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Thomas Dittmar
Kurt S. Zänker *Editors*

Cell Fusion in Health and Disease

II: Cell Fusion in Disease

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Preface

Cell fusion is a specialized cellular event which occurs in multicellular organisms in health and disease. Known as a phenomenon in modern science for over 100 years, cell fusion takes the mandatory center stage in eutherians for the conception, development and physiology of organogenesis or, in pathophysiology, during the process of oncogenesis. The ability of two or more cells to unit and to form a new syncytial cell takes place in metazoans throughout evolution to form muscles, bones and placentae, and, even to form a tumor. This process requires migration, recognition and adhesion between the cells together with the fusion of their plasma membrane and rearrangement of their cytoplasmatic and nuclear contents. Membrane fusion arise during many cellular processes, including membrane traffic, intracellular vesicle fusion, fertilization, and infection by enveloped viruses. Fusion allows to exchange biological materials between different membrane compartments. In order to maintain the functional individuality of each of the intracellular compartments and of the cell itself, membranes do not fuse easily under normal circumstances. The process is subjected to selective control and requires the expression of special (glyco-)proteins and carbohydrates and the formation of a phospholipid interbilayer via an hourglass-shaped structure called a "stalk".

Sperm-egg fusion (fertilization) is the most prominent example of "natural" occurring membrane fusion without the deliberate addition of exogenous fusing agents such as viruses or chemicals in order to create by orchestrated and stepwise processes a zygote. Billions of sperms are deposited at ejaculation in the female reproductive tract, but only one sperm finds and fertilizes the egg. On their way, the spermatozoa ignore the thousands of cells they make contact with during their locomotion to find a single cell, namely the oocyte. Gamete fusion is an extremely important process that must emerge without error to launch life (B.M. Gadella, Utrecht, The Netherlands; J.P. Evans, Baltimore, USA).

For basic research, the nematode *Caenorhabditis elegans* has become an excellent system to study mechanisms and developmental functions in many cell fusion events at the molecular and cellular levels (L. Friedlander-Shani, B. Podbilewicz, Haifa, Israel).

There is still a considerable lack of knowledge, which molecules (fusogens/SNARE proteins) mediate vesicle fusion (B.P. Jena, Detroit, USA), fuse myoblasts to form myotubes in muscles (A. Simionescu, G.K. Pavlath, Atlanta, USA), macrophages to form osteoclasts in bone (A.K. McNally, J.M. Anderson, Cleveland, USA) and cytotrophoblasts to form syncytiotrophoplasts in placentae (B. Huppertz, M. Gauster, Graz, Austria). The chapters written by these well respected authors will throw some lights on the mystery to reveal genuine fusogens.

Until recently, cells were thought to be intregral and discrete components of tissues, and their state was determined by cell differentiation. However, under some conditions, stem cells or their progeny can fuse with cells of other types, mixing cytoplasmic and even genetic material of different (heterotypic) origins (X. Zhou, J.L. Platt, Ann Arbor, USA). The fusion of heterotypic cells could be of central importance for development, for repair of tissues (M. Alvarez-Dolado, M. Martínez-Losa, Valencia, Spain), for the production of fusion vaccines derived from dendritic and tumor cells (W. Lee,

Durjam, USA) and even for cellular reprogramming (D. Sanges, F. Lluis, M.P. Cosma, Barcelona, Spain). The chapters written by these outstanding experts will highlight the process of cell fusion in diverse biological systems. *Volume I* deals with molecular and cellular aspects of cell–cell fusion as a biological meaning to establish pluripotency. or, in other words, when it takes more to make one.

For human health cell–cell fusion is a crucial and highly regulated event in the genesis and homeostasis of both form and function of many tissues. However, cell–cell fusion may also play a critical role in the development of cancer and progression of the disease. Very recently, Gao P. and Zheng J. (Virol J. (2010) 7:238) put forward an attractive working hypothesis that high-risk HPV-16 E5-inducable cell fusion might be a critical initiating event in the early stage of HPV-associated cervical cancer. In general, establishment of a role of cell fusion in cervical carcinogenesis by the HPV-16 E5 fusogenic protein to form tetraploid cells would open an intellectual window to understand additional pathogenic modes of actions for emerging virus-associated cancers.

