 (Metzger *et al*., 2005; Wissmann *et al*., 2007).

Comprehensive comparison of AR-mediated transcription in androgen-sensitive and -resistant derivatives of LnCaP cells identified very specific changes (Wang *et al*., 2009c). In particular, the transcription of genes regulating the M-phase of the cell cycle was increased in androgen-insensitive LnCaP cells and in castration-resistant prostate cancer samples from patients. Among the specific AR-regulated M-phase cell-cycle genes is the ubiquitin-conjugating enzyme E2C, which specifically inactivates the M-phase cell-cycle checkpoint (Wang *et al*., 2009c). In addition, the recruitment of FoxA1, MED1, and GATA2 transcription factors to the E2C promoter was increased in the androgen-insensitive LnCaP cells. In general, AR binding to promoters of M-phase genes was determined by H3K4 methylation and FoxA1 binding. Silencing E2C decreased the growth of castration-resistant cancer cells by blocking S and G2/M cell-cycle progression. Thus, information about the differences in AR-response genes between androgen-sensitive and castration-resistant cancer can potentially be used to develop novel therapeutic approaches specifically targeting castration-resistant prostate cancer.

VIII. SUMMARY AND FUTURE DIRECTIONS

Extraordinary progress in the development of novel genetic technologies in the recent years allowed for the first time a comprehensive analysis of genetic and epigenetic changes in human prostate cancer. This information,

combined with targeted functional studies, helped to identify critical signaling pathways that are causally involved in prostate cancer initiation and progression. Overall, significant evidence has been accumulated that strongly implicates the activation of PI3K signaling, the upregulation of ETS family and c-MYC transcription factors, the overexpression of EZH2 and HEPSIN, and the persistent activation of AR signaling as critical genetic and epigenetic changes that drive prostate cancer initiation and progression. This information provides an opportunity for the development of novel targeted approaches for therapeutic interventions that can block these critical for cancer-inducing pathways.

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Genetic Basis for Susceptibility to Lung Cancer: Recent Progress and Future Directions

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Lung cancer is the leading cause of cancer death worldwide, and cigarette smoking is the major environmental factor for its development. To elucidate the genetic differences in the susceptibility to lung cancer among individuals, genetic factors involved in tobacco-induced lung cancers have been extensively investigated and a number of genetic polymorphisms have been identified to date as candidates. Most of the polymorphisms identified are of genes encoding proteins associated with the activity to metabolize tobacco smoke carcinogens and to suppress mutations induced by those carcinogens, and functional significances have been elucidated for some of these polymorphisms. However, the significance of these polymorphisms in the contribution to lung cancer development still remains unclear. Recently, several novel lung cancer susceptibility genes, including those on chromosomes 5p15.33, 6p21, and 15q24-25.1, have been identified by large-scale genome-wide association (GWA) studies. The 15q25 region contains three nicotine acetylcholine receptor subunit genes, and their polymorphisms have been also reported as being associated with nicotine dependence. The 5p15.33 region is associated with risks specifically for lung adenocarcinoma, the commonest histological type and weakly associated with smoking. This locus has been shown to be associated with risks for a wide variety of cancers, including lung adenocarcinoma. Associations of the 6q21 region have not been consistently replicated among studies. The 6q23-25 and 13q31.3 regions were also identified by recent GWA studies as being associated with risk for lung cancer, particularly in never-smokers. However, contributions of genetic differences on these five loci to the susceptibility to overall lung cancer seem to be small. There are several molecular pathways for the development of lung adenocarcinomas, and environmental factors for their development are still unclear,

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especially those in never-smokers. In addition, geographic differences as well as gender differences in lung cancer risk have been indicated. Furthermore, various genes identified by candidate gene association studies have not been reevaluated for their significance together with genes identified by GWA studies in the same population. Therefore, further studies will be necessary to assess the individual susceptibility to lung cancer based on the combination of polymorphisms in multiple genes, and to establish a novel way of evaluating the individual risk for lung cancer for its prevention.

I. INTRODUCTION: OVERVIEW OF STUDIES ON GENETIC AND ENVIRONMENTAL FACTORS INVOLVED IN LUNG CANCER SUSCEPTIBILITY

Lung cancer is the leading cause of cancer death worldwide (Sun *et al.*, 2007). Therefore, identification of genetic factors as well as environmental factors is very important in developing novel methods of lung cancer prevention. Since cigarette smoking is the major environmental risk factor for the development of lung cancer, genetic factors for tobacco-induced lung cancer have been extensively investigated by candidate gene association studies for many years. Genes involved in the metabolism of tobacco smoke carcinogens and genes involved in the repair of genetic alterations induced by those carcinogens have been the major targets of those investigations. In contrast, recent advances in molecular technology and knowledge of the distribution of genetic polymorphisms in the human genome have made it possible to identify genetic factors responsible for the development of common polygenic diseases, including lung cancer, by a genome-wide approach. Indeed, several loci containing candidate lung cancer susceptibility genes have been identified in recent years by genome-wide association (GWA) studies. One of the chromosomal loci identified was 15q24-25.1, and this region contained three genes encoding nicotinic acetylcholine receptor subunit genes. Since this locus has been also suggested to be associated with nicotine dependence, genetic susceptibility for nicotine addiction has come to be the major genetic factor for the development of lung cancer. However, epidemiologically, the incidence of lung cancers has been increasing in never-smokers, in women, and in Asian population, in recent years; therefore, lung cancers in smokers and those in never-smokers are now considered to be different diseases from each other. For this reason, identification of genetic factors as well as environmental factors for the development of lung cancers in never-smokers has also arisen as a major topic for prevention of these lung cancers. Accordingly, GWA studies for the identification of lung cancer susceptibility genes without association with smoking behavior are now also being extensively conducted. Therefore, when we discuss the genetic basis for susceptibility to lung cancer, three different critical points should be considered, as summarized in Fig. 1, in association with their functional significance in the

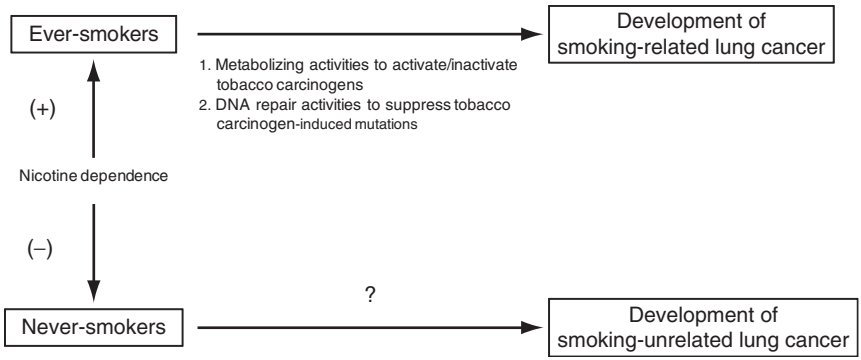

Fig. 1 Three different types of lung cancer susceptibility genes involved in the process of lung cancer development. Details are described in Section I.

susceptibility. The first point is interindividual differences in nicotine dependence, the second point is susceptibility to tobacco-induced (smoking-related) lung cancer, and the third point is susceptibility to lung cancer in never-smokers (smoking-unrelated). Nicotine dependence should be associated with the quantity of the intake of tobacco smoke carcinogens. In the development of smoking-related lung cancers, interindividual differences in metabolizing activities to activate/inactivate tobacco smoke carcinogens as well as DNA repair activities to suppress tobacco smoke carcinogen-induced mutations would play a major role. Since environmental factors for the development of lung cancer in never-smokers are largely unclear at present, GWA studies will be an effective approach to identify responsible genes for their development. In addition, we should accumulate the knowledge for the difference between lung cancers in smokers and those in never-smokers, from the viewpoint of molecular processes for their development. For this reason, in this review chapter, the differences between lung cancers in smokers and those in never-smokers are summarized first, recent progresses in lung cancer susceptibility gene studies are summarized second, and future directions of this field of science are discussed last.

II. DIFFERENCES IN THE PROCESS OF LUNG CANCER DEVELOPMENT BETWEEN SMOKERS AND NEVER-SMOKERS

Lung cancers are divided into the two major categories of small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) from clinicopathological aspects (Sun *et al.*, 2007). NSCLCs are further divided into three

major histological types, adenocarcinoma (ADC), squamous cell carcinoma (SQC), and large cell carcinoma (LCC). However, LCC is thought to be poorly or undifferentiated forms of and more heterogeneous than the other three types of lung cancer, and only limited information is available at present on genetic susceptibility to LCC. Therefore, in this review chapter, LCC is not specifically taken up as a subject for discussion. ADC, SQC, and SCLC are thought to be different in their origins (Govindan, 2010; Subramanian and Govindan, 2008; Travis *et al.*, 2004). Lung epithelial cells consist of monolayer columnar glandular epithelial cells (Fig. 2). Basal cells and neuroendocrine cells in the bronchi are thought to be precursors of SQC and SCLC, respectively. Clara cells in the bronchioles and/or type II pneumocytes in the alveoli are thought to be precursors of ADC. In mice, bronchioalveolar stem cells (BASC) have been identified as being a candidate precursor of ADC (Kim *et al.*, 2005); however, corresponding cells in the human have not yet been identified. Therefore, three major types of lung cancer are thought to be originated from different precursor epithelial cells in the lungs.

It is now widely accepted that cancer is attributed to accumulation of multiple genetic alterations in targeted precursor cells. Therefore, when we discuss genetic susceptibility to lung cancer, it is important to understand the differences and similarities in molecular pathways of cancer development among the three major types. Indeed, accumulated genetic alterations during their development are considerably different among them. Table I summarizes the accumulated genetic alterations in ADC, SQC, and SCLC (Govindan, 2010; Subramanian and Govindan, 2008; Sun *et al.*, 2007; Travis *et al.*, 2004). Ten genes identified to date with frequent genetic alterations in lung cancer cells are chosen as representatives. MYC, p53, PTEN, and PTPRD are genetically altered commonly among ADC, SQC,

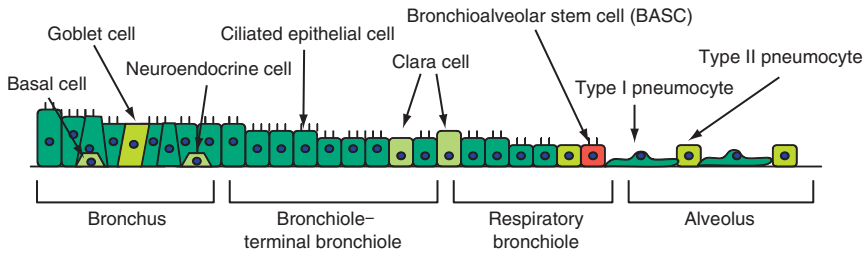

Fig. 2 Component epithelial cells in the pulmonary system. Precursor (progenitor) cells of small cell carcinoma, squamous cell carcinoma, and adenocarcinoma are thought to be neuroendocrine cells, basal cells, and Clara cells/type II pneumocytes, respectively. In mice, bronchioalveolar stem cells (BASCs) were identified as being precursor cells of adenocarcinoma; however, corresponding human cells have not identified to date.

Table I Oncogenes and Tumor Suppressor Genes Genetically Altered in Lung Cancer

Year[a]	Gene	Alteration	ADC	SQC	SCLC
1983	MYC[b]	Amp	+	+	++
1987	KRAS[b]	Mut	+[c]	−	−
1988	RB (RB1)[d]	Del, Mut	−	−	++[c]
1989	TP53[d]	Del, Mut	++	+++	+++
1994	p16 (CDKN2A)[d]	Del, Met, Mut	+[c]	+[c]	−
1998	PTEN[d]	Del, Met, Mut	+	+	++
2002	LKB1[d]	Del, Mut	++	−	−
2004	EGFR[b]	Small Del, Mut	++[c]	−	−
2005	PTPRD[d]	Del, Mut	+	+	+
2007	ALK[b]	Inv	+[c]	−	−

Abbreviations: ADC, adenocarcinoma; SQC, squamous cell carcinoma; SCLC, small cell lung cancer; Amp, amplification; Mut, mutation; Del, deletion; Met, methylation; Inv, inversion.

[a]Year of genetic alterations identified in lung cancer cells.

[b]Oncogene.

[c]Occurrence of genetic alterations in a mutually exclusive manner.

[d]Tumor suppressor gene.

and SCLC. RB and p16 are inactivated in a type-specific and mutually exclusive manner. Namely, RB is specifically inactivated in SCLC whereas p16 is specifically in ADC and SQC. KRAS, LKB1, EGFR, and ALK are specifically mutated in ADC. It is noted that KRAS, EGFR, and ALK are mutated in a mutually exclusive manner in ADC; therefore, in the light of accumulated genetic alterations, ADC can be further divided into at least four different types: KRAS-type, EGFR-type, ALK-type, and non-KRAS/EGFR/ALK-type (Govindan, 2010; Subramanian and Govindan, 2008; Travis *et al*., 2004). EGFR-type is the major type of lung ADC in Asian people (30–50%) and the fraction of EGFR-type in Asian people is higher than that in American/European people (i.e., individuals of European decent; 10–20%), representing a geographic and/or ethnic difference in lung cancer development. Instead, the fraction of KRAS-type in American/European people (20–40%) is higher than that in Asian people (10%). The fraction of ALK-type is ∼5% in both populations.

All three major types of lung cancer are associated with tobacco smoking; however, associations are much stronger in SQC and SCLC than ADC (Sobue *et al*., 2002). Roughly speaking, more than 90% of patients with SQC or SCLC are smokers, whereas only ∼50% of patients with ADC are smokers. Therefore, by considering the incidence of these three types of lung cancer, ∼25% of lung cancer cases are not attributed to smoking. The proportion of patients with lung cancer in never-smokers (less than 100 cigarettes in their life time) is higher in Asian populations than American/European populations. In this subset of lung cancer, mostly classified into

ADC, environmental factors causing the accumulation of multiple genetic alterations have been poorly understood. In particular, EGFR-type and ALK-type are frequent in never-smokers, while KRAS-type and non-KRAS/EGFR/ALK-type are frequent in smokers.

LKB1 alterations preferentially accumulate in the KRAS-type, and both LKB1 and KRAS are genetically altered more frequently in American/European people than in Asian people. In contrast, EGFR mutations occur more frequently in Asian people than in American/European people, as described above. Therefore, even though both KRAS-type and EGFR-type are histologically classified into ADC, they are thought to be different diseases from each other (Govindan, 2010; Subramanian and Govindan, 2008; Travis *et al.*, 2004). From this point of view, it can be said that American/European populations are more susceptible to KRAS-type ADC, while Asian populations is more susceptible to EGFR-type ADC, due to the difference in either or both genetic and environmental factors.

III. CANDIDATE GENE ASSOCIATION STUDIES

Histological heterogeneity of lung cancer has been known for many years. However, lung cancers in never-smokers have not been classified into a different disease until recently. Therefore, in the last two decades, genetic susceptibility for tobacco-induced lung cancer has been extensively investigated by a candidate gene approach focusing on the metabolism of tobacco smoke carcinogens and the suppression of tobacco-induced genetic alterations. Lung cancer cells developed in smokers have been shown to have a unique mutation spectrum, with an excess of G:C to T:A transversions (Hollstein *et al.*, 1991; Le Calvez *et al.*, 2005). Therefore, associations of metabolic enzyme activities as well as DNA repair activities to induce or prevent G:C to T:A transversions have been a focus of genetic susceptibility studies in lung cancer (Govindan, 2010). Benzo[a]pyrene (BP) is a major polyaromatic hydrocarbon (PAH) in tobacco smoke, and benzopyrene-diol-epoxide (BPDE), a metabolite of BP (Alexandrov *et al.*, 2002; Rubin, 2001), forms a DNA adduct and induces G:C to T:A transversions at hot spot codons in the p53 gene in lung cancers of smokers (Le Calvez *et al.*, 2005). Cytochrome P450 (CYP)-related enzymes and glutathione-*S*-transferases (GSTs) are representative metabolic enzymes for tobacco smoke carcinogens because their polymorphisms have been extensively investigated in association with risk for lung cancer, particularly for SQC (Bartsch *et al.*, 2000). CYP1A1 bioactivates PAHs, such as BP, and a single nucleotide polymorphism (SNP) of Ile462Val in the CYP1A1 gene causes the difference in the enzymatic ability. The 462Val allele encodes a protein with a higher activity

to bioactivate PAHs than the 462Ile allele, and individuals carrying the 462Val allele have been shown to have higher risk to lung cancer than those carrying the 462Ile allele. In contrast, GSTs detoxify tobacco carcinogens such as PAH, and individuals lacking GSTM1 (null-type in an insertion/deletion polymorphism) have been shown to have an elevated risk for lung cancer. There have also been extensive works on the role of DNA repair genes as a determinant of inherited susceptibility to lung cancer (Schwartz *et al*., 2007). For instance, in 1999, we first reported the possible contribution of OGG1 SNPs to lung SQC risk (Sugimura *et al*., 1999). 8-oxo-deoxyguanosine (8-oxo-dG) is a major form of oxidative DNA damage induced by reactive free radicals and is highly mutagenic with frequent induction of G:C to T:A transversions both *in vitro* and *in vivo*. The OGG1 gene encodes an oxo-guanine DNA glycosylase that removes 8-oxo-dG from double-stranded DNA, thus preventing the occurrence of G:C to T:A transversions induced by 8-oxo-dG. The risk (326Cys) allele for the Ser326Cys SNP in the OGG1 gene encodes a DNA glycosylase with a weaker activity to repair 8-oxo-dG, in part produced by tobacco carcinogens, than the 326Ser allele (Kohno *et al*., 1998; Yamane *et al*., 2004). TP53 and MDM2 are also representative DNA repair genes associated with lung cancer risk (Bond and Levine, 2007; Imyanitov, 2009; Whibley *et al*., 2009). The risk (72Pro) allele for the TP53-Arg72Pro SNP in the TP53 gene encodes a protein with a weaker apoptotic activity, thus allowing better survival of cells with DNA damages than the 72Arg allele. The risk (G) allele for a T/G SNP in the promoter region of the MDM2 gene (which is called MDM2 SNP309) allows a lower level of expression of MDM2 protein to suppress TP53 function than the T allele.

Table II summarizes lung cancer susceptibility genes identified to date by candidate gene association studies, and confirmed as being consistently associated with lung cancer risk by recent meta-analyses or pooled analyses of various studies (Dai *et al*., 2009; Dong *et al*., 2008; Kohno *et al*., 2006; Li *et al*., 2008; Wilkening *et al*., 2007; Ye *et al*., 2006). However, in most of these analyses, histological differences are not critically analyzed, probably because histological types were not available in some studies selected for meta-analyses. Therefore, the contribution of those polymorphisms to each histological type of lung cancers is not clear at present, although most polymorphisms are thought to be associated with the development of smoking-related lung cancer. In addition, results are different among several meta-analyses due to differences in the studies selected for meta-analyses. Such differences would be due to the quality of each study selected for the analysis. Therefore, to make uniform the quality among studies, several international consortiums have been established to date; thus, more reliable data will be available for various functional SNPs in the near future.

Table II Lung Cancer Susceptibility Genes Identified by Candidate Gene Association Studies (Meta-Analysis)

Gene	Gene product	Function	Polymorphism	SNP ID	Odd ratio	References
CYP1A1	Cytochrome P450	Phase I metabolism	Ile462Val	rs1048943	2.36	Dong *et al.* (2008)
mEH (EPHX1)	Epoxide hydrolase	Phase I metabolism	His113Tyr	rs1051740	0.70	Dong *et al.* (2008)
MPO	Myeloperoxidase	Phase I metabolism	G-463A	rs2333227	0.71	Dong *et al.* (2008)
GSTM1	Glutathione-*S*-transferase	Phase II metabolism	Presence/null	–	1.18	Ye *et al.* (2006)
GSTT1	Glutathione-*S*-transferase	Phase II metabolism	Presence/null	–	1.28	Dong *et al.* (2008)
XPA	Nucleotide excision repair protein	Mutation suppression	G-23A	rs1800975	0.73	Dong *et al.* (2008)
XPC	Nucleotide excision repair protein	Mutation suppression	Lys939Gln	rs2228001	1.30	Dong *et al.* (2008)
XPD	Nucleotide excision repair protein	Mutation suppression	Lys751Gln	rs1052559	1.30	Dong *et al.* (2008)
XRCC1	Base excision repair protein	Mutation suppression	Arg399Gln	rs25487	1.34	Dong *et al.* (2008)
OGG1	Base excision repair protein	Mutation suppression	Ser326Cys	rs1052133	1.32	Li *et al.* (2008)
OGG1 (ADC)					1.43	Kohno *et al.* (2006)
TP53	Transcription factor	Cell cycle/death regulation	Arg72Pro	rs1042522	1.20	Dai *et al.* (2009)
MDM2	Ubiquitine ligase	Cell cycle/death regulation	T309G	rs2279744	1.27	Wilkening *et al.* (2007)

IV. GENOME-WIDE ASSOCIATION STUDIES

Recent GWA studies have lead to the identification of a number of candidate lung cancer susceptibility genes (Table III). Three chromosomal loci, 15q24-25.1, 5p15.33, and 6p21, have been shown to be associated with lung cancer risk in Europeans and Americans (Amos *et al*., 2008; Hung *et al*., 2008; McKay *et al*., 2008; Thorgeirsson *et al*., 2008; Wang *et al*., 2008). The chromosome 15q24-25.1 region contains the nicotinic acetylcholine receptor subunit genes, CHRNA3 and CHRNA5, and their products are expressed in pulmonary epithelial cells including neuroendocrine cells and bind to nicotine. Therefore, the association of this locus with lung cancer risk could be primarily mediated by nicotine dependence as described below. The 5p15.33 region contains the TERT (telomerase reverse transcriptase) gene and the CLPTM1L (cleft lip and palate transmembrane protein 1-like) gene. TERT is known to function in telomere replication and maintenance, and to promote epithelial cell proliferation. CLPTM1L was identified through screening for cisplatin (CDDP) resistance-related genes. Interestingly, this locus is associated with the risk for ADC but not for SQC or SCLC, suggesting the weak association of this locus with lung cancer risk in smokers (Landi *et al*., 2009). Indeed, the 5p15.33 (TERT-CLPTM1L) genotypes were shown to be associated with lung ADC risk in never-smokers (Wang *et al*., 2010). Associations of the 5p15.33 genotypes have been detected not only in lung cancer but also in various other types of cancers, including cancers of the brain, bladder, prostate, uterine cervix, and skin (Rafnar *et al*., 2009; Stacey *et al*., 2009). Therefore, it is likely that genotypes of this locus are associated with the development of a wide variety of cancers. Association with lung cancer risk of a SNP in the CHRNA3 gene at 15q24-25.1 was replicated in a Japanese population, although the frequency of the risk variant in the Japanese is much lower than that in Europeans and Americans (Kohno *et al*., 2010). In a Chinese population, the association of SNPs in this locus with lung cancer risk was also replicated; however, risk variants seem to be different from Europeans and Americans (Wu *et al*., 2009). Interestingly, in Asian populations, the associations of 15q24-25.1 SNPs with lung cancer risk were independent of smoking behavior. Associations with lung cancer risk of SNPs in the 5p15.33 region were validated in both Japanese and Chinese populations (Jin *et al*., 2009; Shiraishi *et al*., 2009), and the TERT gene was indicated to be a more likely target rather than the CLPTM1L gene.

The 6p21 region contains the BAT3 (HLA-B associated transcript 3) and MSH5 (mutS homolog 5) genes. BAT3 protein complexes with a histone acetyltransferase (HAT), p300, which acetylates p53 protein in response to DNA damage. MSH5 is a gene involved in DNA mismatch repair.