At the cutting edge, *Volume II* brings into prominence heterogenic fusion processes in oncogenesis. The editors are very thankful to J.G. Sinkovics (Tampa, USA) that the second volume can start with a chapter, which reflects more then 50 years of clinical and experimental cancer research within a polycontextural and intelligent framework of immunology, cancer vaccines – alone or combined with chemotherapy –, oncolysis and the place of viruses in the "tree of life", mostly addressing sarcomas as a clinical entity. He nicely demonstrates that cell fusion and horizontal exchanges of genes are fundamental attributes and inherent characteristics of the living matter.

Structural studies of viral fusion glycoproteins allows to categorize viral membrane fusogens into three distinct classes. M. Backovic (Paris, France) and Theodore S. Jardetzky (Stanford, USA) describes the newly identified group of class III viral fusion proteins, whose members include fusion proteins form rhabdoviruses, herpesviruses, and baculoviruses. Before embarking on cell fusion in malignancies, we inserted a chapter written by A. Malassiné, G. Pidoux, P. Gerbaud, J.L. Frendo and D. Evian-Brion (Paris, France) on the importance of trophoblast fusion in trisomy 21, demonstrating that cell–cell fusion is increasingly of interest in non cancerous diseases, too.

Myeloma bone disease leads to progressive destruction of the skeleton and is the most severe cause of morbidity in multiple myeloma. Osteolytic lesions are not characterized by a massive presence of osteoclasts, whereas malignant plasma cells may occur as large multinucleated cells. The possibility that myeloma cells fuse and generate polykaryons in vivo is suggested by the in vitro formation of multinuclear cells that express tartrate-resistant acid phosphatase and produce pits and erosive lacunae on experimental osteological substrates (F. Silvestris, S. Ciavarella, S. Strippoli, F. Dammacco, Bari, Italy).

Findings from experimental and clinical cancer research suggest a potentially multifaceted involvement of cell fusion in different stages of tumor progression, including aneuploidy, origin of cancer stem cells (X. Lu, Y. Kang, Princeton, USA), multidrug resistance (C. Nagler, K.S. Zänker, T. Dittmar, Witten, Germany) and the acquisition of metastatic abilities (R. Lazova, A. Chakraborty, J.M. Pawelek, New Haven, USA). These distinguished authors clearly demonstrate that the century-old hypothesis that cell fusion may contribute to the initiation and progression of cancer has revitalized.

Cells of the monocyte/macrophage lineage are important for tumor cell migration, invasion and metastases formation. Fusion between macrophages and cancer cells in vitro and in animal models causes hybrids with increased metastatic potential. Expression of the macrophage antigen CD163 in rectal and breast cancer is associated with early recurrence and reduced survival time (I. Shabo, J. Svanvik, Linköping, Sweden).

Membrane vesicles are membrane-covered cell fragments generated by normal and transformed cells. Autophagosomes are the most prominent double-membrane bound vesicles. Fusion of autophagosomes with lysosomes results in the formation of autolysosomes, where the proteins and organelles are degraded. This degradation pathway is induced under nutrient deprivation, metabolic stress or microenvironment conditions to ensure energy balance, clearance of damaged proteins and

adaptation to stress. Disruption of autophagy is involved in diverse human diseases including cancer. Tumor-derived vesicles may serve as prognostic markers, they were detected in blood plasma and in other body fluids. All of them reflect the special potential of tumor cells for survival and for the expansion of the tumor. The vesicles may facilitate the escape of tumor cells from immune surveillance, they are involved in the establishment of a beneficial environment for newly formed and migrating tumor cells, influencing angiogenesis and the reorganization of the extracellular matrix (E. Pap, Budapest, Hungary).

The editors like to extend their gratitude to all authors, who have presented a review of their respective fields, but have been invited to do so from their unique point of view. All have tried to summarize informations and to provide critical reviews connoting cell–cell fusion as a fundamental biological process, upon which future therapies might be built. If these two volumes serve as a scientific reference from which to plan future research strategies – enlightening cell–cell fusion in health and diseases –, many of which have not yet been anticipated by the editors and the authors, then the publication of these two volumes has fulfilled the intended purpose.

For the current two volumes the Editors want to express a special word of thanks to Springer Publisher (Dordrecht, The Netherlands) and in particular to Tanja van Gaans and Meran Owen who have worked closely with us to achieve a rapid and comprehensive publishing standard at the state-of-the-art of cell–cell fusion in health and disease.

Witten, Germany
Autumn 2010

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Chapter 1
Introduction

Thomas Dittmar and Kurt S. Zänker

Abstract Although cell fusion is an omnipresent process in life, to date considerably less is still known about the mechanisms and the molecules being involved in this biological phenomenon in higher organisms. Cell Fusion in Health and Disease Volume 2 is covering the dark side of cell fusion: namely its role in pathophysiological processes. International leading experts will present up-to-date overviews about cell fusion mediated horizontal gene transfer in bacteria and viruses, class III viral membrane fusion proteins, trophoblast fusion in trisomy 21, and the role of microvesicles in malignancies. Particular attention is paid on cell fusion in cancer and how this biological phenomenon may initiate the origin of (recurrence) cancer stem cells as well as drive the progression of multiple myeloma, colon cancer, breast cancer, and malignant melanoma. Thus, Cell Fusion in Health and Disease Volume 2 represents a state-of-the-art work for researchers, physicians or professionals being interested in reflecting the dark side of cell fusion.

When we talk about cell fusion the possibly most descriptive example for this process in higher organisms is the fusion between the oocyte and the sperm, which gives rise to the fertilized egg cell and the generation of a new life. However, cell fusion does not only play a role in the beginning of life, but is also a prerequisite in a plethora of processes being involved in growth, development and tissue repair. In mammals, trophoblastic cells fuse with each other, thereby giving rise to multinucleated syncytiotrophoblasts, which facilitate and ensure the nutrient exchange between the mother and the foetus. Likewise, myoblasts fuse to form multinucleated skeletal muscle fibres, whereas cells of the monocytic origin fuse to osteoclasts being participated in bone resorption (e.g., bone repair after fracture). Moreover, we know from various studies that bone marrow-derived stem cells as well as cells of the myelomonocytic lineage restore tissue function, e.g., liver, lung, by cell fusion, which raised (and still raise) expectations for autologous stem cell-based tissue regeneration strategies. In addition to these physiologically cell fusion events, artificial cell fusion protocols have been developed to reprogram stem cells, to generate hybridomas and to generate tumor vaccines. Hybridomas, derived from myeloma cell/plasma cell fusions, are the source of monoclonal antibodies. What was once developed for scientific purposes, e.g., Western Blot, immunohistochemistry, is now used in a plethora of approaches ranging from simple diagnostic tests (pregnancy test, drug tests) to routine diagnostic applications (determination of inflammatory markers in serum, blood typing, virus detection in patient samples) to clinical applications (immunosuppression for organ transplantation, use of humanized monoclonal antibodies in cancer therapy). To date, tumor cell-dendritic cell hybrids are the most promising tools for tumor vaccination strategies. Due to fusion of professional antigen

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presenting dendritic cells with tumor cells hybrid cells evolve being capable to initiate a anti-tumor specific immune response because of tumor antigen presentation.

In addition to these cell fusion events being crucial to maintain and ensure the body's homeostasis, the biological process of cell fusion can also be linked to various (malignant) diseases. Without the ability to fuse with the plasma membrane viruses would not be able to deliver their genome into host cells. Thus the identification and characterisation of viral membrane fusion proteins and plasma membrane fusion partners is one promising approach to develop inhibitors, which specifically block the fusion of a virus with its target cells. Such approaches are currently tested, e.g., for blocking Hepatitis C virus as well as HIV infection.