Table III Lung Cancer Susceptibility Genes Identified by Genome-Wide Association Studies (GWAS)

Chromosomal location	Gene	Risk allele	Geographic area	Frequency in control population	Allele OR (95% CI, P)	Case/control	References
15q25.1	*CHRNA3*[a]	rs1051730-T	USA and UK	0.33	1.32 (1.23–1.39, 7.0×10^{-18})	2013/3062	Amos *et al.* (2008)
			Central Europe	0.33	1.30 (1.19–1.43, 5.4×10^{-9})	1922/2520	Hung *et al.* (2008)
			Iceland	0.35	1.31 (1.19–1.44, 1.5×10^{-8})	1024/32,244	Thorgeirsson *et al.* (2008)
			Europe, USA and Canada	0.35	1.31 (1.27–1.36, 1.9×10^{-51})	13,300/19,666	Landi *et al.* (2009)
			Japan	0.02	1.79 (1.19–2.78, 0.0095)	2343/1173	Kohno *et al.* (2010)
5p15.33	*TERT*[b]	rs2736100-G	Europe and Canada	0.49	1.19 (1.11–1.27, 2×10^{-6})	2971/3746	McKay *et al.* (2008)
			Europe, USA and Canada	0.50	1.12 (1.08–1.16, 1.6×10^{-10})	13,300/19,666	Landi *et al.* (2009)
			USA and Europe	0.51	1.15 (1.10–1.20, 1×10^{-10})	9162/11,812	Truong *et al.* (2010)
			Asia, USA and Canada	0.39	1.23 (1.12–1.35, 2×10^{-5})	1686/2101	
			China	0.42	1.16 (1.03–1.30)	1221/1344	Jin *et al.* (2009)
			Japan	0.38	1.38 (1.23–1.56, 6.3×10^{-8})	2343/1173	Kohno *et al.* (2010)
	CLPTM1L[c]	rs402710-C	Europe and Canada	0.68	1.18 (1.12–1.24, 2×10^{-7})	2971/3746	McKay *et al.* (2008)
			USA and Europe	0.65	1.14 (1.09–1.19, 5×10^{-8})	8860/9198	Truong *et al.* (2010)
			Asia, USA and Canada	0.68	1.15 (1.04–1.27, 0.007)	1680/2117	
			China	0.69	1.09 (0.97–1.24)	1221/1344	Jin *et al.* (2009)
			Japan	0.65	1.10 (0.97–1.23, 0.15)	2343/1173	Kohno *et al.* (2010)
		rs401681-C	USA, UK and central Europe	0.55	1.15 (1.09–1.19, 7.9×10^{-9})	5095/5200	Wang *et al.* (2008)
			Europe, USA and Canada	0.56	1.12 (1.09–1.16, 6.7×10^{-11})	13,300/19,666	Landi *et al.* (2009)
			Japan	0.67	1.14 (1.01–1.28, 0.044)	2343/1173	Kohno *et al.* (2010)
6p21.33	*BAT3*[d]*–MSH5*[e]	rs3117582-C	USA, UK and central Europe	0.10	1.24 (1.16–1.33, 5.0×10^{-10})	5095/5200	Wang *et al.* (2008)
			Europe, USA and Canada	0.10	1.22 (1.15–1.29, 4.8×10^{-12})	13,300/19,666	Landi *et al.* (2009)
			Japan	0	–	525/525	Kohno *et al.* (2010)
6p21.31	*HLA-DQA1*[f]	*03	Japan	0.36	1.36 (1.20–1.54, 5.3×10^{-7})	1656/1173	Kohno *et al.* (2010)

[a]Cholinergic receptor, nicotinic, alpha 3.
[b]Telomerase reverse transcriptase.
[c]Cleft lip and palate transmembrane protein 1-like protein.
[d]HLA-B associated transcript 3.
[e]mutS homolog 5.
[f]Major histocompatibility complex, class II, DQ alpha 1.

Therefore, both genes are attractive candidates for lung cancer susceptibility genes; however, a recent pooled analysis from the international lung cancer consortium did not replicate the association of these SNPs with lung cancer risk (Truong *et al.*, 2010). The significance of SNPs at 6p21 on lung cancer risk of Asians has not been fully investigated; however, our recent GWA study on the Japanese using 23,000 microsatellite markers for the screening indicated that the HLA-DQA1 gene, encoding a HLA (human leukocyte antigen)-class II protein, mapped at 6p21.31 is the most significant region at 6p21 (Jin *et al.*, 2009). DQA1*03 of the HLA-DQA1 gene was defined as a risk allele with odds ratio (OR) of 1.36 (95%CI = 1.21–1.54, $P = 5.3 \times 10^{-7}$) by analysis of 1656 ADC cases and 1173 controls. The HLA-DQA1 locus was mapped 1-Mb proximal to the BAT3–MSH5 locus. Therefore, we further examined a SNP in the BAT3–MSH5 locus, rs3117582, which showed a significant association in Europeans and Americans. It was monomorphic for the protective allele in the Japanese. We therefore examined seven SNPs in linkage disequilibrium (LD) with this SNP in Europeans (i.e., $D' = 1$ in the HapMap data); however, associations of these SNPs in the BAT3–MSH5 locus were weaker than genotypes of the HLA-DQA1 locus, and these SNPs comprised a distinct LD block from the locus containing the HLA-DQA1 gene. Therefore, it was concluded that the 6p21.31 region containing the HLA-DQA1 locus is a lung ADC susceptibility locus distinct from the BAT3–MSH5 locus at 6p21.33. In a recent meta-analysis, the associations of the BAT3–MSH5 locus were shown to vary among studies (Broderick *et al.*, 2009). Thus, it is possible that the BAT3–MSH5 locus and also the HLA-DQA1 locus could be affected by the difference in population structure since it is located near/in the locus for major histocompatibility complex, a highly polymorphic locus in the human genome. Therefore, further investigation of this region is warranted to conclude whether and how genotypes in this region are associated with lung cancer risk.

As described above, the incidence of lung cancer in never-smokers is increasing, and lung cancers in never-smokers are now considered to be a different disease from lung cancers in smokers. Therefore, there have been a few GWA studies for the identification of loci associated with lung cancer risk specifically of never-smokers. One of the regions identified is chromosome 6q containing the RGS17 gene, which encodes a member of the regulator of G protein signaling (RGS) family (Amos *et al.*, 2010). RGS17 was identified as a major candidate for familial lung cancer susceptibility gene at chromosome 6q23-25 (Liu *et al.*, 2010a; You *et al.*, 2009), and proliferation and tumorigenesis of human lung tumor cells in nude mice were inhibited by knockdown of RGS17 expression levels. Never-smoking individuals with a risk haplotype of this locus were shown to have a 4.7-fold higher risk than those without risk haplotypes. Another region identified by

a GWA study is chromosome 13q31.3 (Li *et al.*, 2010). This locus was identified by a four-stage association screening of lung cancers in never-smokers, and there was a strong correlation between genotypes of this locus and transcription levels of the GPC5 gene in normal lung tissues, with the high-risk allele linked with a lower level of transcription. Therefore, it was suggested that downregulation of GPC5 might contribute to the development of lung cancer in never-smokers. As described above, the TERT–CLPTM1L locus on chromosome 5p15.33 was also shown to be associated with lung cancer risk in never-smokers (Landi *et al.*, 2009; Wang *et al.*, 2010).

V. ASSESSMENT OF LUNG CANCER RISK IN EACH INDIVIDUAL BY COMBINED GENOTYPES (GENE–GENE INTERACTIONS)

For many years, gene–gene interaction has been investigated among candidate genes with functional polymorphisms. In particular, interactions among CYP-family genes and GST-family genes have been indicated by both molecular epidemiological studies and biological studies (Alexandrov *et al.*, 2002; Bartsch *et al.*, 2000; Schwartz *et al.*, 2007). Biologically, activities of CYP1A1 and GSTM1 are a critical determinant for the dose of carcinogenic BPDE and other DNA-reactive PAH; however, there has been no clear epidemiological evidence indicating the interaction between genotypes of CYP1A1 and GSTM1 on the risk for smoking-associated lung cancer risk, including SQC and SCLC. Since several lung cancer susceptibility genes have been identified by GWA studies, it is now very important to elucidate the interaction among their genotypes in the contribution to lung cancer risk. Then, we will be able to further develop a method to assess individual susceptibility to lung cancer based on the combined genotypes of several lung cancer susceptibility genes. However, up to the present, there have been only a few reports pursuing such an interaction. Here, we briefly summarize the results of three different studies investigating the effect of combined genotypes among genes identified by recent GWA studies on lung cancer risk.

In our recent study, we attempted to evaluate the combined effect among the HLA-DQA1, TERT, and CHRNA3 loci on lung ADC risk, because these three loci showed significant associations with lung ADC risk in the Japanese (Kohno *et al.*, 2010). However, the frequency of the susceptible haplotype in the CHRNA3 gene in the Japanese (0.02) was much lower than in European and American populations (0.4); therefore, interaction of CHRNA3 genotypes with HLA-DQA1 and TERT genotypes was unclear in this analysis. However, when ORs were calculated according to the number of risk alleles

for the HLA-DQA1 and TERT genes, there was an increasing trend with increasing number of risk alleles (per risk-allele OR = 1.43, $P = 7.8 \times 10^{-16}$), reaching up to OD = 4.76 for carriers of all four risk alleles. Namely, individuals homozygous both for the DQA1*03 and minor TERT alleles were defined as high-risk individuals with an OR of 4.76 (95% CI = 2.53–9.47, $P = 4.2 \times 10^{-7}$). These two alleles independently (i.e., without a significant interaction) conferred the risk (P for interaction = 0.88). This result indicates that individuals highly susceptible to ADC can be defined by combined genotypes of HLA-DQA1 and TERT.

Recently, pooled analysis was performed for the replication of lung cancer susceptibility loci at chromosomes 15q24-25.1, 5p15.33, and 6p21 (Truong *et al.*, 2010). Associations between 15q24-25.1 variants and the risk for lung cancer were replicated in white ever-smokers; however, there were no such associations in never-smokers or in Asians. For the chromosome 5p15.33 region, statistically significant associations were confirmed in both whites and Asians. The 6p21 variants were not associated with the risk for lung cancer. Therefore, in this study, associations of the combined genotypes for the 15q24-25.1 locus (rs16969968) and the 5p15.33 locus (rs2736100 and rs402710) with the risk for lung cancer were further analyzed in whites. The OR of lung cancer risk for homozygotes of the three risk variants compared with individuals with no risk allele was 2.64 (95%CI = 1.86–3.74, $P=4 \times 10^{-8}$; per risk-allele OR = 1.15, $P = 1 \times 10^{-26}$). Liu *et al.* recently determined the cumulative association of four loci, 5p15.33, 6p21.33, 6q23-25, and 15q24-25.1, with familial lung cancer risk (Liu *et al.*, 2010a). The results indicate a stronger cumulative association of any combined genotype than any individual genotype with familial lung cancer. The risk for lung cancer was increased to 3- to 11-fold among those who had at least one copy of the risk allele at each locus in comparison with those who did not have any of the risk alleles.

The results of those three studies are consistent and indicate the cumulative effect of the SNPs in three chromosomal regions, 5p15.33, 6p21, and 15q24-25.1, on the genetic susceptibility to lung cancer, although interactions among those SNPs in lung cancer risk are unlikely.

VI. SMOKING-ASSOCIATED DIFFERENCES (GENE–ENVIRONMENT INTERACTIONS)

Cigarette smoking increases the risk for all three major histological types of lung cancers, although the risk is less for ADC than for SQC and SCLC (Govindan, 2010; Sobue *et al.*, 2002; Subramanian and Govindan, 2008;

Sun *et al.*, 2007; Travis *et al.*, 2004). The smoking habit is largely attributed to nicotine dependence, because nicotine is addictive. Therefore, although nicotine itself is not carcinogenic, it has been assumed that nicotine dependence is indirectly associated with lung cancer risk by primarily causing the smoking habit and consequently resulting in the increase of tobacco carcinogen intake (Hecht, 2004). Recent GWA studies have identified an association of a common variant in the chromosome 15q24-25.1 region with lung cancer susceptibility (Amos *et al.*, 2008; Hung *et al.*, 2008; Thorgeirsson *et al.*, 2008). The region of ~200 kb in size with high LD contains six genes, and three of them encode nicotine acetylcholine receptor subunits, CHRNA5/A3/B4. This locus has been identified as being associated with nicotine dependence and smoking quantity by several studies (Lips *et al.*, 2010; Liu *et al.*, 2010b; Spitz *et al.*, 2008; Thorgeirsson *et al.*, 2008). Furthermore, this locus has been also identified as being associated with risk for several smoking-related diseases, such as chronic obstructive pulmonary disease (COPD) (Pillai *et al.*, 2009) and peripheral arterial disease (PAD) (Thorgeirsson *et al.*, 2008). Associations of this locus with smoking quantity and several smoking-related diseases, including lung cancer, support that the CHRNA genotypes are at least, in part, indirectly associated with lung cancer risk through smoking behavior. In contrast, associations with lung cancer risk in never-smokers as well as associations with an earlier age of lung cancer onset indicate the direct association of genotypes with lung cancer risk in a smoking behavior-independent manner. Associations with lung cancer risk after adjusting smoking habit also support the direct effect of genotypes on lung cancer risk. For this reason, associations of the CHRNA3/A5/B4 genotypes with lung cancer risk have been extensively and carefully investigated together with those with smoking behavior and nicotine dependence. However, several inconsistent results have been reported to date; thus, further studies are warranted.

Another example of smoking-associated differences is CYP family–GST family gene polymorphisms associated with smoking-related lung cancers, as described. However, to our knowledge, modifications by smoking behavior and/or smoking quantity of the associations between those genotypes and lung cancer risk have not yet been critically analyzed to date. Importantly, a recent study further indicated the association of other CHRNA genes, CHRNB3 and CHRNA6, on chromosome 8p11, with smoking and nicotine dependence as well as lung cancer risk (Thorgeirsson *et al.*, 2010). Interestingly, in their study, the association was also observed between the chromosome 19q13 region and smoking behavior as well as lung cancer risk. The 19q13 region contains the CYP2A6 gene, whose products have an enzyme activity to oxidize nicotine and to activate procarcinogenic nitrosamines.

VII. NECESSITY OF FURTHER ASSOCIATION STUDIES

To obtain more conclusive information on the genetic basis for susceptibility to lung cancer, we will have to analyze all the polymorphic sequences in the human genome for association with susceptibility. Various SNP array platforms have been developed to date, and the numbers of SNPs analyzable in one platform have been increasing year by year. In 2010, over a million SNPs can be analyzed by a single SNP array. However, it has been assumed that there are at least 10 million SNPs with a minor allele frequency (MAF) $>1\%$ and 5 million SNPs with a MAF $>10\%$ (Chung *et al.*, 2010; Frazer *et al.*, 2009). Therefore, although recent GWA studies have lead to the identification of several lung cancer susceptibility genes, it is still possible that there are several additional SNPs involved in the susceptibility in the human genome. In particular, several functional polymorphisms which have been identified by candidate gene association studies to date are not mounted on major SNP array platforms used in previous GWA studies, such as Affymetrix 500 K/1 M and Illumina HumanHap 300/550. Therefore, at present, it is not possible to obtain association data for those functional polymorphisms together with those for SNPs identified by GWA studies using SNP arrays. For this reason, we recently performed an association study of lung SQC for genes identified by GWA studies (CHRNA3, TERT, and HLA-DQA1) and genes identified by candidate gene association studies (TP53, MDM2, OGG1, CYP1A1, and GSTM1), because associations of these candidate gene polymorphisms were not investigated in recent GWA studies due to the lack of probes to discriminate these polymorphisms in the platforms used for GWA studies (Kohno *et al.*, in press). Genotypes for the TP53 and OGG1 genes showed significant associations with SQC risk in addition to those for the CHRNA3 and HLA-DQA1 genes to similar extents. Therefore, it will be necessary to reevaluate the significance of polymorphisms identified only by candidate gene association studies in several populations together with SNPs identified by GWA studies. In addition, it has been assumed that rare variants with frequencies less than 1% would play much more important role than common SNPs with a MAF $>10\%$ for the susceptibility to various diseases (Ioannidis *et al.*, 2010; Knerr *et al.*, 2010; McClellan and King, 2010). Therefore, a further technological advancement is absolutely required for the assessment of the role of rare variants in cancer susceptibility. Genetic polymorphisms include not only SNPs but also structural variations and copy number variations (CNVs) (Feero *et al.*, 2010; Frazer *et al.*, 2009). However, structural variations and CNVs are not yet easily analyzable at the genome-wide level. A CNV at 1q21.1 was recently shown to be associated with neuroblastoma susceptibility (Diskin *et al.*, 2009); therefore, development of a novel and easy

analytical method for CNVs throughout the human genome will be also necessary to finally identify all types of genetic polymorphisms associated with lung cancer risk.

Allele frequencies of several lung cancer susceptibility genes are different among different ethnic and geographic groups. Therefore, contribution of each susceptibility gene to lung cancer risk, represented by OR, is also considerably different among different ethnic/geographic groups. Figure 3 shows the differences in the frequencies of risk alleles for representative lung cancer susceptibility genes among Japanese, Chinese, Europeans, and Africans. Risk alleles for the CYP1A1 and OGG1 genes are more frequent in Asians than Europeans and Africans. In contrast, the risk allele for the CHRNA5 gene is more frequent in Europeans than Asians. Accordingly, comparative studies of polymorphisms with different allele frequencies and ORs among different ethnic/geographic groups will enable us to clarify the differences in lung cancer susceptibility

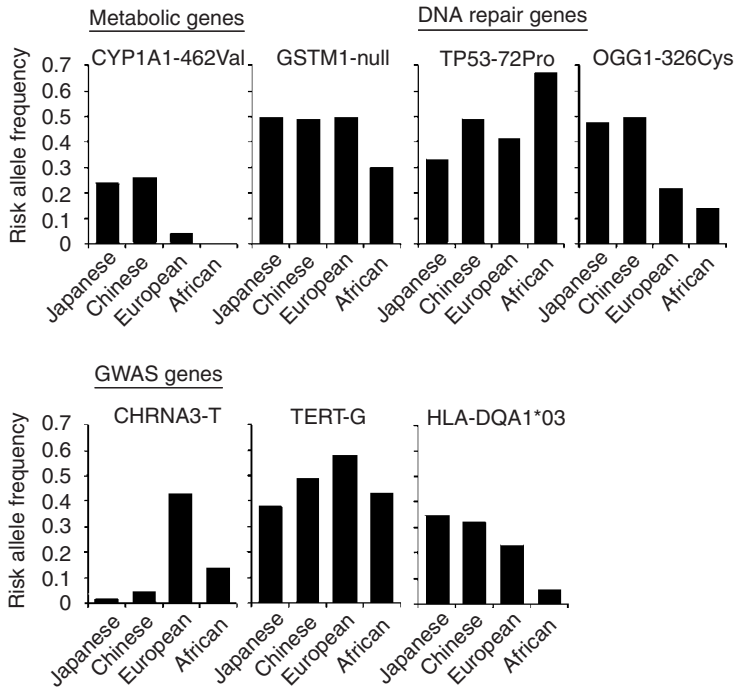

Fig. 3 Frequencies of risk alleles for lung cancer among different ethnic groups. As representatives, allele frequencies of seven genes in Japanese, Chinese, Europeans, and Africans are shown. Allele frequencies determined by the HapMap project, by the International Histocompatibility Working Group projects, by Ye *et al.* (2006), and by us (Kohno *et al.*, 2010; Shiraishi *et al.*, 2009) are combined in each column.

among different populations. For instance, lung ADCs with EGFR mutations in female nonsmokers are more common in Asian than in Americans and Europeans. However, it is still unknown whether or not such a difference is due to the difference in the distribution of risk alleles of some lung cancer susceptibility genes among these populations.

VIII. FUTURE DIRECTIONS

Recent GWA studies have identified three lung cancer susceptibility gene loci at chromosomes 15q24-25.1, 5q15.33, and 6p21. The 15q24-25.1 locus is associated not only with lung cancer but also with smoking behavior and other smoking-related diseases. Associations of the 15q24-25.1 genotypes with lung cancer risk in never-smokers and with lung ADC risk have been inconsistently observed among studies. In addition, the frequency of the risk allele is markedly different among ethnic groups. Therefore, further genetic studies as well as biological studies will be necessary to conclude whether the 15q24-25.1 genotypes play a direct or indirect role in the development of lung cancer, and how commonly/differentially the 15q24-25.1 genotypes contribute to lung cancer risk among different ethnic groups. The 5p15.33 locus is associated with risks not only for lung cancer but also for a variety of cancers, and the risk allele is prevalent among different ethnic groups. Therefore, this locus is likely to be associated with risks in general for a wide variety of cancers, irrespective of ethnic groups. Association of the 6p21 locus, containing the BAT3, MSH5, and HLA-DQA1 genes, with lung cancer has not yet been well reproduced by other genome-wide scale association studies. Therefore, further studies are necessary to obtain more convincing information for this locus in the association with lung cancer risk. Reevaluation of functional polymorphisms identified by candidate gene association studies will also be important for the assessment of individual risk for lung cancer (Wilkening *et al.*, 2009).

Lung cancers in never-smokers have been considered to be a different disease from those in ever-smokers. Associations have been observed between the 5p15.33, 6q23-25, and 13q31.3 genotypes and lung cancer risks in never-smokers. However, lung cancers in never-smokers are more common in women than in men, and also more common in Asian populations than in American and European populations (Govindan, 2010; Reid *et al.*, 2008; Subramanian and Govindan, 2008; Sun *et al.*, 2007). Therefore, it will be very important to elucidate interactions among genotypes, gender, and ethnicity/geography on lung cancer risk in never-smokers. The most frequent type of lung cancer in never-smokers is ADC; however, lung ADC is now considered to be a heterogeneous disease with respect to accumulated genetic alterations in cancer cells. EGFR-types are more frequent in Asian

populations, while KRAS-types are more frequent in American and European populations. However, it is still unknown whether such a difference is due to genetic differences or environmental differences. Identification of lung ADC susceptibility genes in never-smokers will facilitate the identification of environmental factors by subsequent functional analyses of identified genes. The elucidation of associations among such genetic factors, environmental factors other than smoking, and acquired genetic alterations in cancer cells will help us understand the molecular mechanisms underlying lung carcinogenesis in never-smokers and develop methods of its prevention.

Lastly, we point out here that none of polymorphisms have been identified yet to specifically define the risk for tobacco-induced lung cancer, such as SCC and SCLC. The 15q24-25.1 region containing three CHRNA genes is associated with smoking behavior as well as lung cancer susceptibility; however, it is still unclear whether this locus is associated with lung cancer risk among heavy smokers or not. Since only one in 10 smokers is estimated to develop lung cancer (Reid *et al.*, 2008), individual risks for lung cancer by smoking would be different due to genotype differences. Polymorphisms in genes for metabolism of tobacco smoke carcinogens and those for repair of carcinogen-induced genetic alterations have been considered as being candidates for many years. However, their significance is still unclear at present. Therefore, association studies of those genotypes with lung cancer risk by considering the smoking quantity, such as the number of cigarettes smoked per day (CPD), will be also important in assessing the individual risk for lung cancer in smokers. For this reason, the recently identified CYP2A6 gene locus will be another candidate to define lung cancer susceptibility in smokers (Thorgeirsson *et al.*, 2010).

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Functional and Clinical Relevance of Chondroitin Sulfate Proteoglycan 4

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The lack of effective conventional therapies for the treatment of advanced stage melanoma has stimulated interest in the development of novel strategies for the management of patients with malignant melanoma. Among them, immunotherapy has attracted much attention because of the potential role played by immunological events in the clinical course of melanoma. For many years, T cell-based immunotherapy has been emphasized in part because of the disappointing results of the monoclonal antibody (mAb)-based clinical trials conducted in the early 1980s and in part because of the postulated major role played by T cells in tumor growth control. More recently, mAb-based therapies have gained in popularity given their clinical and commercial success for a variety of malignant diseases. As a result, there has been increased interest in identifying and characterizing antibody-defined melanoma antigens. Among them, the chondroitin sulfate proteoglycan 4 (CSPG4), also known as high molecular weight-melanoma associated antigen (HMW-MAA) or melanoma chondroitin sulfate proteoglycan

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(MCSP), has attracted much attention in recent years because of the growing experimental evidence that it fulfills two requirements for immunotherapy to be therapeutically effective: (1) targeting of cancer stem cells (CSC) and (2) development of combinatorial therapies to counteract the escape mechanisms driven by the genetic instability of tumor cells. With this in mind, in this chapter, we have reviewed recent information related to the distribution of CSPG4 on various types of tumors, including CSC, its expression on pericytes in the tumor microenvironment, its recognition by T cells, its role in cell biology as well as the potential mechanisms underlying the ability of CSPG4-specific immunity to control malignant cell growth.

I. INTRODUCTION

Malignant melanoma represents the most common form of fatal skin cancer. Current estimates indicate that its incidence is increasing at a rate of 5% per year (MacKie *et al.*, 2009). In spite of significant improvements in diagnosis (Terando *et al.*, 2003), the mortality rate for melanoma has been continually increasing over the past decade and the disease represents one of the most common fatal malignancies of young adults (MacKie *et al.*, 2009). While early stage melanoma is highly curable with surgery (Crosby *et al.*, 2001), advanced stage melanoma is relatively resistant to conventional therapeutic regimens and is often fatal (Cascinelli *et al.*, 2003; Cooper, 2002; Soengas and Lowe, 2003). Although promising results have been obtained in recent clinical trials with inhibitors of the extracellular signal-related MAP kinase pathway, such as B-Raf and MEK inhibitors (Flaherty *et al.*, 2010), the lack of effective conventional therapies for the treatment of advanced stage disease has stimulated interest in the application of novel strategies for the management of patients with malignant melanoma. Among them, immunotherapy has been emphasized in recent years for a number of reasons. First, the immune system appears to play a role in the natural history of melanoma (Guerry, 1998). Second, the recent success of monoclonal antibody (mAb)-based therapies in patients with some hematological malignancies and some solid tumors (Campoli and Ferrone, 2009), the Federal Drug Administration (FDA) approval of interferon-α2b (Kirkwood *et al.*, 1996) and interleukin-2 (IL-2) (Atkins *et al.*, 1999) for use as adjuvant therapy for patients with advanced stage melanoma as well as the promising results obtained with the CTLA-4 specific mAb ipilimumab in patients with melanoma (Hodi *et al.*, 2010) and the recent FDA approval of PROVENGE® (sipuleucel-T), an autologous cellular vaccine designed to stimulate T-cell immunity to prostatic acid phosphatase in patients with asymptomatic or minimally symptomatic metastatic (hormone refractory) prostate cancer (Cha and Fong, 2010), provide a rationale for the use of immunotherapy to treat patients with melanoma. Third, the identification of well-characterized melanoma antigens (MA) (Ribas *et al.*, 2003; Stevanovic, 2002) and the development of their

corresponding probes, that is, mAb (Weiner *et al.*, 2010) and cytotoxic T lymphocytes (CTL) (Rosenberg and Dudley, 2009), have provided moieties to target melanoma lesions with highly specific reagents. Fourth, the substantial increase in our understanding of the molecular events leading to an immune response as well as the development of effective immunization strategies have facilitated the application of immunotherapy for the treatment of melanoma (Alexandrescu *et al.*, 2010).

To date, a number of MA that meet the criteria to be utilized for immunotherapy, that is, high expression in a large percentage of melanoma lesions and restricted distribution in normal tissues, have been identified and utilized to implement clinical trials in patients with melanoma. During the past 20 years, the use of T cell-defined MA has been emphasized because of the disappointing results obtained with mAb-based immunotherapy in the early 1980s (Milstein and Waldmann, 1999) and because of the general belief that T cells play a major role in tumor growth control (Dudley *et al.*, 2002; Yee *et al.*, 2002). More recently, mAb-based therapies have gained in popularity given their clinical and commercial success for a variety of malignant diseases (Campoli and Ferrone, 2009). The beneficial clinical effects of mAb-based immunotherapy are believed to reflect the ability of antigen-specific mAb to (i) inhibit tumor cell proliferation; (ii) induce tumor cell apoptosis; (iii) trigger antibody-dependent cell-mediated cytotoxicity (ADCC); (iv) mediate complement-dependent cytotoxicity (CDC); and (v) interfere with the function of the targeted antigen and/or affect tumor cell signaling (Campoli and Ferrone, 2009). More recent evidence suggests that tumor antigen (TA)-specific mAb can induce TA-specific T cell immune responses (Campoli *et al.*, 2010). We have focused our studies on antibody-defined MA, since mAb can utilize both immunological as well as nonimmunological mechanisms to control tumor growth. As a result, they are less susceptible to the escape mechanisms caused by defects in TA presentation which are frequent in melanoma cells (Marincola *et al.*, 2000).

The MA that has been the focus of our studies over the past 30 years is the chondroitin sulfate proteoglycan 4 (CSPG4), also known as high molecular weight-melanoma associated antigen (HMW-MAA or melanoma chondroitin sulfate proteoglycan (MCSP) (Campoli *et al.*, 2004). CSPG4 represents an attractive target, since it has a restricted distribution in normal tissues and is expressed in a large percentage of melanoma lesions, on putative cancer stem cells (CSC) and on activated pericytes in the tumor microenvironment. Furthermore, CSPG4 is recognized by both antibodies and T cells. As a result, CSPG4 provides the opportunity to apply combinatorial antibody- and T cell-based immunotherapy to target not only tumor cells including CSC, but also cells in the tumor microenvironment that are required for tumor cell survival and growth.

A considerable amount of information has been accumulated in recent years regarding (1) the distribution and expression of CSPG4 on malignant cells as well as on both normal stem cells and CSC; (2) the role CSPG4 plays in the biology of both normal and malignant cells, and (3) the ability of the adaptive immune system to target CSPG4. In this review, we highlight the properties of CSPG4, that make it a suitable target of immunotherapy for the treatment of patients with malignant disease, mainly emphasizing the information published during the past 5 years and the results of studies in progress in our laboratory. However, we have revisited several concepts regarding CSPG4 that have been derived from previous investigations, since they now need to be reinterpreted in light of the results which have recently become available. Additionally, we have discussed the role CSPG4 plays in the biology of malignant cells with emphasis on the recently identified signal transduction pathways triggered by CSPG4. Finally, we have discussed the potential mechanisms underlying the ability of CSPG4-specific immunity to control malignant cell growth.

II. PHYLOGENETIC EVOLUTION, STRUCTURE, AND TISSUE DISTRIBUTION OF CSPG4

In the early 1980s the interest in the use of CSPG4 as a target of immunotherapy for the treatment of melanoma and the availability of mAb provided the stimulus to investigate CSPG4 expression in melanoma lesions and in normal tissues, the structural profile of CSPG4 as well as the cross-reactivity of human CSPG4-specific mAb with xenogeneic homologues of human CSPG4. More recently taking advantage of the information about the nucleotide sequence of xenogeneic homologues of CSPG4, its phylogenetic evolution has been analyzed. Moreover, the potential role of CSPG4 in the biology of malignant cells has stimulated interest in analyzing CSPG4 expression in various types of malignancies and on CSC.

A. Xenogeneic Homologues of CSPG4

Evidence that CSPG4 homologues are expressed in other animal species has been derived from the cross-reactivity of human CSPG4-specific mouse mAb with normal and malignant lesions as well as cell lines from other animal species and more recently from the homology of CSPG4 amino acid (AA) sequence (Accession # gene: GI:37640684; mRNA: GI:21536290; protein: GI:4503099) (Pluschke *et al.*, 1996) with that of proteins expressed in other animal species. Testing of CSPG4-specific mouse mAb and human single chain

Fv fragments (scFv) has shown cross-reactivity with a fish melanoma cell line (Clauss *et al*., 1990), guinea pig melanoma cell lines and normal tissues (Desai *et al*., 1998; Liao *et al*., 1987), rat glial cells (Ferrone, unpublished results), rabbit smooth muscle cells (Schlingemann *et al*., 1990), swine melanoma cell lines (Clauss *et al*., 1990), canine melanoma cell lines and surgically removed melanoma lesions (Ferrone, unpublished results), and cynomolgus monkey smooth muscle cells (Chattopadhyay *et al*., 1992). In both rabbit smooth muscle cells and cynomolgus monkey tissues, it remains to be determined whether these species express a counterpart to human CSPG4 or merely the antigenic determinant recognized by the CSPG4-specific mAb used for testing. In the case of guinea pig, rat and rabbit, analysis of a panel of surgically removed normal tissues with CSPG4-specific mAb has demonstrated that these potential homologues have a restricted tissue distribution similar to that of CSPG4 in humans (Ferrone *et al*., 1983). It is noteworthy that distinct CSPG4-specific mAb which cross-block each other in their binding to human melanoma cells may display different reactivity patterns with rat glial cells and canine melanoma lesions. These results are compatible with the recognition of distinct, although spatially close determinants by the mAb tested.

Comparison of the predicted AA sequence of human CSPG4 with that of chondroitin sulfate proteoglycans derived from the zebra fish (*Danio rerio*), western clawed frog (*Xenopus* (*Silurana*) *tropicalis*), mouse (*Mus musculus*) chondroitin sulfate proteoglycan designated AN2 (Schneider *et al*., 2001), rat (*Rattus norvegicus*) membrane spanning nerve/glial antigen 2 (NG2) chondroitin sulfate proteoglycan (Stallcup, 2002), chicken (*Gallus gallus*), domestic dog (*Canis lupus familiaris*), cattle (*Bos Taurus*), Camargue horse (*Equus caballus*), rhesus monkey (*Macaca mulatta*), and chimpanzee (*Pan troglodytes*) has shown that these chondroitin sulfate proteoglycans are the potential zebra fish, frog, mouse, rat, chicken, dog, cattle, horse, rhesus monkey, and chimpanzee homologues of human CSPG4. These findings provide a molecular basis for the cross-reactivity of mouse antihuman CSPG4 mAb with tissues and cell lines from other animal species. The CSPG4 genes for frog, mouse, rat, chicken, dog, cattle, horse, rhesus monkey, and chimpanzee CSPG4 encode 506 (Accession number CAL49401), 2327 (Accession number: NP_620570 NP_796079), 2326 (Accession number: NP_112284), 2239 (Accession number: XP_423277), 2734 (Accession number: XP_544783), 2319 (Accession number: XP_613126), 2321 (Accession number: XP_001493494), 2210 (Accession number XP_002804928), and 2322 (Accession number: XP_001144835) AA long proteins, respectively. With the exception of frog chondroitin sulfate proteoglycan, each of the animal homologues shares over 80% AA sequence identity with CSPG4 and with each other. Whether frog chondroitin sulfate proteoglycan truly represents a homologue to human CSPG4 remains to be determined since it only possesses homology with the

extracellular laminin G-type motifs of CSPG4. Of the potential animal homologues, NG2 and AN2 appear to be the closest to CSPG4, since they each share over 90% homology in their AA sequence with that of CSPG4. AA differences between human CSPG4 and its homologues identified in various animal species are spread throughout the full length coding sequence of each protein, suggesting that the primary structure of these proteins is evolutionarily conserved. A unique repeating structural motif (termed a CSPG repeat), which has been identified in the central ectodomain of CSPG4, NG2, and AN2, demonstrates similarity to cadherin repeats (Staub *et al.*, 2002). The latter have been identified in other proteins from cyanobacterium, fly, sea urchin, and worm, suggesting that they are evolutionarily conserved (Staub *et al.*, 2002).

B. Structure, Biosynthesis, and Assembly of CSPG4

CSPG4 represents a highly glycosylated integral membrane chondroitin sulfate proteoglycan, consisting of an N-linked 280 kDa glycoprotein component and a 450 kDa chondroitin sulfate proteoglycan component expressed on the membrane of cells (Ross *et al.*, 1983) (Fig. 1). The 280 kDa and 450 kDa components of CSPG4 contain the same core protein and appear to be expressed independently on the cell membrane (Campoli *et al.*, 2004). The protein can be expressed with or without covalently attached chondroitin sulfate glycosaminoglycan (GAG) and is therefore considered a "part-time" cell surface proteoglycan. Chondroitin sulfate modification of the core protein has been linked to its ability to bind the heparin-binding domain of fibronectin. Like most glycoproteins, both the 280 kDa CSPG4 core protein and the 450 kDa proteoglycan are post-translationally modified in the *trans*-Golgi network through glycosylation and sulfation of carbohydrate moieties (Garrigues *et al.*, 1986; Spiro *et al.*, 1991). Synthesis of CSPG4 occurs through a single precursor protein (Bumol *et al.*, 1984) and this appears to be conserved through phylogenetic evolution, since mutation of the GAG acceptor site in the core protein of the rat homologue of CSPG4, NG2, results in the expression of only a single low mw core protein species in rat cells (Stallcup, 2002).

Although no structural X-ray crystallographic analysis of human CSPG4 or its animal homologues is available, some structural information can be inferred from the AA sequence and from biochemical studies of the molecules (Fig. 2A). The first 29 AA comprise a putative signal sequence. The core protein of CSPG4, NG2, and AN2 contains three major domain structures: a large extracellular domain spanning $\sim$2222 AA, a hydrophobic transmembrane region spanning $\sim$25 hydrophobic AA, and a short cytoplasmic tail spanning $\sim$75 AA. The transmembrane segment of CSPG4 contains one

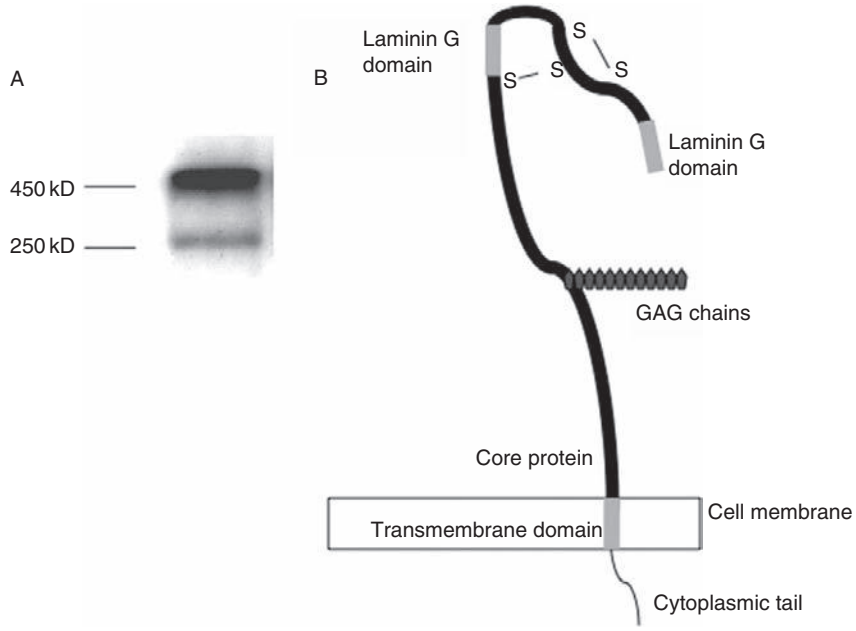

Fig. 1 Structural characteristics of CSPG4. (A) SDS-PAGE analysis of the components immunoprecipitated from radiolabeled human melanoma cells by CSPG4-specific mAb 763.74. (B) Schematic representation of the structure of cell surface CSPG4.

methionine and one cysteine residue followed by several basic arginine and lysine residues that meet the criteria for a transmembrane domain. Whether this transmembrane cysteine residue is involved in intermolecular disulfide bonding or in the function of CSPG4 has not been determined. Nonetheless, the presence of a transmembrane cysteine residue is not unique to CSPG4, since it has been found also in the transmembrane segments of CD44 (Liu and Sy, 1996), fibroblast growth factor (FGF)-binding proteoglycan (Bernfield *et al.*, 1999), and syndecan (Bernfield *et al.*, 1999). There are 15 potential N-linked glycosylation sites throughout the extracellular domain. Predictions of secondary structure suggest that the ectodomain of CSPG4, NG2, and AN2 can be divided into three subdomains based on sequence features. The first subdomain (designated D1) spanning approximately between AA residues 1 and 640 consists of a globular N-terminus containing eight cysteines and four serine/glycine pairs as well as two laminin G-type motifs. Laminin-G domains are widespread among many extracellular and membrane-bound proteins and are implicated in interactions with cellular receptors (integrins, alpha-dystroglycan), sulfated carbohydrates, and other extracellular ligands (Timpl

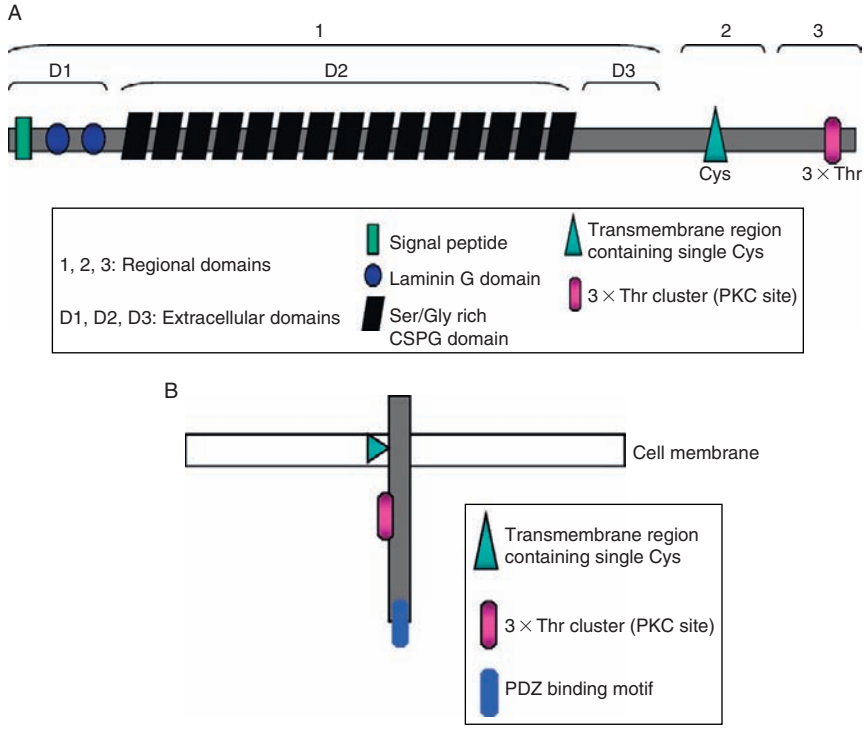

Fig. 2 Analysis of the full-length amino acid sequence of the CSPG4 core protein. (A) Three major domain structures have been identified in the CSPG4 core proteins. They include a large extracellular domain (1), a hydrophobic transmembrane region (2) and a short cytoplasmic tail (3). The ectodomain of CSPG4 can be divided into three subdomains utilizing sequence features as criteria: (D1) the globular N-terminus which also contains a laminin-G domain; (D2) a flexible rod-like central region which contains a novel repeat named CSPG, the latter is believed to contain sites for chondroitin sulfate addition as well as potential collagen V and VI binding sites and (D3) a C-terminal portion in globular conformation. The cytoplasmic domains of CSPG4 contain a PDZ-binding motif, a proline rich region and three potential threonine residues that may be possible candidates for phosphorylation. Notably the rat and mouse homologues, NG2 and AN2, respectively, differ from CSPG4 in the presence of a cluster of six cysteine residues in their D3 subdomain. (B) Schematic representation of the transmembrane region of CSPG4.

et al., 2000). The second subdomain (designated D2), a cysteine-free, serine/glycine-rich domain spanning approximately between AA residues 641 and 1590, consists of an α helical flexible rod-like central region, which contains potential GAG acceptor sites for chondroitin as well as potential type V and VI collagen-binding sites (Stallcup, 2002). The latter may be important for ligand binding. Recent evidence suggests that interactions between CSPG4

and type VI collagen promote growth-stimulatory and prosurvival effects in part by signaling through CSPG4 to sequentially activate Akt and β-catenin and stabilize cyclin D1 (Iyengar *et al.*, 2005). It is noteworthy that the presence of GAG at this potential acceptor site is not required for binding of CSPG4 ligands. The potential significance of this GAG acceptor site remains unclear, although there is some evidence that the GAG chain may be important for targeting the rat homologue of CSPG4, NG2, to specific microdomains of the cell membrane (Stallcup and Huang, 2008). Finally, the third juxtamembrane subdomain (designated D3) spanning roughly between AA residues 1591 and 2221 consists of the C-terminal portion of the ectodomain arranged in a globular conformation containing only two cysteine residues and one serine/glycine pair. Domain D3 contains N-linked oligosaccharides which appear to be required for the rat homologue of CSPG4, NG2, to bind galectin-3 (Wen *et al.*, 2006). Moreover, at least for NG2, this subdomain contains sites for binding α1 integrin as well as sites for proteolytic cleavage and shedding of soluble NG2 (Stallcup and Huang, 2008). Although little is known about the mechanisms of shedding as well as the function of shed CSPG4 and NG2, shedding of CSPG4 and NG2 is greatly enhanced when cells are incubated with interferon-α A, -α D, -β, and -γ or in several types of tissue injury (Asher *et al.*, 2005; de Castro *et al.*, 2005; Giacomini *et al.*, 1984; Jones *et al.*, 2002; Larsen *et al.*, 2003; Maio *et al.*, 1989; Nishiyama *et al.*, 1995; Tsujisaki *et al.*, 1987).

The cytoplasmic domain of CSPG4, like that of NG2 and AN2, spanning roughly between AA residues 2247 and 2322, contains some recognizable motifs that appear to be important, at least for NG2, for their function (Fig. 2B). Specifically, at the extreme C-terminus of the cytoplasmic domain of CSPG4, NG2, and AN2, there is a PDZ-binding motif (QYWV) that appears to mediate the interaction of NG2 with the scaffolding proteins multi-PDZ domain protein 1 (MUPP1) (Barritt *et al.*, 2000), glutamate receptor interacting protein 1 (GRIP1) (Stegmuller *et al.*, 2002), and syntenin-1 (Chatterjee *et al.*, 2008). Moreover, the cytoplasmic domain of CSPG4, like that of NG2 and AN2, contains three threonine residues with surrounding amino acid sequences conforming to the motif for potential protein kinase C (PKC)-α phosphorylation sites (Makagiansar *et al.*, 2004). The latter is suggested by the phosphorylation of NG2 Thr-2256 and Thr-2314 by PKCα and extracellular signal-regulated kinase (ERK), respectively (Makagiansar *et al.*, 2007). Although a classical PXXP SH3 binding domain is not present in the C-terminal domain of CSPG4, NG2, or AN2, this region is very rich in prolines. It is noteworthy that NG2 and AN2 differ from CSPG4 in the presence of a cluster of six cysteine residues in their extracellular domain. The significance of this difference is unknown.