The normal view of viral infections assumes that one virus (or more) infects (fuses with) only one target cell, whereas the virus-mediated cell fusion is neglected in this context (although viruses, e.g., Sendai virus, were the first tools for study the process of cell fusion and to characterize hybrid cells). Recent studies indicate that the virus-mediated cell fusion seems to be a common phenomenon in viral infections and that such processes might cause cancer due to induction of chromosomal instability. If so, this would mean that cell fusion (possibly driven by viruses) might also contribute to cancer stem cells, which have been defined as the seed for tumor growth. Whether such a process would also explain the phenomenon that tumor cells are highly fusogenic is, however, unknown. Nonetheless, the fusion of cancer cells with other (normal) cells can give rise to hybrids exhibiting new properties, such as an increased proliferation rate, an enhanced metastatic capacity, as well as an increased drug resistance towards chemotherapeutic/cytotoxic compounds. Macrophage antigens have been identified on tumor cells and both in vivo and in vitro studies revealed that tumor cell/macrophage hybrid cells possesses an enhanced metastatic capacity. Stem cell/tumor cell fusions have also been observed both in vivo and in vitro. Because tumor cell/normal cell hybrids might exhibit an increased drug resistance concomitantly with an enhanced malignity it was suggested that cancer relapses might originate from fusion events. If so, this indicates that not only tumor initiation, but also metastasis formation and cancer relapses can be linked to this biological process.

In addition to cell–cell fusion events further fusion related processes have been associated to cancer. These include microvesicles and autophagy. Microvesicles are plasma membrane fragments being shed from almost all cell types including tumor cells following activation or apoptosis. Elevated amounts of microvesicles are found in the blood of cancer patients and, because microvesicles harbor a multitude of biologically active (oncogenic) proteins and RNA species, it is currently assumed that microvesicles might be a mode of intercellular tumor cell communication. The phenomenon of autophagy (or autophagocytosis) describes the degradation of a cell's own components through the lysosomal machinery. The role of autophagy in cancer is unclear. On the one hand, autophagy can act as a tumor suppressor by degrading damaged organelles. On the other hand, autophagy can promote survival of cancer cells under conditions of poor nutrient supply as well as protecting tumor cells against therapy-induced apoptosis.

This short introduction indicates that cell fusion is not limited to a few physiologically processes, but is a common biological phenomenon, whereby cell fusion plays a pivotal role both in health and disease. Because of the complexity of cell fusion the book will be divided into two volumes. The first volume will summarize cell fusion in health, whereby the second volume will give an overview about cell fusion process being related to (malignant) disease.

We further realize this book as a platform for a summary of the latest findings on cell fusion-mediating molecules in mammals. In contrast to *C. elegans* or *D. melanogaster*, where cell fusion and the molecules to be involved in are well-characterized, only a handful of fusogenic proteins (e.g., syncytin, SNAREs, CD200, CD44, CD47 and PTPNS1) have been identified in mammals. However, as mentioned above for virus membrane fusion proteins, the knowledge about these cell fusion-mediating proteins is crucial for developing specific cell fusion inhibitors. Studies on viruses indicate that such approaches are feasible, thereby impairing viral infections. If we conclude that malignant cells could evolve from cell fusion events than the inhibition of this biological process might be one approach to

prevent cancer formation and/or impair cancer progression, which in turn perquisites the knowledge about the process itself and the molecules to be involved-in.

We are thankful that so many internationally recognised experts accepted our invitation to contribute to this exciting book project. We sincerely thank them all for their interest in this important topic and that they, despite other duties and responsibilities, found the possibility to present excellent and comprehensive overviews of the most important recent findings in their field of scientific engagement within this topic. We would also like to thank Tanja van Gaans and Meran Owen from Springer Publishers (Dordrecht, The Netherlands) for their kind assistance and excellent collaboration on this project, as well as for giving the opportunity to realize this book project.

We hope that this book may encourage new scientific approaches within the field of cell fusion in health and disease as well as closer interdisciplinary collaborations on this fascinating and important issue in the future.