C. Expression of CSPG4 in Normal and Malignant Cells

1. EXPRESSION IN NORMAL TISSUES

As noted above, the similarities in structure of CSPG4 with NG2 have provided a useful backdrop to elucidate the tissue distribution and, as will be discussed below, the biologic function of CSPG4. Originally, CSPG4 distribution in normal tissues in adults was thought to be restricted, having been initially detected only in melanocytes, endothelial cells, and pericytes (Ferrone *et al.*, 1983; Schlingemann *et al.*, 1990, 1991). More recently, it has become apparent that CSPG4 distribution is broader, being expressed in a number of normal and malignant cells. Immunohistochemical (IHC) staining with mAb has demonstrated that besides being expressed on melanocytes, endothelial cells, and pericytes (Ferrone *et al.*, 1983; Schlingemann *et al.*, 1990, 1991), CSPG4 is expressed on restricted areas of the interfollicular epidermis as well as the basal layer of normal oral mucosa, with the exception of foreskin and perineum (Ghali *et al.*, 2004; Legg *et al.*, 2003), the basal layer of the outer root sheath and the follicular papilla of the hair follicle (Ghali *et al.*, 2004; Legg *et al.*, 2003), chondrocytes in normal and osteoarthritic adult articular cartilage (Midwood and Salter, 1998), smooth muscle cells (Tordsson *et al.*, 2000), angiomyolipomas (Schlingemann *et al.*, 1990), differentiated myofibers of the sarcolemma and neuromuscular junction of human postnatal skeletal muscle (Petrini *et al.*, 2003), and microglial and mesangial cells of the renal glomerulus (Pouly *et al.*, 1999). It should be noted that whether these tissues express human CSPG4 or merely the antigenic determinant of the mAb utilized remains to be determined.

Whether CSPG4 is also expressed on oligodendrocyte progenitor cells (OPC), glial and muscle progenitor cells, brain capillary endothelial cells, chondroblasts of the limbs and spinal column, embryonic cardiomyocytes, and smooth muscle cells in developing macrovasculature like NG2 (Stallcup and Huang, 2008) remains to be determined since no IHC staining of human tissues have been performed to date. Additional studies have demonstrated that CSPG4 is expressed by smooth muscle cells in the intestinal muscle layer as well as intestinal subepithelial myofibroblasts (Terada *et al.*, 2006). The latter are thought to have an important role in protecting and maintaining the integrity of the epithelial cell layer and also in the process of wound healing. More recently, CSPG4 has been shown to be expressed by human bone marrow mesenchymal stromal cells, the multipotent cells capable of differentiating into various mesenchymal cells such as chondrocytes, osteoblasts, myoblasts, and adipocytes (Kozanoglu *et al.*, 2009). It is noteworthy that in spite of this apparent broad distribution of CSPG4 in normal tissues, CSPG4-specific humoral and cellular immunity, whether spontaneous or induced by immunotherapy, does not appear to cause toxicity in patients with melanoma and in healthy individuals (Erfurt *et al.*, 2007; Mittelman *et al.*, 1992). As will be discussed below, this finding is paralleled by similar results in mice.

In fetal tissues, CSPG4 is also expressed on epidermal melanocytes as well as in the vascular paracellular clefts of placental villi from the first trimester of human pregnancy (Challier *et al.*, 2001). Whether CSPG4 plays a functional role during the development of fetal melanocytes is not known.

It has been known for some time that CSPG4 can be found on pericytes both *in vivo* and *in vitro* (Schlingemann *et al.*, 1990). Furthermore, CSPG4 has been shown to represent a useful phenotypic marker for 'activated' pericytes, since it is preferentially expressed on pericytes in conditions associated with neovascularization *in vivo* (Schlingemann *et al.*, 1991). Recent studies have shown that upregulated CSPG4 expression is effective in mediating the targeting of activated pericytes by cellular and humoral immunity. In this regard, immunization of tumor-bearing mice with a recombinant *Listeria monocytogenes* that expresses and secretes a fragment of CSPG4 induces CSPG4-specific CTL (Maciag *et al.*, 2008). Because of their cross-reactivity with AN2, the mouse counterpart of CSPG4, CTL infiltrate and destroy mouse-activated pericytes in the tumor microenvironment. Similarly, administration of the CSPG4-specific mAb 225.28 which cross-reacts with AN2, in combination with metronomic chemotherapy, that is, continuous administration of cyclophosphamide at a low dose which does not cause toxicity, destroys pericytes, and inhibits neoangiogenesis (Kerbel and Kamen, 2004). As a result, the growth of transplanted tumor cells, even those that do not express CSPG4 is inhibited (Maciag *et al.*, 2008). Similar observations have been made in mice injected for several months systemically with CSPG4-specific mAb that cross-react with mouse AN2. It is noteworthy that these beneficial effects are not associated with side effects caused by neoangiogenesis inhibition such as prolongation of the time required for wound healing as well as fertility, gestation length, and pup mass at term (Maciag *et al.*, 2008). The lack of detectable effects on resting pericytes may reflect AN2 expression at levels below those required for recognition by CTL and antibodies. These results altogether are compatible with the possibility that targeting the tumor microenvironment with CSPG4-specific immunity may improve its therapeutic efficacy since activated pericytes present in regenerating tumor vasculature are crucial for malignant cell survival and proliferation. Additionally, targeting pericytes in the tumor stroma might cause a certain degree of vasculitis that could promote the infiltration of the tumor by CSPG4-specific T cells and improve the efficacy of cancer immunotherapy.

2. EXPRESSION IN PATHOLOGIC CONDITIONS

a. Genetic and Inflammatory Disorders

As mentioned above, it has been suggested that CSPG4 represents a phenotypic marker for "activated" pericytes (Schlingemann *et al.*, 1991). In this regard, CSPG4-expressing pericytes have been found in biopsy samples from patients with autoimmune Raynaud's phenomenon, in both early

fibrotic and early nonfibrotic scleroderma skin and on stellate-shaped cells of unknown histogenesis in myxomatous tissue of human coronary artery lesions (Schlingemann *et al.*, 1991). As will be discussed later, these findings suggest that CSPG4 may play a significant role in promoting the proliferation, migration, and angiogenic potential of CSPG4-expressing cells. CSPG4 is also expressed at a low level on keratinocytes of patients with oral lichen planus and oral hyperkeratosis lesions (Kose *et al.*, 2007) as well as on muscles of patients with merosin-negative congenital muscular dystrophy and Duchenne muscular dystrophy as well as on chronically inflamed synovia (Pouly *et al.*, 1999). Whether CSPG4 is expressed because of gene dysregulation in patients' cells and/or whether CSPG4 plays a role in the pathogenesis of the aforementioned diseases is not known.

b. Tumors of Melanocytic Origin

A large number of surgically removed lesions of melanocytic origin have been analyzed in IHC staining reactions with CSPG4-specific mouse mAb. Nevertheless, it should be stressed that no large systemic quantitative study has been performed assessing CSPG4 expression in primary and metastatic melanoma lesions. Many of the published studies have utilized different CSPG4-specific mAb as well as methodologies to detect CSPG4 expression. Moreover, very few of these studies have stratified the analyzed melanoctyic lesions using definitive histopathologic criteria. Therefore, the available data should be interpreted with caution. Future well-designed studies should be directed at assessing the frequency of CSPG4 expression in different types of primary and metastatic melanocytic tumors from different anatomic sites utilizing well-defined histopathologic criteria. In general, CSPG4 has been found to be expressed on greater than 90% of the surgically removed benign nevi and melanoma lesions analyzed, with a limited degree of intra- and interlesional heterogeneity (Ferrone *et al.*, 1983; Natali *et al.*, 1981, 1983). Furthermore, its expression in malignant lesions does not vary following treatment with chemotherapeutic agents (Ferrone *et al.*, 1993).

The frequency of CSPG4 expression is similar in primary lentigo maligna, nodular, and superficial spreading melanoma lesions but is lower in primary acral lentiginous melanoma (ALM) lesions (Kageshita *et al.*, 1991; Nishi *et al.*, 2010). This difference may reflect silencing of the CSPG4 gene because of methylation of its promoter (Luo *et al.*, 2006). In mucosal melanoma, the level of CSPG4 expression has been found to be lower than in nodular melanoma lesions (Kageshita *et al.*, 1994); however, the number of mucosal melanoma lesions analyzed thus far (10 primary and 6 metastatic) is too low to draw definitive conclusions regarding the level of CSPG4 expression. In uveal melanoma, information about the level of CSPG4 expression is conflicting with studies demonstrating CSPG4 at lower (Natali *et al.*, 1983; van der Pol *et al.*, 1987) or similar (Bomanji *et al.*, 1987; Li *et al.*, 2003) levels

with primary cutaneous melanoma lesions. CSPG4 is also expressed in desmoplastic melanoma (DM), a spindle cell vertical growth phase melanoma with various degrees of desmoplasia that can develop *de novo* or in a preexisting radial growth phase lesion (Goto *et al*., 2010). In the latter case, it has clinical and histologic features of lentigo maligna, ALM, or mucosal-lentiginous melanoma. Histologically, DM is characterized as a mainly intradermal ill-defined lesion composed of elongated hyperchromatic spindle cells distributed singly or in bundles, fascicles, or nests, between variably increased collagen fibers of the papillary and the reticular dermis (Barnhill and Gupta, 2009). Both primary and metastatic DM lesions usually show immunoreactivity with S-100 protein and NKI/C3 but not with HMB-45, MART-1, NKI/beteb, and Leu-7 (CD57), or neuron specific enolase (NSE)-specific antibodies. Nevertheless, negativity does not exclude the diagnosis when the clinical picture and/or histology are characteristic. In this regard, CSPG4 has been shown to be more sensitive than HMB-45 and MART-1 for IHC diagnosis of primary and metastatic DM lesions (Goto *et al*., 2010).

CSPG4 expression has not been found to vary between primary and metastatic lesions in all types of melanoma with the exception of ALM. In the latter, CSPG4 expression is significantly higher in metastatic than in primary lesions (Kageshita *et al*., 1991, 1993; Nishi *et al*., 2010). These findings in conjunction with CSPG4's role in cell migration, suggest that CSPG4 may play a role in the metastatic potential of ALM cells (Kageshita *et al*., 1991, 1993). Interestingly, CSPG4 expression has been found to correlate with the clinical course of the disease only in ALM (Kageshita *et al*., 1993). In the latter case, CSPG4 expression in primary lesions is associated with a worse prognosis (Kageshita *et al*., 1993).

c. Tumors of Nonmelanocytic Origin

As noted above, it was formerly thought that CSPG4 expression had a restricted tissue distribution in tumors of known neuroectodermal origin. However, in recent years, the mounting evidence that CSPG4 plays a role in cell adhesion, motility, and invasion, all of which contribute to the disordered behavior of malignant cells, has stimulated interest in investigating CSPG4 expression in tumors of different histotype. Staining of various types of tumors with CSPG4-specific mAb has shown that its expression is not restricted to melanoma. For solid tumors, besides being expressed on tumors of neuroectodermal origin such as astrocytomas, gliomas, and neuroblastomas, CSPG4 is also expressed by squamous cell carcinoma of the head and neck (SCCHN), basal breast cancer, mesothelioma, pancreatic carcinoma, some types of renal cell carcinoma, chordoma, chondrosarcoma, and soft tissue sarcomas (Schwab *et al*., 2009; Wang *et al*., 2010a,b). The frequency of CSPG4 expression in each type of tumor is at least 50% of the surgically removed lesions analyzed. However, these data have to be interpreted with

caution, since the number of lesions analyzed in each type of tumor is relatively small. It is of interest that in soft tissue sarcomas, the level of CSPG4 mRNA in primary lesions was associated with risk of metastases; furthermore, the level of CSPG4 mRNA in metastatic lesions was correlated with the prognosis of the disease (Benassi *et al.*, 2009).

In regard with hematologic malignancies, CSPG4 is expressed on blast cells in both childhood and adult acute lymphoblastic leukemia (ALL) and childhood acute myeloid leukemia (AML) (Behm *et al.*, 1996; Borkhardt *et al.*, 2002; Hilden *et al.*, 1997; Mauvieux *et al.*, 1999; Schwartz *et al.*, 2003; Smith *et al.*, 1996; Wuchter *et al.*, 2000). Interestingly, CSPG4 cell surface expression has been correlated with poor outcome and with balanced 11q23 translocations (Smith *et al.*, 1996). Moreover, CSPG4 expression in pediatric patients with ALL has been found to be a useful marker to identify patients with t(4:11)-mixed lineage leukemia (MLL) rearrangements, the most important criteria for high-risk stratification in protocols for childhood ALL. It is noteworthy that CSPG4-specific mAb have been shown to inhibit leukemic cell growth and enhance the antiproliferative effect of cytarabine in xenograft animal models (Drake *et al.*, 2009). These findings in conjunction with the sensitivity of leukemic cells to treatment with mAb such as the CD20-specific mAb rituximab (Dillman, 2003; Lin *et al.*, 2003), argue in favor of the possibility that CSPG4-specific mAb may have therapeutic potential in the treatment of both child ALL and AML.

d. Expression on CSC in Basal Breast Carcinoma, SCCHN, and Melanoma

According to the CSC theory, the failure of current cancer treatments to abrogate recurrence and metastasis reflects their inability to adequately target CSC (Lobo *et al.*, 2007; Visvader and Lindeman, 2008). In this regard, CSC are characterized by their enhanced chemo- and radioresistance and high tumorigenicity in immunodeficient mice and play an important role in tumor maintenance and progression. The central role CSC play in tumor maintenance and progression has emphasized the need to develop novel therapeutic strategies to eradicate CSC for successful treatment of malignant disease. The interest in applying immunotherapy to target CSC and the potential of CSPG4 as a target of immunotherapy has prompted investigations to determine whether CSPG4 is expressed on CSC. As we have recently described (Wang *et al.*, 2010a,b), in SCCHN and in basal breast cancer, CSPG4 is expressed on CSC, identified by the high expression of aldehyde dehydrogenase 1 family member A1 (ALDH1), a detoxifying enzyme responsible for the oxidation of intracellular aldehyde. In melanoma, the assessment of CSPG4 expression on CSC has to be addressed with caution given the conflicting information about the identification of CSC in this disease. The markers reported to identify CSC include the ATP-Binding

Cassette Subfamily B member 5 (ABCB5), CD133, CD271, nestin, and JARIDB (Boiko *et al.*, 2010; Klein *et al.*, 2007; Monzani *et al.*, 2007; Roesch *et al.*, 2010; Schatton *et al.*, 2008). The validity of these markers to identify CSC in melanoma has been questioned by Quintana *et al.* (2008) who have reported that melanoma does not follow a CSC model and that tumorigenic potential is frequently present in all melanoma cells. These authors as well as Clarke *et al.* (2006) have reported that the tumorigenicity of melanoma cells in immunodeficient mice, which represents the standard assay to assess tumor self-renewal, is influenced by several variables. Among them are the duration of the xenotransplantation assay and the degree of immunodeficiency of the mouse strain used for the xenotransplantation experiments. These variables are likely to play a role in the different results published in the literature.

At present, it is difficult to evaluate the validity of the markers reported in the literature to identify CSC in melanoma, since with very few exceptions, reagents have not been exchanged among laboratories and few studies have attempted to reproduce results obtained in other laboratories, utilizing the experimental conditions described in the original publication. To the best of our knowledge, only Shackleton and Quintana (Shackleton and Quintana, 2010) have reported to have not been able to reproduce the results recently published by Weissman and his associates (Boiko *et al.*, 2010). Specifically, the $CD271^+$ cell subpopulation isolated from surgically removed melanoma lesions was not enriched in tumorigenic potential. However, this comparison has to be interpreted with caution, since the experimental conditions used in the two studies were different in terms of tumor digestion protocol, mice used for xenotransplantation, and anatomic site used for injection of melanoma cells. Thus far, we have been unable to confirm the specificity of the ABCB5-specific mAb used by Frank *et al.* (2003; Schatton *et al.*, 2008) to identify melanoma CSC. Clearly there is debate as to whether the CSC theory can account for melanoma cell heterogeneity as well as to whether melanomas are organized into subpopulations of tumorigenic and nontumorigenic cells. In order to provide an explanation for the lack of reproducibility of CSC studies in melanoma, Herlyn and his associates (Roesch *et al.*, 2010) have redefined the role of melanoma CSC as cells capable of continuously maintaining a tumor cell population but not capable of tumor initiation. Studies aimed at comparing the markers as well as the reagents and techniques utilized to identify CSC are unmistakably needed to more appropriately define and characterize tumor cell populations in melanoma. In our experience, melanoma cells that express both ABCB5 and high levels of ALDH1A1 meet the criteria to be classified as CSC, since they are tumorigenic at low numbers in SCID mice. These cells demonstrate CSPG4 expression. It is noteworthy that, although the identity of CSC in melanoma is still an open question, CSPG4 is more than likely to be expressed on these cells given its expression on more

than 95% of cells in melanoma cell lines and in melanoma cell populations isolated from surgically removed primary and metastatic lesions.

III. FUNCTIONAL PROPERTIES OF CSPG4

Early investigations had shown that NG2, the rat homologue of CSPG4, promotes progenitor and tumor cell motility, adhesion, and growth, resulting in melanoma and glioma cell growth and metastasis (Stallcup and Huang, 2008). More recently, *in vitro* and *in vivo* assays with CSPG4-specific mAb have shown that the functional properties of CSPG4 are similar to those displayed by NG2.

A. Role in Cell Proliferation

The potential role of CSPG4 in melanoma cell proliferation is suggested by the enhanced proliferation rates of melanoma cells transfected to express CSPG4 or NG2, *in vitro* and when inoculated into SCID mice (Stallcup, 2002; Wang *et al.*, 2010b). Conversely, CSPG4-specific mAb, like NG2-specific mAb, can reverse the aforementioned effects (Harper and Reisfeld, 1983; Stallcup, 2002; Wang *et al.*, 2010b). This finding may reflect the modulation of cellular responses to growth factors by CSPG4, since NG2 is capable of binding platelet-derived growth factor AA (PDGF-AA) and basic fibroblast growth factor (bFGF) (Goretzki *et al.*, 1999; Grako and Stallcup, 1995; Grako *et al.*, 1999; Nishiyama *et al.*, 1996). Furthermore, CSPG4- and NG2-specific mAb inhibit proliferation of cell lines derived from different embryological origins such as melanoma, head and neck squamous cell carcinoma, basal breast carcinoma, and osteosarcoma (Wang *et al.*, 2010a,b) (Fig. 3) and of oligodendrocyte precursors as well as mitosis of rat smooth muscle cells in response to bFGF and PDGF-AA (Stallcup, 2002). Therefore, both CSPG4 and NG2 may act as an auxiliary cell surface receptor for growth factors, potentiating their ability to activate intracellular signal transduction cascades (Goretzki *et al.*, 1999; Grako *et al.*, 1999).

B. Role in Cell Migration and Invasion

Cell surface proteoglycans represent a diverse set of adhesion receptors. It has been well established that both the GAG constituent and the core protein of proteoglycans are responsible for binding a number of different ligands,

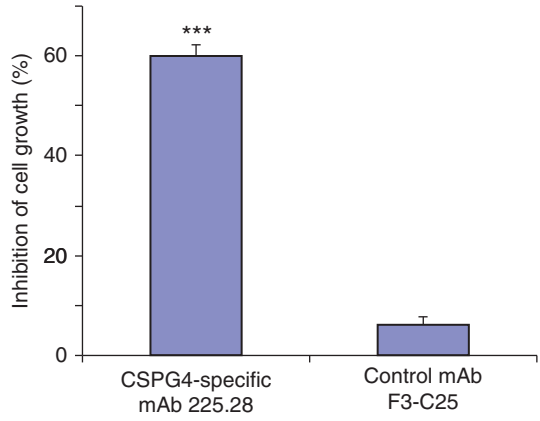

Fig. 3 Inhibition by CSPG4-specific mAb 225.28 of osteosarcoma cell *in vitro* growth. Human osteosarcoma MG-63 cells, which express CSPG4, were incubated with either CSPG4-specific mAb 225.28 (0.5 mg/ml) or control mAb F3-C25 (0.5 mg/ml) in a 3D (matrigel) setting for 10 days at 37 °C. PBS, which was used as the solvent for both mAbs, was used as a reference for 100% cell growth. Cells in each well were harvested from matrigel using Cell Recovery Solution (BD Pharmingen) and counted by two individuals using Trypan Blue. The results are expressed as % inhibition of cell growth, utilizing the values obtained for cells grown in PBS, as a 100% reference. The values shown are the mean of three independent experiments (*** indicates $p < 0.001$).

not limited to, but including adhesion molecules, chemokines, cytokines, extracellular matrix (ECM) components and growth factors (Bernfield *et al*., 1999). For some time CSPG4 has been implicated in numerous aspects of melanoma cell biology including adhesion, spreading, and migration (Burg *et al*., 1998; de Vries *et al*., 1986; Harper and Reisfeld, 1983; Iida *et al*., 1995). As shown in Fig. 4A, human M14 melanoma cells which express CSPG4 following transfection with CSPG4 cDNA displayed an approximately fourfold higher migratory ability than the parental M14 cells which do not express CSPG4. Moreover, CSPG4-specific mAb inhibit melanoma cell adhesion, chemotactic responses to fibronectin, and cytoplasmic spreading on ECM proteins such as collagen and fibronectin (Burg *et al*., 1998; de Vries *et al*., 1986; Harper and Reisfeld, 1983; Iida *et al*., 1995) as well as osteosarcoma MG-63 cell migration (Fig. 4B). These findings are consistent with the possibility that CSPG4 interacts with ECM components and promotes cell adhesion and migration, thereby influencing their metastatic potential. Two lines of evidence further support this possibility. First, CSPG4-specific mAb inhibit melanoma cell attachment to capillary endothelium and spreading on various ECM components including collagen and collagen-fibronectin complexes (Ferrone *et al*., 1993; Harper and Reisfeld, 1983). Second, in parallel with the effect of NG2- and AN2-specific mAb on

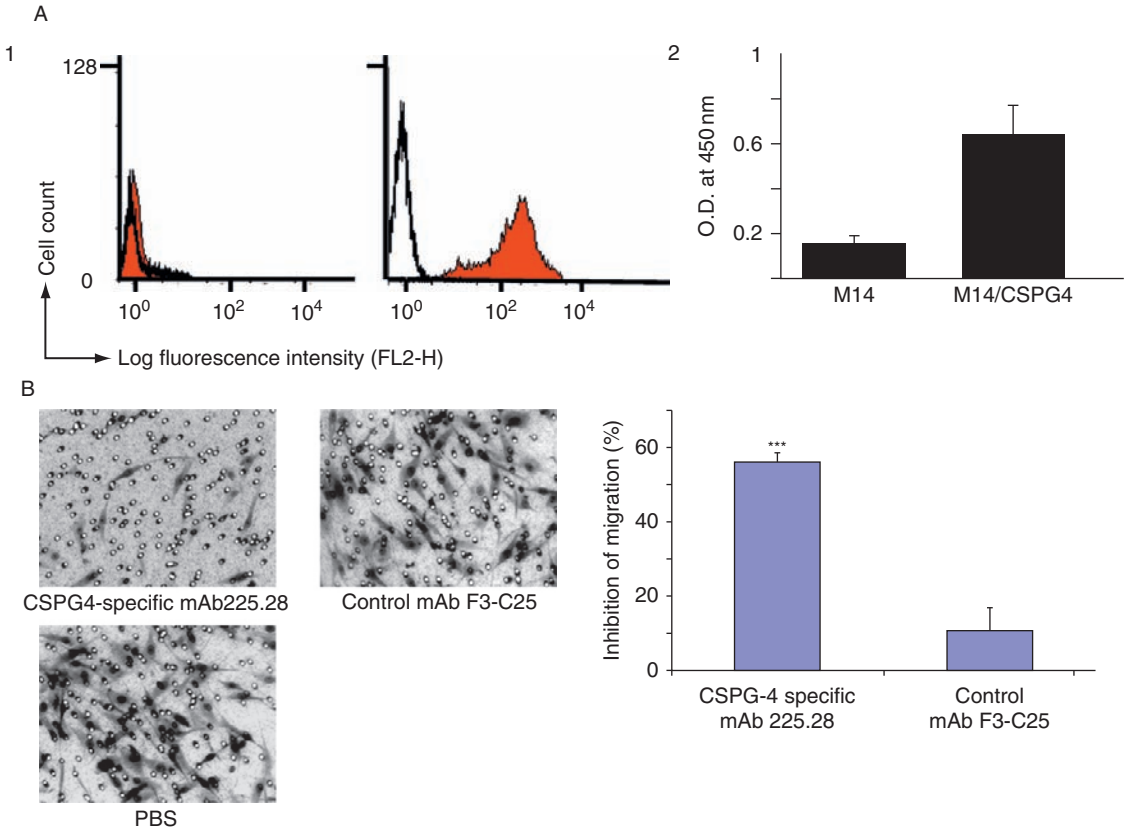

Fig. 4 Enhancement by CSPG4 of melanoma and osteosarcoma cell migration *in vitro*. (A) Induction of CSPG4 expression on human melanoma M14 cells by transfection with CSPG4 cDNA resulted in a fourfold increase in their migratory ability over the mock-transfected parental cells. (1) CSPG4 expression (closed histogram) was detected on M14 melanoma cells transfected with CSPG4 mRNA (right panel) but not on the mock-transfected parental cells (left panel), as determined by flow cytometry analysis of cells stained with CSPG4-specific mAb 763.74. An isotype-matched irrelevant mAb (open histogram) was

oligodendrocyte precursor cells (Diers-Fenger *et al*., 2001; Fang *et al*., 1999; Stegmuller *et al*., 2002), CSPG4-specific mAb inhibit the cytoskeletal reorganization frequently observed in migrating melanoma cells, indicating that CSPG4-specific mAb may have direct effects on the migratory ability of melanoma cells.