Chapter 2
Horizontal Gene Transfers with or without Cell Fusions in All Categories of the Living Matter

Joseph G. Sinkovics

Abstract This article reviews the history of widespread exchanges of genetic segments initiated over 3 billion years ago, to be part of their life style, by sphero-protoplastic cells, the ancestors of archaea, prokaryota, and eukaryota. These primordial cells shared a hostile anaerobic and overheated environment and competed for survival. "Coexist with, or subdue and conquer, expropriate its most useful possessions, or symbiose with it, your competitor" remain cellular life's basic rules. This author emphasizes the role of viruses, both in mediating cell fusions, such as the formation of the first eukaryotic cell(s) from a united crenarchaeon and prokaryota, and the transfer of host cell genes integrated into viral (phages) genomes. After rising above the Darwinian threshold, rigid rules of speciation and vertical inheritance in the three domains of life were established, but horizontal gene transfers with or without cell fusions were never abolished. The author proves with extensive, yet highly selective documentation, that not only unicellular microorganisms, but the most complex multicellular entities of the highest ranks resort to, and practice, cell fusions, and donate and accept horizontally (laterally) transferred genes. Cell fusions and horizontally exchanged genetic materials remain the fundamental attributes and inherent characteristics of the living matter, whether occurring accidentally or sought after intentionally. These events occur to cells stagnating for some 3 milliard years at a lower yet amazingly sophisticated level of evolution, and to cells achieving the highest degree of differentiation, and thus functioning in dependence on the support of a most advanced multicellular host, like those of the human brain. No living cell is completely exempt from gene drains or gene insertions.

2.1 Acquisition and Horizontal Transfer of *Vibrio cholerae* Virulence Gene

2.1.1 Hamburg 1892

In 1892 cholera struck the city of Hamburg. Unfiltered water from the river Elbe carried the pathogen into the city's drinking and cooking water supply. Allegedly "Russian immigrants brought in the disease to Prussia". Hamburg's neighboring small town Altona received its water supply from a different source. In Hamburg the number of sick afflicted with cholera reached 16,956; of these patients 8,605 died ("8,605 von 16,956 Erkrankten starben"). In Altona there were only a few cases of cholera ("Altona blieb deshalb 1892 von der Cholera weitgehend"). German steamships (Cavour,

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Elbe, Leibnitz, Normannie, Rugia) leaving Hamburg spread the disease to Antwerp, London, New Orleans, New York, and Zion. The disease was transmissible by the traveling sick or "germ carrier".

Arrived in Hamburg from Munich Herr Professor Max Josef von Pettenkofer, the pride of Germany ("der große Hygieniker"), who made Munich the cleanest city in Europe (probably in the world). Professor Pettenkofer's theory was that the multiple causations of epidemics, like the one in Hamburg, were "Miasmen", in this case organic poisons from the buried corpses of slaughtered animals in and around slaughterhouses ("Miasmen-Lehre", "Boden und Grundwasser in ihren Beziehungen zu Cholera", "Boden und sein Zusammenhang mit der Gesundheit des Menschen", "der Erreger wirkt ausschliesslich durch verseuchtes Grundwasser"). In order to prove that no special bacteria caused cholera, even before the Hamburg outbreak, Professor Pettenkofer and two members of his staff (two assistants) swallowed the liquid contents of a vial containing cultured cholera vibrio bacteria. On his request, the vial was sent to Professor Pettenkofer by Professor Koch. The three self-infected volunteers became ill with diarrhoea but survived. They were claiming gloriously thereafter that it was not the cholera vibrio that caused cholera and/or the deadly epidemic now in Hamburg.