The ability of CSPG4/ECM interactions to enhance the migratory potential of melanoma cells is supported by the localization and distribution of CSPG4 on melanoma cell membranes. Early studies demonstrated that both CSPG4 and NG2 expression is restricted to cell surface microspike domains on migrating cells *in vitro* (Garrigues *et al*., 1986). These microspike domains are actin-rich structures, resembling filopodia, and are important for the formation of adhesive contacts with components of the ECM in migrating cells. Microspike formation requires dynamic reorganization of the actin cytoskeleton that involves a number of intracellular signaling pathways (Weed and Parsons, 2001). Therefore, CSPG4 may enhance the migratory potential of melanoma cells by initiating intracellular signaling pathways. In this regard, stable transfection with CSPG4 cDNA of radial growth phase melanoma WM1552c cells, which lack endogenous CSPG4 expression, leads to colocalization of CSPG4 and $\alpha_4\beta_1$ integrin, extensive microspike formation as well as enhanced $\alpha_4\beta_1$ integrin-mediated spreading and enhanced phosphorylation of both focal adhesion kinase (FAK) and ERK 1 and 2 (ERK 1/2) (Yang *et al*., 2004). Moreover, CSPG4 and NG2 interactions with ECM components, such as laminin, tenascin, and types II, V, and VI collagen, trigger cytoskeletal rearrangements that lead to melanoma cell spreading and migration (Fang *et al*., 1999) as well as to the activation of FAK and ERK 1/2 signaling cascades (Stallcup and Huang, 2008; Yang *et al*., 2004). Additional support for the notion that CSPG4/ECM interactions may activate intracellular signaling pathways is provided by two lines of evidence derived from the analysis of the biologic function of NG2 and AN2. First, mouse melanoma cells transfected with NG2 cDNA exhibit an increased ability to form tumors as well as to metastasize (Burg *et al*., 1998). Second, cells transfected to express NG2 show an increased ability to migrate in response to type VI collagen (Stallcup and Huang,

used as a specificity control. (2) Migration was determined by an *in vitro* cell migration assay (transwell system) and quantitated with MTT test. (B) Inhibition by CSPG4-specific mAb 225.28 of the migration of CSPG4-expressing human osteosarcoma cells MG-63. Cells were seeded and incubated with either CSPG4-specific mAb 225.28 (0.5 mg/ml), control mAb F3-C25 (0.5 mg/ml) or PBS in a migration assay. Cells in each well were imaged with a Zeiss inverted fluorescence microscope (AxioVision Software) ($\times 200$) (left panel). The results are expressed as percentage of inhibition of migration, utilizing the values obtained in PBS without mAb as a 100% reference. The values shown are the mean of three independent experiments (*** indicates $p < 0.001$) (right panel).

2008). These results are consistent with a role for CSPG4 in cell motility/invasion and suggest that CSPG4 may act to promote the formation of initial adhesive contacts at the leading edge of migrating cells.

It is noteworthy that cell migration and invasion into surrounding normal tissue is accomplished by a host of enzymes that break down the ECM, including plasmin (Ranson and Andronicos, 2003) and membrane type matrix metalloproteinases (MT-MMPs) (Polette and Birembaut, 1998). Interestingly, the formation of CSPG4–MT3-MMP complexes is crucial for the ability of melanoma cells to degrade type I collagen *in vitro* (Iida *et al.*, 2001). The interaction of CSPG4 with MT3-MMP requires the presence of chondroitin sulfate on CSPG4, since its removal can effectively inhibit invasion and proteolysis of type I collagen. This finding is not unique to CSPG4, since other members of the MMP protease family have been documented to bind other GAG such as heparin and this interaction can potentiate the activation of certain MMPs (Crabbe *et al.*, 1993). Therefore, CSPG4 may be involved in the recruitment of MT-MMPs to the cell membrane. This recruitment is expected to facilitate the invasion of aggressive primary tumors within the dermis by activating specific MMPs at sites of contact of melanoma cells with underlying ECM. As a result, the invasive potential of tumor cells may be enhanced (Iida *et al.*, 2001) (Fig. 5).

1. SIGNAL TRANSDUCTION NETWORKS TRIGGERED BY CSPG4–ECM INTERACTIONS

CSPG4/ECM, like NG2/ECM interactions result in the extension of filopodia and lamellipodia as well as retraction of fibers at the leading edge and rear of migrating cells, respectively (Campoli *et al.*, 2004; Stallcup and Huang, 2008). These morphological changes are highly suggestive of the involvement of the Rho family small GTPases. Specifically, the Rho GTPase family members RhoA, Rac, and Cdc42 are known to be involved in the regulation of the actin cytoskeleton as well as integrin-based cell matrix adhesion, while Rac and Cdc42 also regulate lamellipodia and filopodia formation, respectively (Bosco *et al.*, 2009; Huveneers and Danen, 2009). Engagement of CSPG4 as well as of NG2 by the corresponding antibodies has been shown to trigger signal transduction pathways through the activation of Rho GTPase family proteins including Rho, Rac, and Cdc42 as well as the adaptor protein $p130^{Cas}$ (Eisenmann *et al.*, 1999; Stallcup and Huang, 2008). Rac and Cdc42 interact with p21-activated kinases (PAK) to initiate downstream signaling events associated with cell motility, while the adaptor protein $p130^{Cas}$ couples transmembrane signaling to cell motility (Bokoch, 2003). It is noteworthy that Rho GTPases are known growth stimulators that act by modulating key cell cycle regulators, such as cyclin D1 and the transcription factor nuclear factor-

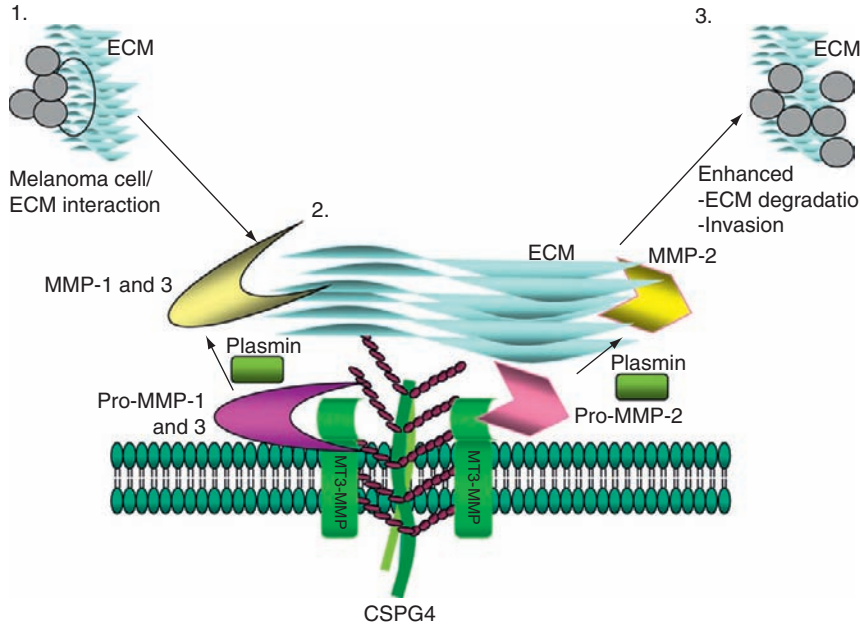

Fig. 5 Enhancement by CSPG4 of matrix metalloproteinase (MMP) activity and tumor cell ECM invasion. It is postulated that CSPG4 may enhance the invasive potential of tumor cells through the following mechanisms: (1) interactions of CSPG4 on tumor cell membranes with ECM components result in the recruitment of and activation of MT-MMP3; (2) CSPG4 interaction with plasminogen and MT-MMP3 may increase the formation of active MMP-1, -2, and -3; and (3) increased MMP activity enhances the ability of tumor cells to degrade ECM resulting in increased invasion.

kappaB (NFkB) as well as signal transducer and activator of transcription 3 (STAT3) (Fletcher *et al*., 2009; Grivennikov and Karin, 2010; Piche and Li, 2010; Raptis *et al*., 2009; Williams *et al*., 2008). Therefore, the activation of Rho GTPases through CSPG4 provides a potential link between CSPG4 and NFkB as well as STAT3 activation. Interestingly, both the NFkB and STAT3 signaling cascades have been recognized to play important roles in inflammation-induced carcinogenesis and in antitumor immunity. Both proteins are not only persistently activated in cancer cells and essential for transducing cytoplasmic signals from extracellular stimuli, but also function as nuclear transcription factors required for regulating genes involved in tumor cell proliferation, survival and invasion, in addition to genes encoding key cancer-promoting inflammatory mediators (Grivennikov and Karin, 2010). Recent evidence also suggests that aberrant STAT3 expression plays important roles in the pathogenesis of many malignancies, by promoting cell cycle progression and survival as well as by stimulating angiogenesis. Specifically, STAT3 activation can lead to the

transcriptional activation of specific proteins, such as the antiapoptotic proteins BCl-xL and survivin; the cyclin proteins D1, D2, and E; the paracrine cellular growth, motility, and morphogenic factor hepatocyte growth factor; and the oncogene c-myc as well as the downregulation of the tumor suppressor protein p53 (Yu *et al.*, 2009). Moreover, STAT3 controls the transcription of the vascular endothelial growth factor (VEGF) gene, which is important for neovascularization. Inhibition of STAT3 signaling blocks VEGF- and bFGF-induced cell migration and proliferation of endothelial cells (Yu *et al.*, 2009). Lastly, STAT3 also suppresses the production of proinflammatory cytokines and chemokines by tumor cells and might thus aid in immune evasion (Yu *et al.*, 2009). Along these lines, STAT3 has been shown to play a role in the generation of a group of $CD11b^+$, $Gr\text{-}1^+$ cells known as myeloid suppressor cells that are also thought to play an important role in tumor unresponsiveness by suppressing TA-specific immune responses (Lee *et al.*, 2010).

CSPG4 has also been shown to enhance via CSPG4-$\alpha_4\beta_1$ integrin interactions the spreading of cells adherent to collagen via $\alpha_4\beta_1$ integrin. This enhanced cell spreading is accompanied by increased phosphorylation of FAK compared to cells adherent via integrin alone (Yang *et al.*, 2004). The interaction between CSPG4 and $\alpha_4\beta_1$ integrin is not surprising, since the importance of adhesion receptors such as integrins and cell surface proteoglycans in modulating melanoma cell adhesion, migration, and invasion *in vitro* has been well documented (Iida *et al.*, 1995; Knutson *et al.*, 1996; Schon *et al.*, 1996). This phenomenon is not unique to melanoma cells, since CD44, a related cell surface proteoglycan, activates β_1 integrin-mediated signaling and plays a role in the regulation of adhesion and proliferation of both chronic myelogenous leukemia progenitors (Lundell *et al.*, 1997) and endothelial cells (Kraal and Mebius, 1997). It still remains to be determined whether cytoskeletal reorganization mediated by CSPG4 is dependent on its interactions with integrin. In this regard, engagement of CSPG4 with collagen in the absence of integrin interaction has been shown to have no effect on FAK activation, indicating that CSPG4 enhances integrin-mediated FAK activation by an indirect mechanism (Yang *et al.*, 2004). Alternatively, CSPG4 may independently trigger pathways related to the cytoskeleton, since engagement of CSPG4 and NG2 with various ECM components, such as type I collagen, type VI collagen, matrigel, and vitronectin, results in the enhanced phosphorylation (activation) of both FAK and src in human melanoma cell lines (Yang *et al.*, 2004) (Fig. 6A). Conversely, CSPG4-specific mAb inhibits the activation of FAK, PKC-α, β-catenin, p-PDK1, p-Akt, and p-Erk1/2. Representative results obtained with the human osteosarcoma MG-63 cell line are shown in Fig. 6B. Regardless of the mechanism, FAK activation triggers a number of downstream pathways important for cell spreading, resistance to apoptosis, and cell proliferation (Liu *et al.*, 2000; Parsons, 2003). Specifically, downstream proteins that are activated

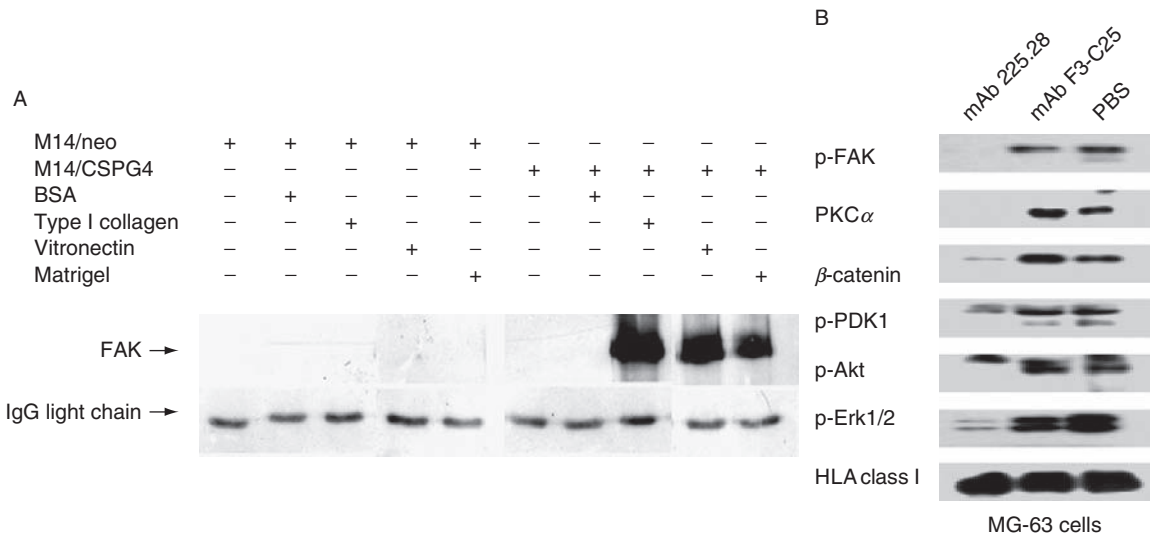

Fig. 6 Signaling transduction pathways associated with CSPG4 and ECM components. (A) FAK activation upon interaction of CSPG4, expressed on M14/CSPG4 cells, with type I collagen-, vitronectin-, or matrigel-coated plates. Lysates were prepared and immunoprecipitated with phospotyrosine-specific mAb. Immunoprecipitates were blotted with FAK-specific mAb. (B) Inhibition by CSPG4-specific mAb 225.28 of multiple CSPG4 associated signaling pathways in human osteosarcoma MG-63 cells *in vitro*.

by FAK include $p130^{Cas}$, growth factor receptor-bound protein 2 (Grb2), RAS, src, phosphatidylinositol 3-kinase (PI 3-kinase), and cytoskeletal proteins paxillin, talin, and tensin. All of them are involved in the mediation of cytoskeletal reorganization and ultimately cell migration (Iida *et al.*, 1995, 1996, 1998; Liu *et al.*, 2000; Parsons, 2003; Sieg *et al.*, 2000). Notably, the activation of GRb2 by CSPG4 provides a link between CSPG4 and B-RAF, a proto-oncogene that has been implicated in the pathogenesis of nonfamilial cutaneous melanoma. In this regard, Grb2 along with Son of Sevenless (SOS), a guanine nucleotide release protein, is specifically involved in the cell growth-regulatory MAPK/ERK pathway as well as in the activation of RAS at the plasma membrane (Giubellino *et al.*, 2008). Activated RAS recruits RAF to the plasma membrane where it in turn becomes activated. RAF phosphorylates and activates MEK, which in turn phosphorylates and activates ERK 1/2. Activated ERK 1/2 mediates various cellular responses, such as proliferation, survival, and differentiation. It still remains to be determined whether ERK 1/2 phosphorylation is mediated by the activation of FAK, since in some cases, CSPG4 expression can lead to enhanced ERK 1/2 phosphorylation which is independent of FAK phosphorylation (Yang *et al.*, 2004).

At present, CSPG4 appears to enhance the activation of two key pathways that are involved in melanoma cell invasion and metastasis. First, CSPG4 indirectly enhances FAK activation by an integrin-dependent mechanism thereby activating an intracellular signal transduction cascade that is critical in mediating cytoskeletal reorganization and cell migration. Second, through a separate yet undefined mechanism CSPG4 can enhance the activation of ERK that may ultimately increase the oncogenic behavior of melanoma cells. Consequently, it is our hypothesis that one important function of CSPG4 may be the enhancement of the efficiency of signal transduction initiated by other receptors/stimuli. Specifically, CSPG4 may provide cells with a selective advantage in the tumor microenvironment by possibly decreasing the amount of ligand required for melanoma cell adhesion, growth, invasion, and survival. Understanding the molecular mechanisms underlying CSPG4 signaling in melanoma cells may have a significant influence on our ability to determine the means by which CSPG4-targeted therapies impact melanoma cell growth and metastasis. A summary of putative interactions of CSPG4 with FAK, STAT3, and NFkB and ERK pathways is shown in Figs. 7–11.

2. STRUCTURAL BASIS OF SIGNAL TRANSDUCTION ACTIVATION BY CSPG4

Analysis of the differential effect of mAb recognizing distinct determinants of CSPG4 on the functions attributed to this antigen suggests that the extracellular portion of CSPG4 possesses different functional domains. For

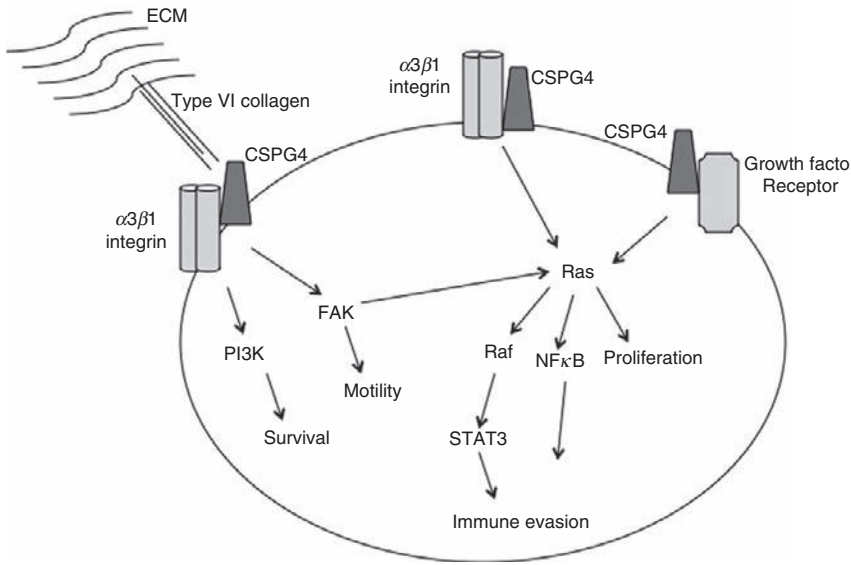

Fig. 7 Putative model for CSPG4 transduction in malignant cells. On the apical cell surface, the thr-2314 phosphorylated CSPG4 activates α3β1 integrin to promote enhanced proliferation. CSPG4 also promotes cell proliferation via potentiation of growth factor receptor signaling. On leading edge lamellipodia, thr-2256 phosphorylated CSPG4 activates α3β1 integrin signaling to promote enhanced motility. CSPG4-mediated integrin signaling can also enhance cell survival via the PI3/AKT pathway. Last, CSPG4 provides a linkage between the cell surface and the extracellular matrix via its interaction with type VI collagen. (Adapted from Stallcup and Huang, 2008).

instance, CSPG4-specific mAb 9.2.27 induces clustering of CSPG4 and increases $\alpha_4\beta_1$ integrin signaling, while CSPG4-specific mAb 763.74 inhibits these effects (Iida *et al*., 1998). Furthermore, mAb 9.2.27 inhibits FAK activation (Yang *et al*., 2004), cell adhesion, and cytoplasmic spreading on ECM proteins, such as collagen, or on collagen-fibronectin complexes (Harper and Reisfeld, 1983), while CSPG4-specific mAb 149.53 has no detectable effect.

Analysis of the structural features that trigger CSPG4 signal transduction pathways indicates that the chondroitin sulfate moieties on CSPG4 may regulate integrin function, since $\alpha_4\beta_1$ integrin interacts with CSPG4 via a 16–20 AA long peptide located in the α_4 integrin subunit (Iida *et al*., 1992). Furthermore, inhibition of the interactions between this peptide and chondroitin sulfate prevents ECM-induced activation of $\alpha_4\beta_1$ integrin signaling and melanoma cell adhesion to surfaces coated with an $\alpha_4\beta_1$ integrin peptide ligand (Iida *et al*., 1992). It has been suggested that chondroitin sulfate may help to modulate integrin function by clustering fibronectin and integrin

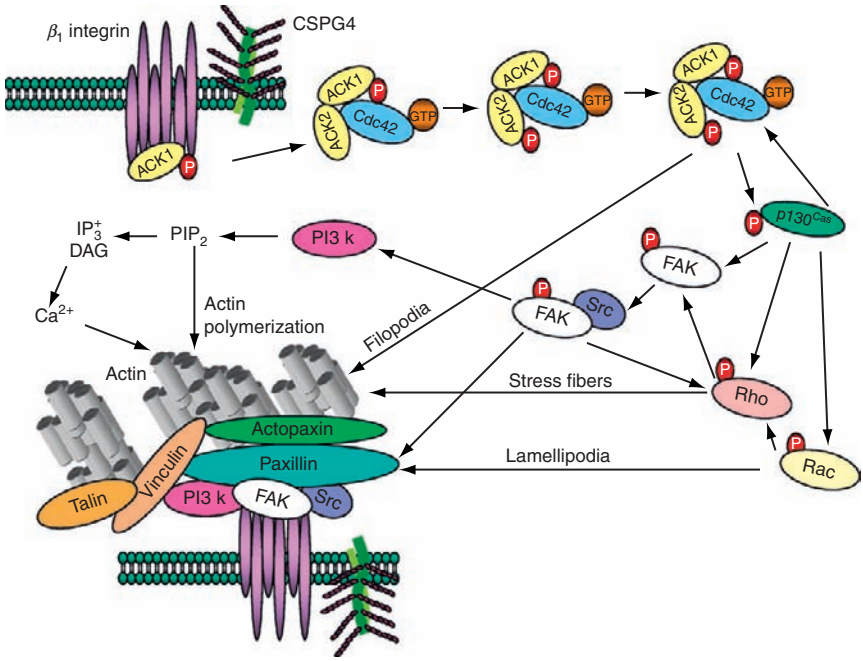

Fig. 8 Putative model for CSPG4-mediated FAK activation in malignant cells. The ECM, CSPG4, integrins, and the cell cytoskeleton interact at sites called focal contacts. The series of signaling events triggered by interactions of CSPG4 with β1 integrin and the ECM is postulated to occur through three phases, early, intermediate, and late. In the early phase, Ack-1 is activated and in turn triggers the activation of Rho GTPase Cdc42. In the intermediate phase, $p130^{Cas}$ is activated and, functioning as a positive feedback switch, turns on additional GTPases, such as Rho and Rac and the downstream FAK. In the late phase, FAK, in conjunction with Rho and Rac, activates various cytoskeletal components including paxillin, vinculin, talin, etc., and PI3-K. Alpha-actinin is a cytoskeletal protein that binds to vinculin and cross-links actin in actomyosin stress fibers and tethers them to focal contacts. Phosphorylation of Alpha-actinin by FAK1 reduces the cross-linking of stress fibers and prevents maturation of the focal contacts. Vinculin transiently recruits the Actin-related protein complex (Arp2/3) to new sites of integrin aggregation. Arp2/3 complex nucleates new Actin filaments from the sides of preexisting filaments. This interaction requires phosphorylation of the Arp2/3 complex by p21-activated kinase 1 (PAK1) that leads to polymerization of Actin. The culmination of these events leads to rearrangement and polymerization of the actin cytoskeleton and eventually to cell motility/migration.

receptors on the cell surface, since it also binds to the carboxyl terminal heparin binding domain of fibronectin (Iida *et al.*, 1992). The CSPG4 chondroitin sulfate chains may also be important in localizing the mature proteoglycan to retraction fibers, since migrating cells that express a chondroitin sulfate-free NG2 core protein assume a less polarized shape (Burg *et al.*, 1996; Lin *et al.*, 1996; Stallcup and Huang, 2008; Tillet *et al.*, 1997).

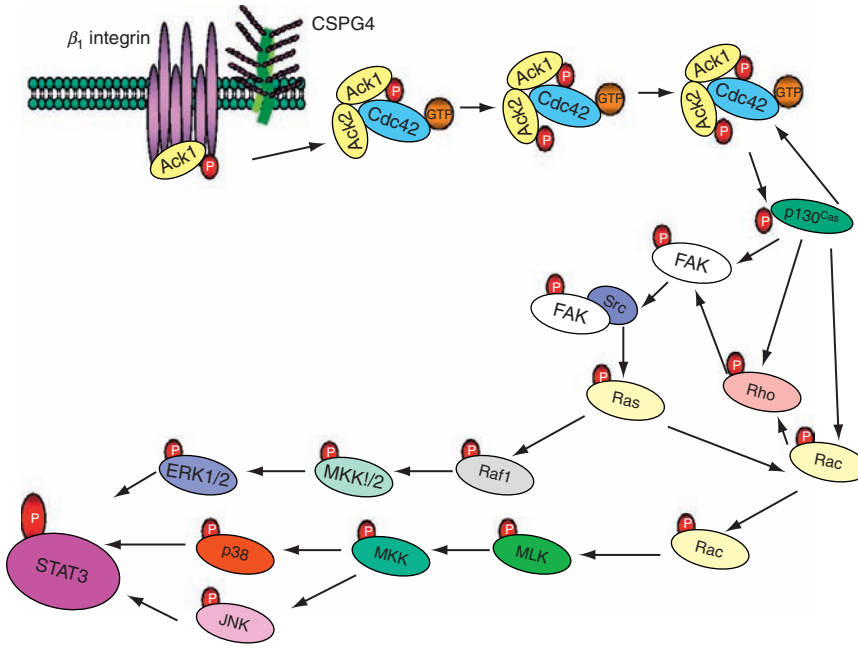

Fig. 9 Putative model for CSPG4-mediated STAT3 activation in malignant cells. The activation of STAT3 through interactions of CSPG4 with β1 integrin and the ECM is postulated to occur via activation of GTPases Ras and Rac through the intermediates $p130^{Cas}$ and FAK. Ras and Rac efficiently modulate STAT3 transcriptional activity by inducing its simultaneous tyrosine and serine phosphorylation via the MAP family of kinases, Src and JAK. The JNK (c-Jun N-terminal Kinase)/ERK (Extracellular-Signal Regulated Kinase) pathway mediates serine phosphorylation (Ser727); cooperation of both tyrosine as well as serine phosphorylation is necessary for full activation of STAT3. STAT3 is also activated in response to the small guanine nucleotide-binding protein Rac1. The Rac functions in growth factor-induced activation of STAT3 in two ways. It is thought to localize STAT3 to kinase complexes at the cell surface through Ras and also promotes activation of kinases, like MLKs (Mixed-Lineage Kinases), JAK2, TYK2 that phosphorylate STAT3 at Tyr705. Following tyrosine phosphorylation, STAT3 proteins dimerize, translocate to the nucleus, and activate specific target genes. This transcriptional activation by STAT3 proteins requires the recruitment of coactivators such as CBP (CREB-binding Protein)/p300. STAT3 proteins recognize a conserved element in the promoter of p21/WAF1 (wild-type p53-activated fragment-1) and increase the mRNA expression of this cell cycle regulatory gene. Effectively, STAT3 activates several other genes involved in cell cycle progression such as Fos, Cyclin-D, CDC25A, c-Myc, or Pim1 and upregulates antiapoptotic genes such as BCL2 (B-Cell CLL/Lymphoma-2), and BCLXL. Thus, many STAT3 target genes are key components of the regulation of cell cycle progression from G1 to S phase.

In contrast, migrating cells expressing an unmodified chondroitin sulfate proteoglycan NG2 core protein exhibit “leading” edges with actin-rich lamellipodia and “trailing” edges with NG2-positive retraction fibers. These results suggest that CSPG4, like NG2, may regulate cytoskeletal

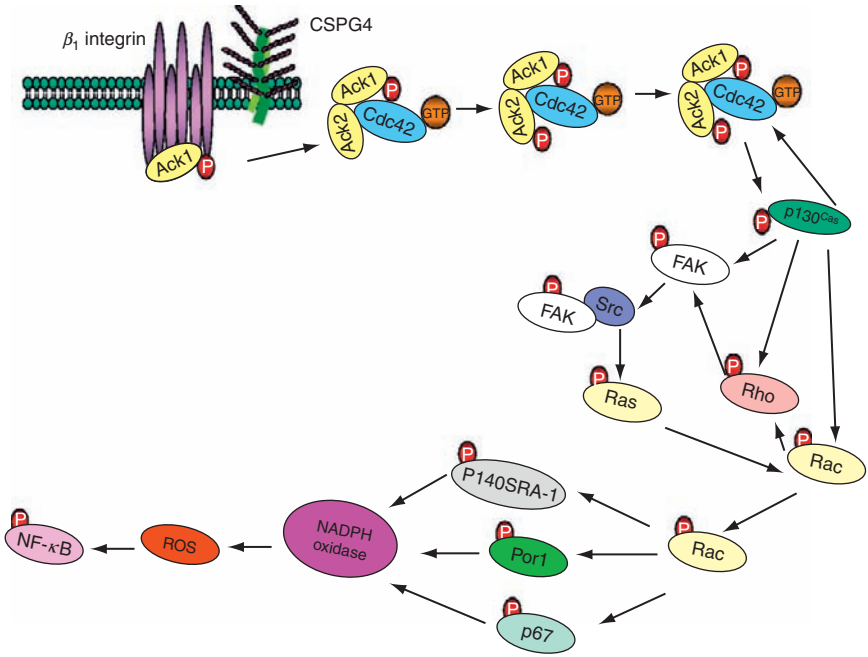

Fig. 10 Putative model for CSPG4-mediated NFkB activation in malignant cells. The activation of NFkB through interactions of CSPG4 with β1 integrin and the ECM is postulated to occur via activation of GTPases Ras and Rac through the intermediates $p130^{Cas}$ and FAK. Subsequently, Rac binds p67(Phox) to increase activation of the NADPH oxidase system and production of ROS (reactive oxygen species), which mediate activation of NFkB-dependent gene expression, Rac modulation of cell cycle progression and inhibition of Rho activity. Rac may also activate additional intermediates like the Rac-interacting protein, POR1 (Partner of Rac1), and the Rac1-associated protein, p140SRA1, both of which play a role in activation of NADPH oxidase as well as in membrane ruffling. Alternatively, Rac and CDC42 can also activate NFkB through their ability to stimulate PI3K and activate Akt. Once activated, the NFkB family of transcription factors, which consists of a heterodimeric protein composed of different combinations of members of the Rel family of transcription factors, is involved in cell survival and proliferation, tumorigenesis, and inflammatory responses.

rearrangement at the trailing edges of motile cells, since retraction fibers represent a specialized mechanism for facilitating release of the trailing edge from the substratum (Mitchison, 1992; Sheetz, 1994).

The cytoskeletal reorganization observed in migrating melanoma cells in response to CSPG4/ECM interactions indicates that CSPG4 may interact with the cytoskeleton directly and/or with components of the cytoskeletal-signaling complex. Additional adaptor proteins are likely to be required to connect CSPG4 to intracellular signaling pathways, since its core protein has no apparent catalytic domains. As noted above, the three threonine residues

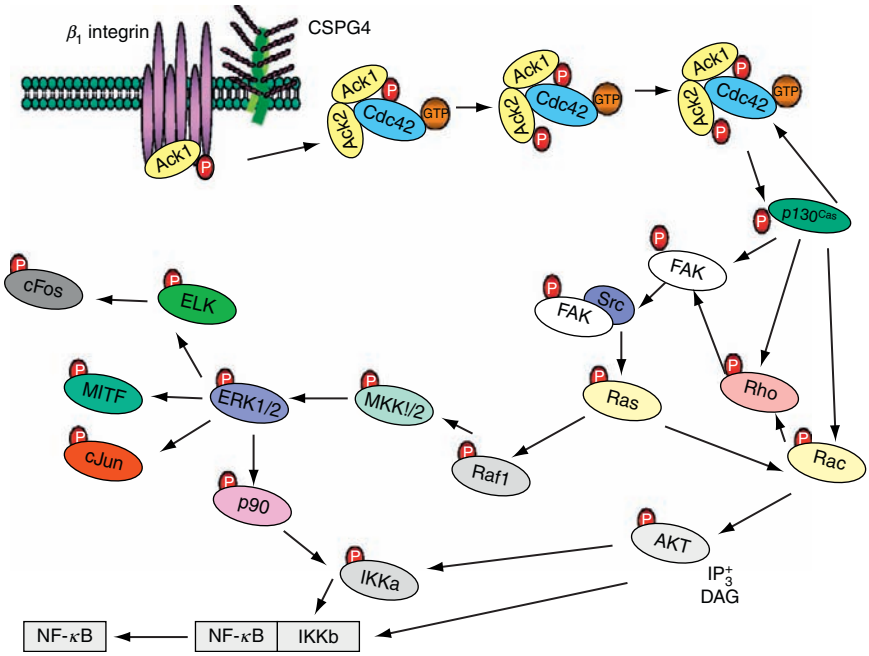

Fig. 11 Putative model for CSPG4-mediated MAPK pathway activation in malignant cells. The activation of the MAPK pathway through interactions of CSPG4 with β1 integrin and the ECM is postulated to occur via activation of GTPases Ras and Rac through the intermediates $p130^{Cas}$ and FAK. Ras and Rac then recruit and activate Raf, the MAP3K of the ERK cascade. Raf phosphorylates the MAP2 kinase, MEK, which transmits the signal to ERK. The ERK pathway comprises three Rafs (A, B, and C), two MEKs (1 and 2), and two ERKs (1 and 2). The three Rafs have different abilities to respond to Ras and to phosphorylate MEK, B-Raf ranking first in both cases, followed by C-Raf and A-Raf. Raf goes on to activate ERK1/2 via MEK1/2. ERK once activated translocates to the nucleus to phosphorylate and activate several nuclear targets. The major target of activated ERKs is RSK (90 kDa Ribosomal protein S6 Kinase). Activated RSKs appear to play a major role in transcriptional regulation, translocating to the nucleus and phosphorylating such factors c-Jun, c-Fos, DPC4 (deleted in pancreatic carcinoma 4), p53, ATF2 (activating transcription factor-2), NFAT4 (nuclear factor of activated T-cell-4), NFAT1 (nuclear factor of activated T-cell-1), STAT1 (signal transducers and activators of transcription-1), HSF1, SHC, and Bcl2 (B-cell CLL/lymphoma-2). Another important target of ERK is NFkB, which binds to its consensus sequence and positively regulates the transcription of genes involved in immune and inflammatory responses, cell growth control, and apoptosis. This activation of transcription factors goes on to promote cellular proliferation, migration, survival, and tumorigenesis.

present in the cytoplasmic tail of CSPG4 may serve as potential phosphorylation sites facilitating the interactions with adaptor proteins. Such a possibility is supported by the requirement of the carboxy-terminal half of NG2-cytoplasmic domain for NG2-mediated cell spreading and migration (Stallcup and Huang, 2008). This segment contains the multi-PDZ binding

domain, the proline rich segment and two of the threonines that are potential phosphorylation targets (Stallcup and Huang, 2008). Moreover, *in vitro* studies have demonstrated that PKCα and ERK can directly phosphorylate Thr 2256 and Thr 2314, respectively, on NG2 cytoplasmic domain (Makagiansar *et al.*, 2004, 2007). Phosphorylation of NG2 at Thr 2256 is accompanied by redistribution of the proteoglycan on the cell surface, by polarization of the cell membrane and by significant increases in cell motility (Makagiansar *et al.*, 2004, 2007). In contrast, phosphorylation of NG2 at Thr 2314 is accompanied by increased cellular proliferation (Makagiansar *et al.*, 2004, 2007). That NG2 phosphorylation brings about these effects is demonstrated by the inability of the mutated Thr 2256 and Thr 2314 transfectants to increase their motility or their proliferation in response to PKCα activation (Makagiansar *et al.*, 2004, 2007).

It is noteworthy that there appears to be a balance between cell proliferation and migration, since PKCα-mediated phosphorylation at Thr 2256 appears to stimulate cell motility while inhibiting cell proliferation. Conversely, ERK-mediated phosphorylation at Thr 2314 blocks cell motility while promoting cell proliferation (Makagiansar *et al.*, 2004, 2007). Considering that growth factor-dependent phosphorylation of both Thr 2256 and 2314 on NG2 appears to depend on PKCα activation, under what circumstances Thr 2314 phosphorylation leads to increased cell proliferation is unclear. It has been suggested that Thr 2314 phosphorylation may be explained by the ability of non-growth factor-driven mechanisms to activate ERK independently of PKCα activation via the activation of ERK via a G protein-coupled receptor-dependent pathway (Pierce *et al.*, 2001). Alternatively, integrin-mediated activation of the FAK-src-p130^{Cas} pathway could serve to stimulate ERK independently of PKCα (Defilippi and Cabodi, 2006). In the case of integrin-mediated signaling, both CSPG4 and NG2 are able to activate β1-integrin signaling by forming a complex with α3β1-integrin when the proteoglycan is expressed in *cis* fashion on the same cells as the integrin, as well as, in the case of NG2, when present in a soluble exogenous form (Fukushi *et al.*, 2004; Iida *et al.*, 1995; Yang *et al.*, 2004). At present, it is not clear how integrin activation by CSPG4 or NG2 might be able to stimulate proliferation in one case (Thr 2314 phosphorylation) and motility in the other one (Thr 2256 phosphorylation). It has been suggested that differential localization of CSPG4 or NG2-integrin complexes may be determined by the respective phosphorylation pattern with the result that integrin signaling occurs in distinctly different microdomains of the cell (Stallcup and Huang, 2008). In this regard, NG2 phosphorylated Thr 2256 is localized along with β1-integrin in lamellipodia so that integrin activation and the resulting downstream signaling intermediates are localized to a cellular microdomain that is critical to cell motility (Stallcup and Huang, 2008). In contrast, the NG2 phosphorylated Thr 2314, while

colocalized with β1-integrin, is associated with increased tyrosine phosphorylation on apical microprotrusions and not lamellipodia (Stallcup and Huang, 2008). These observations are consistent with the concept that integrin-mediated signal transduction can activate both motility and proliferation via intermediates such as FAK and Crk-associated substrate at specific cell membrane locations (Cox *et al.*, 2006).

C. Role in Angiogenesis

Angiogenesis involves a number of cellular processes including endothelial cell and pericyte proliferation and invasion (Ozerdem *et al.*, 2001). The role CSPG4 may play in regulating angiogenesis and tumor cell invasion is suggested by several lines of evidence. First, as already described, CSPG4 is expressed at high levels on both "activated" pericytes and pericytes in angiogenic vasculature within the tumor microenvironment (Schlingemann *et al.*, 1990). Pericytes are known to be associated with endothelial cells in developing vasculature and are thought to play a role in the regulation of angiogenesis by controlling endothelial cell proliferation and invasion, directing the outgrowth of microvessels and stabilizing capillaries. Second, CSPG4, like NG2, is widely expressed by angiogenic blood vessels in normally developing tissues, as well as in neovasculature found in tumor stroma and granulation tissue of healing wounds, but is not detectable on quiescent vasculature (Schlingemann *et al.*, 1990, 1991; Stallcup and Huang, 2008). Third, NG2-positive tumors have been found to have significantly increased neovascularization rates and vascular volumes (Chekenya *et al.*, 2002; Kirsch *et al.*, 2004). Fourth, NG2 enhances the ability of plasminogen activator to convert plasminogen into its active form plasmin and its anti-angiogenic proteolytic fragment angiostatin (Kirsch *et al.*, 2004). Last, NG2 can sequester angiostatin and block its ability to inhibit endothelial cell proliferation and angiogenesis (Kirsch *et al.*, 2004). Taken together, these findings in conjunction with the potential ability of CSPG4 to activate STAT3 signaling, are suggestive of CSPG4's involvement in angiogenesis.

IV. CLINICAL APPLICATIONS OF CSPG4

The expression of CSPG4 in a high percentage of melanoma lesions with low intra- and interlesional heterogeneity, its restricted distribution in normal tissues, and the availability of CSPG4-specific mAb have provided the rationale to utilize CSPG4 as a marker for immunoscintigraphy of malignant disease lesions and as a target of immunotherapy.

A. I*n Vitro* and I*n Vivo* Diagnostic Applications of CSPG4

A number of single-center and multicentric trials, which have enrolled more than 1000 patients with melanoma, have shown that CSPG4 represents a useful marker to visualize and localize cutaneous melanoma lesions with radiolabeled mAb (Ferrone *et al.*, 1993). However, there is conflicting information regarding the value of CSPG4 in immunoscintigraphy of uveal melanoma due most likely to the variable level of CSPG4 expression observed in uveal melanoma lesions (Bomanji *et al.*, 1987; Li *et al.*, 2003; van der Pol *et al.*, 1987). Despite the value of CSPG4 in immunoscintigraphy, its use as a marker to detect melanoma lesions has not become a widely accepted technology. This conclusion is mainly due to the low sensitivity of immunoscintigraphy and to the high background because of nonspecific accumulation of mAb in normal organs. Recently, this limitation has been, at least in part, overcome and tumor targeting has been improved both in mice transplanted with human melanoma cells and in patients with melanoma utilizing CSPG4-specific scFv fragments (Kang *et al.*, 1999, 2000). Whether the sensitivity of immunoscintigraphy with radiolabeled mAb can be enhanced by combination with more advanced imaging procedures such as positron emission tomography (PET), as suggested by the recent results obtained in an animal model system (Paul *et al.*, 2009) and as shown with other types of tumors (Ghesani *et al.*, 2003), remains to be determined.

Early studies demonstrated that CSPG4 is present in sera from healthy donors as well as from patients with malignant melanoma and that CSPG4 serum levels were increased in patients with stage IV melanoma (Ferrone *et al.*, 1993). No follow-up studies were performed to determine whether CSGP4 expression could predict outcomes in patients with melanoma. In recent years, the use of serum tumor markers in the clinical management of several malignancies has rekindled interest in determining the utility of CSPG4 serum level as a biomarker in predicting outcomes in patients with melanoma. Preliminary studies have suggested that changes in CSPG4 serum levels may be of prognostic significance in patients with resected melanomas who were immunized with a cell-based melanoma vaccine. The median 75 percentile recurrence-free survival was significantly longer in immunized patients whose CSPG4 serum levels decreased while on the treatment regimen (Vergilis *et al.*, 2005). Given the current interest in the identification of serologic biomarkers to monitor patients with early- as well as late-stage malignant disease and response to immunotherapy, it may be useful to determine whether these results can be reproduced on a large-scale basis.

Growing evidence in various types of carcinomas suggests that circulating tumor cells may help predict recurrence of disease and response to therapy

(Nagaiah and Abraham, 2010). These findings in conjunction with the high level of CSPG4 expression on melanoma cell membranes and the availability of mAb with high association constant have provided the rationale to evaluate the usefulness of CSPG4 as a marker to isolate melanoma cells from patients' peripheral blood. The results obtained by Ulmer *et al.* (2004) and by one of us (Kitago *et al.*, 2009) indicate that CSPG4-specific mAb bound to immunomagnetic beads are useful to isolate melanoma cells suitable for genomic and transcriptome characterization from melanoma patients' peripheral blood. Furthermore, the enumeration of melanoma cells isolated with mAb-coated immunomagnetic beads provides clinically significant information, since detection of two or more melanoma cells was correlated with reduced survival in patients with metastatic melanoma.

B. Targeting CSPG4 with Antibody-Based Immunotherapy

In the 1980s, CSPG4 was used as a target of antibody-based passive immunotherapy in patients with advanced melanoma. In these trials, CSPG4-specific mAb alone or mAb conjugated to radioisotopes or toxins were administered to patients with advanced melanoma (Larson *et al.*, 1985; Schroff *et al.*, 1985; Spitler *et al.*, 1987). Although CSPG4-specific mAb have been shown to control tumor growth in animal model systems (Bumol *et al.*, 1984; Matsui *et al.*, 1985; Morgan *et al.*, 1987), in the above clinical trials only minor clinical responses were observed in only a few patients. However, it is worth noting that targeting CSPG4 with CSPG4-specific mAb in patients with melanoma has not been associated with significant normal-organ-related accumulation or toxicity (Ferrone *et al.*, 1993; Reisfeld and Cheresh, 1987). Nevertheless, the enthusiasm for antibody-based immunotherapy of melanoma with CSPG4-specific mAb progressively waned. This topic needs now to be revisited in light of several recent findings in animal model systems and in patients with melanoma which indicate that CSPG4-specific mAb may inhibit melanoma cell growth. First, a retrospective study reviewing follow-up data on a group of 300 patients previously enrolled in an immunoscintigraphy trial with CSPG4-specific mAb demonstrated a significant prolongation of survival in patients who had received multiple injections of CSPG4-specific mAb (Bender *et al.*, 1997). Second, induction of CSPG4-specific antibodies in patients with stage IV melanoma immunized with the anti-idiotypic mAb MK2-23, which mimics the determinant defined by the CSPG4-specific mAb 763.74 (Kusama *et al.*, 1989), is significantly associated with regression of metastatic lesions in a few patients (Mittelman *et al.*, 1994) and with a statistically significant survival prolongation in

patients who had developed CSPG4-specific antibodies (Mittelman *et al.*, 1992). These findings are likely to reflect the antitumor activity of the elicited antibodies, since CSPG4-specific antibodies isolated from mice and rabbits immunized with CSPG4 peptide mimics can inhibit the functional properties as well as the growth of human melanoma tumors grafted in immunodeficient mice (Luo *et al.*, 2005, 2006; Wagner *et al.*, 2005, 2008). Third, similar results have been obtained with CSPG4-specific mAb. The latter have been shown to be able to control the growth of human CSPG4 expressing melanoma tumors and to inhibit metastasis of human basal breast cancer cells and melanoma cells in SCID mice (Wang *et al.*, 2010a,b). The mechanism(s) underlying these findings is (are) likely to be changes in the biology of melanoma cells, since the CSPG4-specific mAb utilized in these studies are poor mediators of complement- and cell-dependent cytotoxicity of melanoma cells (Imai *et al.*, 1982; Reisfeld and Cheresh, 1987; Wang *et al.*, 2010a,b). On the basis of the results recently published by Bluemel *et al.* (2010), the inability of the tested CSPG4-specific mAb to mediate immune lysis of target cells may reflect the location of the corresponding epitopes in subdomains of CSPG4 which are distant from the target cell membrane. If this interpretation is correct, mAb which recognize epitopes located in the subdomain of CSPG4 close to the target cell membrane should be developed. mAb with this specificity are expected not only to inhibit the signal transduction pathways relevant to CSPG4, but also to mediate complement- and cell-dependent cytotoxicity of CSPG4 bearing tumor cells. As a result, the efficacy of mAb-based immunotherapy targeting CSPG4 may be improved.