Arrived in Hamburg Herr Professor Robert Koch. He saw the vibrio under the microscope in the intestinal tract of the dead in 1883 in Alexandria, Egypt (like Filippo Pacini in 1854 in Firenze), but could not culture it. In Egypt, the cholera epidemic in 1883 claimed over 58,000 (100,000?) lives. It was later in India in the same year that Koch produced pure cultures of *V. cholerae* "swarming on gelatine plates." The pathogen was "ein kurzes, kommaähnlich gekrümmtes Bakterium." "Die Identifizierung des Cholera Erregers" and the established fact ". . .daß Koch den Choleraerreger gefunden hatte" followed. In Hamburg Professor Koch educated the inhabitants of the city that drinking water ("Übertragung von Cholera durch Trinkwasser") carried the germ of the disease, advocated strict isolation of the sick, introduced disinfection ("Desinfektionskolonne mit Chlorkalkkarren während der Cholera-Epidemie in Hamburg 1892"), quarantined the ports of the city, and demanded that the city's water supply be sand-filtered. The epidemic was halted[1].

2.1.2 The Vibrio and the Disease

In their aquatic habitat, *Vibrionaceae* enzymatically degrade chitins of crab and crustacean shells [1]. Of the 205 *Vibrio cholerae* serogroups, only O1 and O139 cause cholera epidemics; other members of the group may cause diarrhoeal illness, but not cholera [2, 3].

The non-choleragenic serogroup members produce vibrio cytolysins (VCC) and hemolysins. Anion channels opened up by VCC in the membranes of enterocytes trigger an outpouring of chloride, sodium and water; such efflux results in the watery diarrhoea and dehydration of the host [4]. The infamous El Tor biotype belongs to the O1 serogroup. The El Tor vibrios carry multiple antibiotic-resistance gene clusters within transposon-like structures (cassettes; constins, operons). The genes confer resistance of the vibrio to streptomycin, sulfamethoxazole, trimethoprim, and chloramphenicol (STX). The STX genes reside within a large integrating conjugative element (ICE). The transfer of these excised gene clusters occurs by conjugation and integration (not by phage-mediation) [5–7]. In two chromosomes (one large, one small) vibrios carry 142 and 42 single copy genes, and gene clusters for virulence and the inserted genomes of its prophages, respectively. Smaller genetic entities in the cytoplasms are the plasmids, frequently the vectors of horizontal gene transfers ("rampant" such activity) [8].

[1]Max von Pettenkofer Institut für Hygiene und Mikrobiologie. Pettenkoferstrasse 9a, München D-80336; Robert Koch Institute, Nordufer 20, Berlin, D-13353; Richard J. Evans: Death in Hamburg. Oxford: Clarendon Press, 1987 xxii 676 pp.

The pathogenic cholera vibrios (*V. cholerae* or *V. mimicus*) replicate in the small intestine, and release their AB-type toxin, thus inducing the profuse secretory diarrhoea leading to electrolyte losses, profound dehydration, lipopolysaccharide (LPS) endotoxin shock [9] and death. Cell surface gangliosides bind the pentameric subunit CTB. The cholera toxin CTA after transgressing the plasma membrane of the host cell is taken up by the endoplasmic reticulum (ER). In the ER, the monomeric subunit A1 chain is unfolded and directed to pass through the protein-conducting channel into the cytosol. Escaping degradation by proteasomes, the toxin refolds and acts [10]. The innate proinflammatory responses induced by the cholera toxins consist of the activation of the Akt cascade resulting in the overproduction of interleukin-1α (IL-1α), IL-6, and tumor necrosis factor-α (TNF-α) and the translocation from cytoplasm to nucleus of nuclear factor kappa B lineage lymphocyte (NF-κB) [11] The major immunosuppressive effect of the cholera toxin is inhibition of IL-12 production in dendritic cells (DCs), thus it is the abrogation of a Th1-type immune response in the gut. This is further achieved by the inhibition of interferon (IFN) regulatory factor-8 (IRF8), thus plasmacytoid DCs remain undifferentiated non-IFN producers. Even if IL-12 were produced, CTAB inhibit the expression of IL-12R (beta receptors β1 and β2). Immune T cells, if generated, do not release interferon-γ (IFN-γ). The immunoglobulin response is mainly that of IgE; thus further toxin outpour is not neutralized, but it is reacted to with anaphylactoid events culminating in shock [12]. The beta subunit of CTB induces a tolerogenic response mediated by both antigen-specific FoxP3$^+$ regulatory T cells (T_{reg} cells) and by transforming growth factor-beta- (TGF-ß) and IL-10-producing T cells [13]. The major *V. cholerae* toxins are the cholera toxin (CTAB), neuraminidase, chitinase and LPS endotoxin. In *V. cholerae* antigen- (toxin-) exposed mice, peritoneal macrophages died apoptotic deaths [14].