C. Targeting CSPG4 with T Cell-Based Immunotherapy

As already mentioned, the observed clinical benefits of CSPG4-specific humoral immunity is likely to reflect its ability to influence the biology of melanoma cells and not to mediate cell and/or complement-dependent lysis of melanoma cells. However, it cannot be discounted that CSPG4-specific cellular immunity plays a role in controlling melanoma cell growth, since immunization of melanoma patients with anti-idiotypic mAb which mimics determinants of CSPG4 can induce HLA class I antigen-restricted, CSPG4-specific CTL (Murray *et al.*, 2004; Pride *et al.*, 1998). These findings do not appear to be unique to the anti-idiotypic mAb MF11-30, which mimics the determinant defined by the CSPG4-specific mAb 225.28 (Kusama *et al.*, 1989), since immunization of melanoma patients with an anti-idiotypic mAb that mimics GD2 ganglioside also results in the generation of GD2-specific CTL (Basak *et al.*, 2003). To date, there is substantial preclinical and clinical evidence to support the possibility that TA-specific mAb can

function as opsonins and promote the immunogenicity of TA both in patients and in mouse model systems. As discussed elsewhere (Campoli *et al.*, 2010; Ferris *et al.*, 2010), multiple mechanism(s) are utilized by anti-idiotypic mAb or TA-specific anti-anti-idiotypic antibodies to prime antigen-specific CD4(+) and/or CD8(+) T cells. First, the anti-idiotypic mAb itself, which may possess partial AA sequence and/or structural homology with the target TA, can be taken up by antigen presenting cells (APC) and presented to T cells. Second, TA-specific anti-anti-idiotypic antibodies may mediate complement- and/or cell-dependent lysis of tumor cells or may effect direct tumor cell killing. Complexing of the released TA with the corresponding antibody will enhance its uptake, internalization, and presentation to CD8(+) T cells by dendritic cells, a process termed "cross-presentation." It is noteworthy that the activation and clonal expansion of CSPG4-specific CD8(+) T cells benefits from the activation of CD4(+) T cells. In this regard, CD4(+) T helper cell epitopes have been located in the extracellular domain of CSPG4 (Erfurt *et al.*, 2007, 2009). Further, HLA class II antigen-restricted, CSPG4-specific CD4(+) T cells have been found in patients with melanoma and with a higher frequency in healthy donors. The mechanism(s) and the clinical significance of this difference are not known. Nevertheless, natural CSPG4-specific immunity present in both healthy donors and patients with melanoma is not associated with any clinical signs of autoimmunity. This finding may reflect the lack of recognition by CD4(+) T cells of most somatic cells because of the lack of HLA class II antigen expression under basal conditions and their markedly lower CSPG4 expression as compared to normal tissues (Erfurt *et al.*, 2007), a situation which parallels several other TA such as CD20, HER2, and VEGF that have been successfully targeted in antibody-based immunotherapies (Forstpointner *et al.*, 2004; Hurwitz *et al.*, 2004; Slamon *et al.*, 2001). These findings, in conjunction with the preclinical and clinical evidence that TA-specific mAb can promote the immunogenicity of TA as well as enhance TA-specific immunity in TA-specific mAb-treated patients, provides further justification to target CSPG4 with mAb in patients with tumors expressing CSPG4.

V. CONCLUSION

In the past 10 years, there has been significant progress in the characterization of the expression of CSPG4 by normal and malignant cells, its role in the biology of melanoma cells and its recognition by humoral and cellular immunity. The information we have acquired has greatly contributed to our understanding of the unique features of this antigen and of its potential as a

target of immunotherapy. Nonetheless, a number of questions remain; we address some of them in this section of this review.

The data we have summarized clearly indicates that CSPG4 has a broader distribution than originally reported (Ferrone *et al.*, 1983) since it is expressed on various types of tumors of different embryological origin. At least in some tumors, CSPG4 is expressed not only on differentiated tumor cells, but also on cells with the phenotype of CSC. Because of its potential role in migration and metastasis of malignant cells, CSPG4 is likely to be involved in the functional properties of CSC. This possibility is supported by the ability of CSPG4-specific mAb to inhibit experimental lung metastasis as well as spontaneous metastases in a post surgery-recurrence/metastasis tumor model system (Wang *et al.*, 2010a).

A number of questions also remain regarding the biological function of CSPG4. First, if CSPG4 and integrins cooperate in transducing ECM signals into melanoma cells, how is this cooperation regulated? Second, since NG2 has been suggested to enhance the proliferative capacity of tumor cells by facilitating angiogenesis in rat models (Stallcup and Huang, 2008), does CSPG4 have the same effect in human melanoma cells as well as in other types of malignant cells? Third, if CSPG4 has multiple functions in melanoma cells, are they mediated by distinct domains of the antigen with respect to specific extracellular ligands or intracellular substrates? In this regard, it will be interesting to see if CSPG4 truly possesses distinct functional domains or its ability to modulate different physiologic functions is merely due to the different functional properties of chondroitin sulfate-free and -associated CSPG4. If distinct functional domains do exist, are they involved in different biologic processes? Do these functional domains have distinct extracellular ligands or intracellular substrates?

The molecular mechanisms by which CSPG4 activates intracellular signaling networks also need to be more clearly defined. In this regard, it is not clear how CSPG4 influences intracellular signaling transduction pathways into cells. What adaptor proteins are involved in transducing CSPG4 signals? How is this process regulated? It has yet to be established as to whether the CSPG4 can be phosphorylated and this should clearly be a focus of future studies. Moreover, if CSPG4 can promote MT-MMP and MMP activity, does this enhance the invasive and/or metastatic properties of melanoma as well as other types of malignant cells? Is the ability of CSPG4 to activate MT-MMP directly involved in wound healing and angiogenesis during tissue remodeling? In addition, can CSPG4 promote angiogenesis in a manner analogous to that of NG2? The expression of CSPG4 on activated pericytes in normal and tumor angiogenic vasculature suggests that this molecule may play an important role in regulating angiogenesis. Last, by which mechanism(s) do CSPG4-specific mAb influence melanoma cell biology? Can CSPG4-specific mAb interfere with CSPG4-ECM

interactions? Do they interfere with the cellular signal transduction pathway (s) required for CSPG4 to exert its function possibly as an integrin coligand/coreceptor to bind to certain ECM components?

CSPG4 displays several features that make it an attractive target for the application of immunotherapy in patients with malignant disease. First, CSPG4 can be targeted with both mAb and with T cells, therefore providing the opportunity to combine mAb- and T cell-based immunotherapeutic strategies. This combinatorial therapeutic strategy is expected to be able to counteract some of the escape mechanisms tumor cells utilize to avoid recognition and destruction by the host immune system. These escape mechanisms include, but are not limited to, downregulation and/or loss of the targeted TA, and/or defects in the expression and/or function of HLA class I antigens as well as antigen processing machinery components which are required for the presentation of CSPG4 derived peptides to cognate CTL. Second, CSPG4 is expressed not only on differentiated malignant cells, but also on cells with the phenotype of CSC, at least in basal breast carcinoma and SCCHN. More importantly, the data obtained in experimental models of basal breast carcinoma and SCCHN indicate that CSPG4 can mediate the destruction of CSC (Wang, 2010, unpublished results). If the results derived from experiments performed with human cell lines implanted in immunodeficient mice are paralleled in a clinical setting, CSPG4-specific immunotherapy may overcome one of the major limitations of the current available antineoplastic therapies, that is, their inability to eradicate CSC, which according to the CSC theory are responsible for recurrence and metastasis of malignant lesions (Lobo *et al*., 2007; Visvader and Lindeman, 2008). Third, CSPG4 is expressed on activated pericytes in the tumor microenvironment. More importantly, CSPG4-specific immunotherapy has an antiangiogenic effect which can inhibit the growth of tumor cells even those which do not express CSPG4. Therefore, one can expect that this type of immunotherapy may counteract the escape mechanisms driven by the genetic instability of tumor cells. It is noteworthy that both the antiangiogenic and the antitumor effects of CSPG4-specific immunotherapy can be enhanced by combination with metronomic chemotherapy (Kerbel and Kamen, 2004). It is expected that the reduction of pericyte coverage of blood vessels in the tumor microenvironment caused by CSPG4-specific immunotherapy may enhance the sensitivity of endothelial cells to cytotoxic drugs. Fourth, the role of CSPG4 in the biology of malignant cells is consistent with the marked antitumor effects caused by inhibition of its function. Identification of the signaling pathways involved in these effects provides a background to design combinatorial strategies to enhance the efficacy of CSPG4-specific immunotherapy. Finally, CSPG4 appears to have a stable expression on tumor cells, therefore minimizing the escape mechanisms caused by loss of the targeted TA. In the future, it will be important to

determine if we can enhance the therapeutic efficacy of conventional therapeutic regimens by the concomitant administration of CSPG4-specific mAb. Further, given the promising results of early clinical studies with B-Raf and MEK inhibitors, it will be important to determine if the therapeutic efficacy of CSPG4-specific mAb-based immunotherapy can be enhanced by the administration of small-molecule inhibitors that target signal transduction pathways. Must these inhibitors target different or the same signal transduction pathways mediated by CSPG4? Can antibody-drug (cytotoxic agents) conjugate increase the magnitude and duration of response to CSPG4-specific mAb and to cytotoxic agents and reduce systemic toxicity caused by cytotoxic agents? The answers to these questions will not only greatly improve our understanding of the role of CSPG4 in malignant cell biology, but will help to improve the design of therapies for the treatment of CSPG4 expressing malignant tumors.

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KRAS Signaling Pathway Alterations in Microsatellite Unstable Gastrointestinal Cancers

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Microsatellite instability (MSI) occurs in about 15% of gastrointestinal cancers and it is associated with specific clinic, pathologic, and molecular features of the tumors. MSI-high (MSI-H) carcinomas also follow specific tumor development pathways. This review is focused on the molecular profile of alterations in members of the KRAS signaling pathway (*EGFR*, *KRAS*, *BRAF*, *PIK3CA*, *RASSF1A*, and *MLK3* genes) in MSI gastrointestinal carcinomas. Alterations in these genes characterize more than half of gastrointestinal cancers and frequently occur simultaneously in the same tumor, pinpointing the KRAS signaling pathway as one of the most frequently altered pathways in this subset of cancers. Nowadays, many and novel inhibitors targeting molecules of this signaling pathway are being described; therefore, it is worthwhile to test their efficacy in MSI gastrointestinal cancers in order to develop new and more directed targeted therapies for patients affected by this disease.

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I. MISMATCH REPAIR, MSI, AND RELATED TARGET GENES

About 15% of gastrointestinal carcinomas are characterized by the accumulation of numerous mutations across the genome, mainly occurring in repetitive sequences (microsatellites), due to a defective mismatch repair (MMR) system and creating a molecular phenotype designated as microsatellite instability-high (MSI-H) (Ionov *et al.*, 1993).

The MMR system is composed of at least seven proteins, h-MLH1, h-MLH3, h-MSH2, h-MSH3, h-MSH6, h-PMS1, and h-PMS2, which associate with specific partners to form functional heterodimers that recognizes base-pair mismatches and small nucleotide insertion/deletions (1–4 base pairs) that occur during DNA replication (Jiricny, 2006; Rustgi, 2007; Thomas and Boland, 2006). h-MLH1 and h-MSH2 are essential components of the human MMR machinery and form five functional heterodimeric complexes: h-MSH2/h-MSH3, h-MSH2/h-MSH6, h-MLH1/h-PMS1, h-MLH1/h-PMS2, and h-MLH1/h-MLH3 (Jiricny, 2006). Genetic and epigenetic alterations occurring at these MMR system effectors, namely in h-MLH1 and h-MSH2, and less frequently in h-MSH6 and h-PMS2, are the main mechanisms by which MMR system failure occurs in MSI-H gastrointestinal cancers (Peltomaki, 2001).

In cells with a defective MMR system, mutations accumulate not only in noncoding repetitive sequences but also in coding sequences. In the case of instability affecting coding repeat sequences, immediate functional consequences are expected to occur in such genes. Target genes of MMR deficiency include among many others, *TGFBRII*, *IGFIIR*, *BAX*, *TCF-4*, *h-MSH3*, *h-MSH6*, *CASPASE-5*, *GRB-14*, *RHAMM*, *RAD50*, *RIZ*, *E2F4*, *PTEN*, *MBD4*, *CHK1*, *FAS*, *APAF-1*, *BCL-10*, and *EPHB2* (Davalos *et al.*, 2006; Duval and Hamelin, 2002). *TGFBRII*, among all described MMR target genes, is the one harboring the highest mutation frequency (about 80%) and these alterations are known to associate with enhanced cell proliferation rate and higher angiogenic potential (Jakowlew, 2006).

II. MSI-H PHENOTYPE-RELATED MOLECULAR AND CLINICOPATHOLOGIC FEATURES

A. Gastric Cancer

The MSI-H phenotype generally characterizes 15–25% of GC cases and it comprises a group of stomach tumors displaying specific clinicopathologic features (Beghelli *et al.*, 2006; Corso *et al.*, 2009; Ottini *et al.*, 2006).

The mechanism leading to MMR deficiency in 75% of MSI-H GC cases is *h-MLH1* promoter hypermethylation (Gu *et al*., 2009; Pinto *et al*., 2000). Moreover, *Helicobacter pylori* infection was recently described to be able to downregulate major DNA repair pathways (Machado *et al*., 2009, 2010). Although somatic mutations in MMR genes have also been described in MSI-H GC, they were proved to constitute a consequence rather than a cause of the mutator phenotype (Pinto *et al*., 2008).

In terms of clinicopathological features, most MSI-H GCs are of intestinal histotype, are located in the distal part of the stomach, and occur more frequently in older women (Corso *et al*., 2009; dos Santos *et al*., 1996; Oliveira *et al*., 1998; Seruca *et al*., 1995). Further, this phenotype delineates a subset of tumors with lower ability to invade serosal layers that preferentially spread to the periphery of the stomach via the lymphatic stream to the nodes, therefore generating an overall favorable long-term prognosis even in patients with advanced disease (Corso *et al*., 2009; dos Santos *et al*., 1996; Oliveira *et al*., 1998; Seruca *et al*., 1995). Data on long-term survival demonstrated that MSI-H advanced GC patients show higher survival rates in comparison to patients with other types of GC even if they are in the same disease stage (Beghelli *et al*., 2006). Overall, patients with GC displaying MSI-H phenotype have better survival than those with a microsatellite stable (MSS) phenotype. Altogether, this data most probably identify a particular subset of MSI-H tumors biologically less aggressive and with a favorable prognosis (Corso *et al*., 2009).

MSI-H GC from patients with familial history of GC were recently found to display similar clinicopathologic characteristics to those of MSI-H sporadic GC cases (Leite *et al*., 2010, in press; Pedrazzani *et al*., 2009).

B. Colorectal Cancer

MSI-H colorectal cancers (CRCs) arise either in a sporadic or in a hereditary context (Lynch syndrome) (Lynch *et al*., 1991).

In MSI-H sporadic CRCs, methylation of the *h-MLH1* promoter region is the main mechanism responsible for MMR system failure and occurs in about 75% of the cases (Deng *et al*., 2004; Lubomierski *et al*., 2005). Another epigenetic mechanism involved in the somatic deregulation of MMR proteins expression was recently described (Valeri *et al*., 2010). In this work, the authors reported that overexpression of miR-155 significantly downregulates the core MMR proteins, h-MSH2, h-MSH6, and h-MLH1, inducing a mutator phenotype and MSI. Furthermore, they have demonstrated an inverse correlation between the expression of miR-155 and the expression of h-MLH1 or h-MSH2 proteins in a number of MSI tumors with a previous unknown cause of somatic MMR inactivation (Valeri *et al*., 2010).

In Lynch syndrome, the most common causal genetic events underlying MMR deficiency are germline mutations at MMR genes, namely, *h-MLH1*, *h-MSH2* (*h-MLH1* and *h-MSH2* accounts for almost 90% of all identified mutations), *h-MSH6,* and *h-PMS2* (Castells *et al.*, 2009; Lynch and de la Chapelle, 2003; Peltomäki and Vasen, 2004).

Recently, deletions of the terminal end of the *EPCAM* gene have been identified in a small number of families with Lynch syndrome whose tumors demonstrate loss of h-MSH2 (Ligtenberg *et al.*, 2009). In these cases, a failure in transcriptional termination of *EPCAM* results in the generation of fusion transcripts with the adjacent *h-MSH2* gene downstream, giving rise to methylation of the *h-MSH2* promoter, particularly in epithelial tissues where *EPCAM* is expressed at high levels (Ligtenberg *et al.*, 2009). Constitutional epimutations of the *h-MLH1* gene have also been identified in mutation-negative individuals with a clinical diagnosis of Lynch syndrome (Gazzoli *et al.*, 2002; Goel *et al.*, 2010; Hitchins *et al.*, 2005; Miyakura *et al.*, 2004; Niessen *et al.*, 2009; Suter *et al.*, 2004). This defect is characterized by soma-wide promoter methylation and transcriptional silencing of a single allele of the *h-MLH1* gene (Hitchins *et al.*, 2005; Miyakura *et al.*, 2004; Suter *et al.*, 2004). Despite the importance of these findings, the frequencies of germline epimutations of *h-MLH1* and *h-MSH2* seem to be rather infrequent in a cohort of Lynch syndrome-suspected patients (0.6% and 0.9%, respectively) although quite high in the genetically proven Lynch syndrome cases (about 16% of all mutations) (Niessen *et al.*, 2009).

Due to the presence of the mutator phenotype, MSI-H CRCs arising either in a sporadic or in a hereditary context are associated with unique clinical and pathological features namely, proximal location, poor differentiation, and frequent presence of a mucinous component (Cai *et al.*, 2008). The presence of tumor-infiltrating lymphocytes is prevalent in MSI-H tumors and it has been suggested as a useful marker in the identification of the MSI-H phenotype (Banerjea *et al.*, 2009; Chang *et al.*, 2009; Drescher *et al.*, 2009; Greenson *et al.*, 2009). MSI-H CRCs have been found to harbor a lower tumor stage at diagnosis and rarely metastasize to distant lymph nodes or distant organs (Gryfe *et al.*, 2000; Malesci *et al.*, 2007), showing a good prognosis and a better overall and relapse-free survival (Clark *et al.*, 2004; Gryfe *et al.*, 2000). Additionally, several clinical studies have demonstrated that patients with MSI-H CRCs do not benefit from the treatment with 5-fluorouracil (5-FU)-based adjuvant chemotherapies (Arnold *et al.*, 2003; Carethers *et al.*, 1999, 2004; Des Guetz *et al.*, 2009a,b,c; Ribic *et al.*, 2003; Sinicrope and Sargent, 2009).

Nevertheless, and besides the abovementioned shared clinical and pathologic characteristics between hereditary and sporadic MSI-H CRCs,

differences also exist, mainly the sporadic setting of MSI-H tumors arising in the proximal or distal colon.

In the sporadic setting of MSI-H CRCs, cases localized in the distal colon were shown to form a distinct subgroup of tumors with particular clinicopathological and molecular features that differ from those localized in the proximal MSI-H CRCs. Patients with MSI-H CRCs in distal location were significantly younger and of the male gender, and tumors displayed preferentially differentiated histology, smaller tumor size, distant metastasis, stability at BAT25 and BAT26, and showed h-MLH1 protein expression by immunohistochemistry as compared with proximal MSI-H CRCs. In addition, distal MSI-H CRCs demonstrated significantly worse 3-year overall and disease-free survival rates when compared to proximal MSI-H CRCs (87.0% vs. 97.4%; 81.6% vs. 95.9%). For stage III–IV CRCs, distal MSI-H CRCs also showed significantly worse 3-year overall and disease-free survival rates than proximal MSI-H CRCs (72.2% vs. 90.5%; 58.3% vs. 94.4%) (Kim *et al.*, 2010; Tanaka *et al.*, 2007).

Sporadic MSI-H CRCs are more frequent in older patients and are preferentially associated with the female gender. On the contrary, the cancers from the hereditary setting are associated with a younger age at onset and are more frequently found in males. Furthermore, lymphocytic infiltration, tumor budding (dedifferentiation), and the presence of coexisting adenomas are more evident in HNPCC. Furthermore, the cytological features of Lynch syndrome cancers recapitulate the basophilia and nuclear characteristics of conventional adenomas (Jass, 2004). MSI-H sporadic CRCs are mucinous, show poor differentiation, tumor heterogeneity, and glandular serration, and exhibit coexisting serrated polyps are more evident in sporadic MSI-H CRCs (Jass, 2004). Moreover, sporadic MSI-H CRCs are also characterized by cytoplasmic eosinophilia and nuclei that are large, round, vesicular, and contain a prominent nucleolus (Jass, 2004). Therefore, the crucial distinction between MSI-H hereditary and MSI-H sporadic CRCs should be achieved by integrating all available data including family history, age at onset of malignancy, clinicopathological, and molecular features (Jass, 2004).

III. MSI-H-RELATED EPIGENETIC FEATURES: CPG ISLAND METHYLATOR PHENOTYPE

The CpG island methylator phenotype (CIMP) was firstly described in CRC by Toyota *et al.* (1999) as being a mechanism whereby some CRCs were found to accumulate high rates of aberrant promoter methylation.

In this pioneer work, the authors analyzed the methylation status of several CpG islands in a series of CRCs, colorectal adenomas, and normal colorectal mucosa. They found that the majority of the CpG islands analyzed were methylated in both normal mucosa and in cancers, which is suggestive of an age-related phenomenon. Although, some of the CpG islands studied showed a cancer-specific methylation pattern and characterized a particular group of tumors. These findings raised the hypothesis that CIMP could contribute to CRC. Furthermore, the demonstration that the mismatch-repair gene *h-MLH1* was frequently inactivated due to promoter methylation in the CIMP positive group of CRCs was a strong argument favoring the involvement of an epigenetic mechanism working in CRC development (Toyota *et al.*, 1999). In fact, many other genes involved in cancer have a CpG island within their promoter region and so they are potential targets for transcriptional repression through methylation in a CIMP context (Toyota *et al.*, 1999).

CIMP was shown to be associated with several clinicopathologic and molecular features of CRCs. It is more frequently found in tumors located in the proximal colon (Toyota *et al.*, 1999). It is associated with female gender (Ogino *et al.*, 2006), older age, poor differentiation, and mucinous histology (Whitehall *et al.*, 2002). At the molecular level, CIMP was shown to be more frequent in sporadic cancers characterized by the presence of oncogenic BRAF and MSI-H (Ahuja *et al.*, 1997; Weisenberger *et al.*, 2006). Moreover, CIMP has been observed to occur in nonmalignant serrated lesions and implicated in the development of sporadic MSI-H CRCs in close association with the presence of oncogenic BRAF (O'Brien *et al.*, 2006).

On the other hand, Lynch syndrome cancers seldom display CIMP, but whenever present, this methylator phenotype affects genes different from those that are aberrantly methylated in the sporadic counterpart (Herman *et al.*, 1998; Kuismanen *et al.*, 2000; Wheeler *et al.*, 2000; Yamamoto *et al.*, 2002). For example, the frequency of DNA methylation at *hMLH1*, *CDKN2a*, and *THBS1* genes was found to be significantly lower in MSI-H cancers from Lynch patients than in MSI-H sporadic cancers (Herman *et al.*, 1998; Kuismanen *et al.*, 2000; Wheeler *et al.*, 2000; Yamamoto *et al.*, 2002).

CIMP phenotype was also reported to occur in about 31% of MSI-H gastric carcinomas (An *et al.*, 2005). Genes such as *hMLH1*, *MGMT*, *CDH1*, and *COX2* were shown to be frequently methylated (Carvalho *et al.*, 2003). Similar to CRC, in gastric carcinomas, MSI-H status is also closely associated with *hMLH1* promoter hypermethylation and CIMP status (An *et al.*, 2005; Carvalho *et al.*, 2003). Furthermore, hypermethylation at promoter regions might be a particularly important epigenetic determinant in gastric cancer development, since it has been reported to increase along progression from chronic gastritis, intestinal metaplasia, and adenomas to carcinomas of the stomach (Fleisher *et al.*, 2001).

IV. MSI-H-RELATED GENETIC FEATURES: THE KRAS SIGNALING PATHWAY

MSI-H gastrointestinal cancers display particular epigenetic and genetic backgrounds associated with specific molecular pathways, therefore reflecting their different clinic and pathologic features. A disturbed KRAS signaling pathway represents an important event in the initiation and progression of GC and CRCs, as well as, an important therapeutic biomarker.

A. Molecular Alterations in the KRAS Signaling Pathway

Activation of the KRAS signaling pathway is a frequent event in the carcinogenesis process of gastrointestinal malignancies. The KRAS signaling pathway is a very broad pathway that is involved in the control of several other signaling pathways by interacting with a wide spectrum of direct downstream effector molecules (Fig. 1). The mutational activation of *KRAS* and other molecules belonging to the pathway, such as epidermal

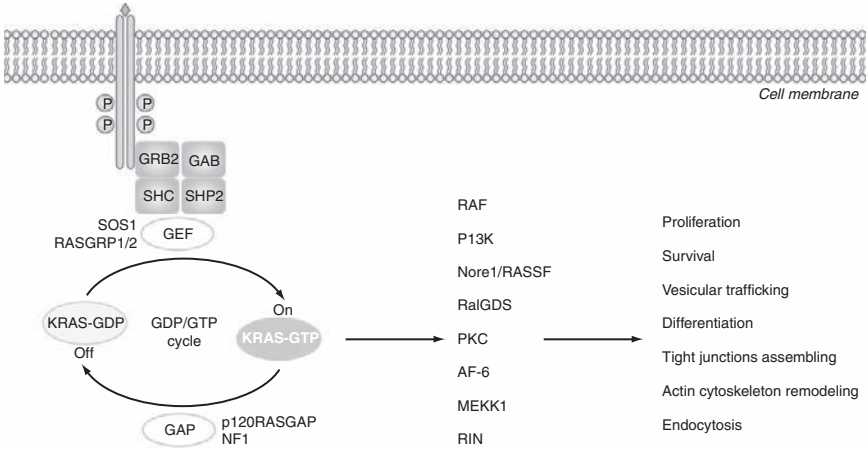

Fig. 1 KRAS activation, downstream targets, and cellular effects. At the plasma membrane, cell surface receptors are activated by extracellular stimuli. Activated cell surface receptors interact with a variety of adaptor proteins (GRB2, GAB, SHC, and SHP2) that mediate the subsequent activation of GEFs (SOS1 and RASGRP1/2). Active GEFs catalyze the transition from a GDP to an active GTP-bound state. Active KRAS interacts with several downstream effectors (RAF kinases, PI3K, Nore1/RASSF, RalGDS, PKC, AF-6, MEKK1, and RIN) and by this way, it is involved in the control of a variety of cellular functions.

growth factor receptor (*EGFR*), phosphatidylinositol 3-kinase (*PI3K*) and mixed-lineage kinase (*MLK3*), and RAS-association domain family isoform 1 A (*RASSF1A*), constitute important events in the activation of this signaling pathway in these types of cancer (Fig. 1).

In CRCs, KRAS, a small guanosine triphosphatase (GTPase) and BRAF, a serine/threonine protein kinase, which are key players of the mitogen-activated protein kinase (MAPK) signaling pathway, are often mutated (Rajagopalan *et al.*, 2002). Oncogenic mutations in these genes are preferentially found as alternative molecular alterations (Rajagopalan *et al.*, 2002) and due to this fact, they were initially thought to signal through the same pathway. However, their mutational frequency is not homogenous among the different subsets of CRC, in particular, in the MSI-H subset of carcinomas (Domingo *et al.*, 2004a; Rajagopalan *et al.*, 2002), suggesting that rather than leading to similar functional roles, *KRAS* and *BRAF* oncogenes may play different and independent functions in the development and progression of MSI-H CRCs.

KRAS mutations characterize both sporadic and hereditary MSI-H tumors but they are more frequent in the later (40%) rather than in the former (20%) (Deng *et al.*, 2004; Domingo *et al.*, 2004a; Lubomierski *et al.*, 2005; Oliveira *et al.*, 2004). Moreover, the *KRAS* mutation frequency and pattern differ among the different subsets of MSI-H CRCs (hereditary and sporadic). In CRC, the most frequent amino acid changes observed were G12D, G12V, and G13D (Oliveira *et al.*, 2004). *KRAS* mutations in codon 12 were more common in sporadic cases, whereas mutations in codon 13 were predominant in the MSI hereditary setting (Oliveira *et al.*, 2004). Although, the reasons underlying this observation are not yet elucidated, it is likely that different *KRAS* mutations are differentially selected for tumor development/progression depending on distinct tumor contexts.

BRAF mutations occur preferentially in the sporadic setting of MSI-H CRCs (40%) (Domingo *et al.*, 2004a; Oliveira *et al.*, 2003) where they strongly correlate with the presence of a genome-wide CIMP phenotype and *h-MLH1* promoter hypermethylation (Deng *et al.*, 2004; Lubomierski *et al.*, 2005; Nosho *et al.*, 2008; Ogino *et al.*, 2009; Shen *et al.*, 2007; Weisenberger *et al.*, 2006). In addition, the presence of mutant *BRAF* was demonstrated to be a poor prognostic indicator, being found associated with advanced phases of the disease (Zlobec *et al.*, 2009) and leading to abrogation of the good prognosis associated to MSI-H CRCs (Ogino *et al.*, 2009). On the other hand, *BRAF* mutations were very rarely found in Lynch syndrome associated carcinomas (Deng *et al.*, 2004; Domingo *et al.*, 2004a; Lubomierski *et al.*, 2005; Oliveira *et al.*, 2004), and therefore the detection of a positive *BRAF* mutation in a CRC is indicative of its sporadic origin and suggests the use of *BRAF* mutation testing as an exclusion criteria when selecting hereditary MSI cancers (Domingo *et al.*, 2004b, 2005).

In the case of cancers arising in the stomach, *KRAS* mutations were described in about 4% of the cases, while *BRAF* mutations were extremely rare (Oliveira *et al.*, 2003). In GC, *KRAS* mutations were only observed in the MSI subset, in about 30% of the cases (Brennetot *et al.*, 2003).

In addition to *KRAS* and *BRAF* mutations, alterations in other KRAS downstream effectors, such as PI3K activation through *PI3K* catalytic alpha subunit (*PIK3CA*) mutations and *RASSF1A* inactivation through promoter hypermethylation, are also found in CRCs (Samuels *et al.*, 2004; van Engeland *et al.*, 2002) and represent another example of a disturbed KRAS signaling pathway.

PIK3CA oncogenic mutations were described to characterize both sporadic and hereditary MSI CRCs, occurring at similar frequencies in both tumor settings. In the sporadic setting, *PIK3CA* mutations were described to occur in about 16% of the cases, whereas they occur preferentially associated with the presence of *KRAS* or *BRAF* oncogenic mutations (Campbell *et al.*, 2004; Samuels and Velculescu, 2004; Velho *et al.*, 2005). *PIK3CA* gene mutations were also described to occur in about 15% of hereditary MSI colorectal carcinomas (Ekstrand *et al.*, 2009; Miyaki *et al.*, 2007), and were also found to occur in cases harboring *KRAS* oncogenic mutations (Ekstrand *et al.*, 2009). Both in sporadic and hereditary tumors, *PIK3CA* oncogenic alterations were associated with an invasive phenotype (Miyaki *et al.*, 2007).

PIK3CA mutations were also described in about 4–11% of GC cases (Lee *et al.*, 2005; Li *et al.*, 2005; Velho *et al.*, 2005). A higher frequency was initially described by Samuels *et al.* (2004) (25%), but this study was performed in a small series of tumors ($n = 12$) (Samuels *et al.*, 2004). A deeper analysis, performed later, has demonstrated that *PIK3CA* alterations occur specifically in the MSI subset of GC (Li *et al.*, 2005; Velho *et al.*, 2005). Moreover, *PIK3CA* gene mutations and *KRAS* oncogenic alterations were described as alternative genetic events in this subgroup of GC (Velho *et al.*, 2005).

Hypermethylation of the promoter region of *RASSF1A* gene was reported to occur in 52% of sporadic MSI CRCs and in 30% of MSI hereditary carcinomas (Oliveira *et al.*, 2005). Interestingly, and despite similar high frequencies of *RASSF1A* promoter hypermethylation were found in both CRC settings, sporadic MSI cancers accumulate overall significantly more not only epigenetic but also genetic alterations in genes belonging to the KRAS signaling pathway (Oliveira *et al.*, 2005). *RASSF1A* promoter hypermethylation was also reported as a frequent epigenetic alteration in MSI gastric malignancies and it was found to occur mainly in tumors lacking *KRAS* mutations (Oliveira *et al.*, 2005).

Nevertheless, alterations in molecules functioning upstream of KRAS, such as the receptor tyrosine kinase EGFR, as well as in other molecules involved in mediating KRAS signal, independently of being its direct targets,

such as the MLK3, are also important players in the deregulation of this signaling pathway in MSI carcinomas.

EGFR overexpression was reported to occur in 30–85% of human CRCs (Jorissen *et al.*, 2003; Shia *et al.*, 2005), while mutations in the kinase domain are rare in this type of cancer (Barber *et al.*, 2004; Ogino *et al.*, 2005). In GC, although the frequency of EGFR overexpression is high, the mechanisms responsible to this overexpression remain undiscovered in most cases (Becker *et al.*, 2006; Takehana *et al.*, 2003). *EGFR* increased copy number was reported in about 13% of GC, which was mainly attributed to polysomy of chromosome 7 rather than to *EGFR* gene amplification (Moutinho *et al.*, 2008). Furthermore, mutations at the *EGFR* kinase domain were found very rarely in GC (Mammano *et al.*, 2006; Moutinho *et al.*, 2008).

Recently, a novel genetic mechanism underlying EGFR overexpression was described in MSI-H CRCs (Yuan *et al.*, 2009). High levels of EGFR expression were detected in MSI CRC cell lines due to the presence of a novel deletion in a polyA(13) repeat element within the 3′-untranslated region (3′-UTR) of the *EGFR* gene. Approximately 64% of MSI-H CRC cell lines and 69% of MSI-H CRC cases presented deletions at this repeat sequence (Yuan *et al.*, 2009). This alteration was shown to enhance *EGFR* mRNA stability, thus leading to EGFR protein overexpression (Yuan *et al.*, 2009). Our group has recently investigated the frequency of this alteration in a cohort of 63 MSI-H GCs and respective normal mucosa. We verified that about 32% of the GCs studied displayed tumor-specific deletions at the *EGFR* polyA(13) repeat element (unpublished data). In addition, this type of *EGFR* deletions was found to occur either as isolated genetic alterations or in concomitance with mutations of *KRAS* and/or *PIK3CA* genes, suggesting a cumulative effect of both oncogenic events in MSI-H GCs.

The high frequency of deletions in *EGFR* polyA(13) repeat element found in MSI-H gastrointestinal cancers, and its association with EGFR protein overexpression pinpoints EGFR as a good candidate for target therapy in MSI-H gastrointestinal cancers. Further studies are needed in order to determine the relationship between the presence of this *EGFR* mutation and the potential use of anti-EGFR monoclonal antibodies in the treatment of MSI-H cancers, perhaps by adapting the least successful conventional chemotherapy regimens. Nevertheless, a cautionary note should be added since deletions at the *EGFR* polyA(13) repeat may also occur in association with *KRAS* mutations (unpublished data) which is by itself a negative predictor of response to anti-EGFR monoclonal targeted therapies (Lièvre *et al.*, 2006, 2008).

MLK3 is a serine/threonine protein kinase that regulates the MAPKinase pathway activating ERK, p38, and JNK, in response to extracellular signals (Chadee and Kyriakis, 2004; Gallo and Johnson, 2002). In a recent paper by our group (Velho *et al.*, 2010), we described for the first time *MLK3*

mutations in a series of gastrointestinal cancers (Velho *et al*., 2010). *MLK3* gene mutations were significantly associated with MSI phenotype, occurring in 21% of the MSI tumors (Velho *et al*., 2010). Interestingly, cells expressing mutant forms of this gene showed transforming capacity *in vitro*, and were able to develop locally invasive tumors when subcutaneously injected in mice. Therefore, *MLK3* was described as a new candidate biomarker and/or an attractive potential target for future therapy in MSI gastrointestinal tumors (Velho *et al*., 2010).

B. Functional Effects of KRAS and BRAF Oncogenes in MSI-H Colorectal Cancer

Due to the specific *KRAS* and *BRAF* mutational profile observed either in MSI-H CRCs or in nonmalignant lesions, it is possible to hypothesize that oncogenic signals from these mutant genes may not rely on the same signaling pathway and thus they may harbor distinct functional roles in MSI-H CRC development and progression. Furthermore, and as mentioned earlier, MSI-H CRCs are described as being originated through the serrated carcinogenesis pathway in which BRAF, in association with CIMP, seems to be a key player (O'Brien *et al*., 2006). Data from *in vitro* studies support the assumption of a different functional role of these oncogenes. By inhibiting BRAF protein expression by RNA interference, our group showed that BRAF provides proliferation signals through ERK activation, by increasing the levels of Cyclin D1 and decreasing the levels of p27Kip1 (Preto *et al*., 2008). Further, we demonstrated that *BRAF* mutation provides survival signals through Bcl-2 in MSI-H CRC cell lines (Preto *et al*., 2008). In addition, we also demonstrated that BRAF inhibition was not sufficient to decrease proliferation and induce apoptosis in *KRAS* mutant MSI-H CRC cell lines (Preto *et al*., 2008). Overall, these results suggest that KRAS-mediated oncogenic signaling do not entirely follow through the KRAS/BRAF/MAPkinase signaling cascade and strengthen the idea that KRAS and BRAF activation, in MSI-H CRCs, most likely activate distinct signaling pathways by interfering with different cellular functions.

V. A SERRATED CARCINOGENESIS PATHWAY IN THE ORIGIN OF SPORADIC MSI CRC

In 1988, Fearon and Vogelstein proposed an explaining mechanism for CRC development known as the adenoma–carcinoma model (Fearon and Vogelstein, 1990). This was a linear, multistep sequence of accumulation of

genetic alterations, where inactivating mutations of *APC* gene generated the adenoma formation. Acquisition of further genetic alterations, namely *KRAS* oncogenic activation and *p53* inactivating mutations, would then function as driving forces toward progression from adenoma to carcinoma (Fearon and Vogelstein, 1990) (Fig. 2).

Currently, it is widely accepted that the abovementioned adenoma–carcinoma concept oversimplifies the heterogeneity of the molecular, clinical, and pathological basis of CRC, and therefore cannot be applied to all types of CRCs. For instance, *APC*, *KRAS*, and *p53* mutations, the typical molecular alterations of this Vogelstein pathway, were found to occur in the same tumor only in 7.7% of CRCs (Imai and Yamamoto, 2008; Samowitz *et al.*, 2007; Smith *et al.*, 2002). A much higher percentage of concomitant mutations would be expected if the adenoma–carcinoma pathway was the main sequence leading to CRC initiation and development. Furthermore, the

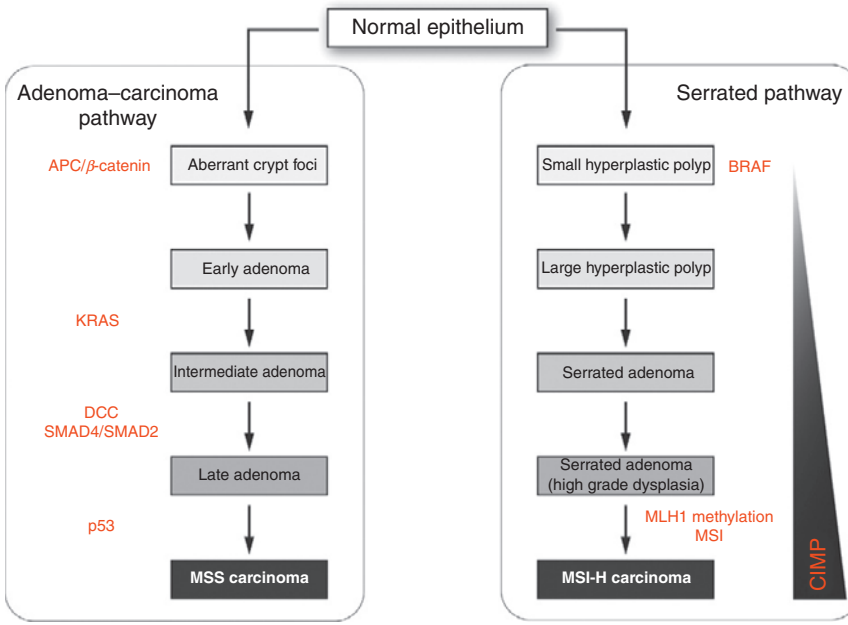

Fig. 2 Pathways for colorectal cancer development. (A) In the adenoma–carcinoma sequence, inactivating mutations of *APC* gene are the onset of adenoma formation. Acquisition of further genetic alterations, namely *KRAS* oncogenic activation and *p53* inactivating mutations, would then function as driving forces toward progression from adenoma to a MSS carcinoma. (B) *BRAF* mutation is a specific marker for the serrated polyp pathway that has its origin in a hyperplastic polyps and a potential end point as a MSI carcinoma. CIMP develops early in this sequence and MSI develops late due to *h-MLH1* silencing by promoter methylation.

molecular features of sporadic MSI-H/*BRAF* mutant/CIMP-positive CRCs do not fit within the adenoma–carcinoma pathway, since the frequency of *KRAS* mutations is low in this tumor setting and the inactivation of *APC* and *p53* genes is very rare (Jass, 2007; Samowitz *et al*., 2007). These evidences demonstrate that CRCs are not homogenous subsets with respect to their molecular, clinical, and pathological features, and so alternative genetic pathways to CRC initiation and development have been proposed (Jass, 2006) in order to improve our knowledge about the biology of CRC and to better explain its diversity.

In this regard, a serrated pathway for CRC origin has emerged (Jass, 2005). The progressive stages of the serrated neoplasia pathway are separate and distinct from those of the traditional adenoma–carcinoma sequence (Jass, 2005; O'Brien *et al*., 2006). In this pathway, hyperplastic polyps (HPs), traditional serrated adenomas (TSAs), and sessile serrated adenomas (SSAs) are assumed to be the precursor lesions of tumor development (O'Brien *et al*., 2006). For a long time, these serrated polyps were seen as inoffensive lesions of the colon with no potential to become malignant. However, molecular studies performed on these type polyps demonstrated that they already harbor alterations that are commonly found in CRCs, such as *BRAF* and *KRAS* mutations, CIMP positivity, *h-MLH1* hypermethylation, and MSI-H phenotype (Kambara *et al*., 2004; O'Brien, 2007; O'Brien *et al*., 2006; Yang *et al*., 2004), and thus the possibility of progression to cancer was strengthened. In fact, mutant *BRAF* is a specific marker for the serrated polyp pathway that has its origin in a HP with the potential to originate a MSI-H carcinoma (Kim *et al*., 2008; O'Brien *et al*., 2006). Further, *BRAF* mutations are frequently associated with the presence of CIMP-H (Kambara *et al*., 2004; Kim *et al*., 2008; Velho *et al*., 2008), and both events arise early in the serrated sequence (Wynter *et al*., 2004; Yang *et al*., 2004). Aberrant, widespread methylation of CpG islands increases with the histological progression of serrated adenomas (Dong *et al*., 2004). Loss of microsatellite stability due to *h-MLH1* promoter hypermethylation occurs at late stages and is the key event inducing MSI and malignant transformation (Kambara *et al*., 2004; Velho *et al*., 2008) (Fig. 2).

KRAS mutations might also be involved in the development of sporadic MSI-H tumors through the serrated pathway, since mutations in this gene are also found in colonic serrated lesions (O'Brien *et al*., 2006; Velho *et al*., 2008). However, this may represent an alternative serrated pathway that diverges from the one in which *BRAF* gene is involved. First, the type of serrated lesions in which *KRAS* mutations occur is different from the ones that harbor *BRAF* mutations. *BRAF* mutations are more frequently found in sessile-serrated adenomas while *KRAS* mutations occur more frequently in the other types of serrated lesions, such as TSAs and mixed hyperplastic and adenomatous polyps and goblet cell-serrated polyps (O'Brien *et al*., 2006;

Velho *et al.*, 2008; Yang *et al.*, 2004). Second, the presence of *KRAS* mutations is not associated with the presence of CIMP-H and *h-MLH1* hypermethylation (O'Brien *et al.*, 2006; Velho *et al.*, 2008), which implies a different molecular environment involved in the progression from nonmalignant to a malignant state. Both evidences suggest that sporadic CRCs originated in a *KRAS* mutant context or in a *BRAF* mutant background can represent distinct molecular, clinical, and pathologic entities.

VI. CONCLUSIONS

An altered KRAS signaling pathway represents an important factor in the development and progression of gastrointestinal carcinomas. Mutations of *KRAS*, *PIK3CA,* and *MLK3* genes, as well as *RASSF1A* promoter hypermethylation are commonly found in MSI sporadic gastrointestinal carcinomas and in hereditary MSI CRCs. The pattern of *BRAF* oncogenic mutations show a more restricted pattern being only found in MSI sporadic CRC. Moreover, the results obtained from the KRAS-related genes mutation detection in premalignant lesions as well as those obtained from the functional analysis of *KRAS* and *BRAF* mutant CRC cell lines support that KRAS- and BRAF-dependent signaling may lead to different carcinogenic pathways. Moreover, concomitant alterations in genes from the KRAS signaling pathway are frequently observed, suggesting a strengthening of the signaling or a possible dependency of gastrointestinal cancers on this molecular pathway. This knowledge has potential clinical implications and may be translated into the development of novel and more directed targeted therapeutic strategies in a near future.

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