Overcoming the immunosuppressive and tolerogenic effects of CT, the host mobilizes immune reactions against the vibrio and its toxins. Patients recovering from cholera withstand a second exposure to *V. cholerae*. These individuals possess memory B and T cells in Peyers' patches and in the circulating blood. Antibodies reacting to re-exposure are of the IgA and IgG classes and react with LPS and CTAB [15]. During the acute disease and in convalescence, patients begin circulating CD4$^+$ and CD8$^+$ gut-homing T, and CD19$^+$ B cells, Upon stimulation with vibrio cell membrane or TCP (toxin co-regulated pilus) antigens, these cells respond by clonal expansion and release of INF-γ (for Th1-type immunity) and/or IL-13 (for Th2-type immunity) [16]. These immune reactions serve as reasoning for the development of preventive cholera vaccines. The vaccines offer good to partial protection, but their immune efficacy weakens without re-vaccination [17–20]. The Peru-15 (CholeraGarde; AVANT) live-attenuated oral vaccine was safely and effectively administered to Bangladesh infants and toddlers [21–23]. The College of Medicine, University of Central Florida is developing a united malaria-cholera vaccine [24].

Bicarbonates stimulate the ToxT regulatory protein, thus the transcription of CT and TCP. The contents of the small intestine are bicarbonate-rich. Ethoxyzolamide inhibits carbonic anhydrase and negates the stimulatory effect of bicarbonates on ToxT [25]. Resveratrol (3,4′5-trihydroxystilbene) inhibited cholera toxin-induced damage in Vero cells. The toxin was precipitated and its endocytosis was inhibited. In the cells, the toxin's effect on cyclic adenosine mono- or diphosphates (AMP/ADP), such as AMP accumulation and ADP-ribosyltransferase activity, were suppressed [26]. The expression of cholera toxin in the cell is regulated by the transcriptional cascade of ToxT. Cis-palmitoleic acid reduces the expression of both virulence factors of the cholera toxin (CT) and prevents ToxT from binding DNA [27].

There exist natural vibrio isolates that harbor a provirus CTXφ, which does not possess the *ctxA* and *ctxB* genes. In addition, these prophages lack the upstream control region normally located 5′ of *ctxA*, and the promoter region and coding sequences of *ctxB*. In these phages, the ancestral precursor of the CTXφ phage was found still in existence. Therefore the *ctxAB* genes do not behave as vertically transmitted genuine phage genes; they are acquired genes and as such they were gained through horizontal transfer [28, 29]. The cluster of these genes and their regulatory sequences must have been acquired simultaneously. Horizontal transfer of the CTXφ genes must have occurred repeatedly.

The CTXφ phage enters the vibrio through its type IV pilus, TCP (*vide supra*). The cluster of vibrio genes encoding the pilus can be transmitted between vibrios by unknown mechanisms [30]. The OrfU (open reading frame) protein binds CTXφ phage particles to TCP, as their attachment receptor. The pili serve the bacterium as essential colonization factors in the human small intestine. The bacterial flagellae penetrate the mucosal layers of the small intestine. Flagella loss (Fgl^{-}) releases the anti-sigma and alternative sigma factors and these repress quorum sensing regulators with the release of virulence factors [31]. Excessive colonization frequently assumes the form of biofilms [32, 33]. The CTXφ phages infecting classical and El Tor vibrios are distinct, but diverged from a common ancestor. It is the *orfU* and *zot* (zona occludens toxin) genes of these CTXφ lineages that show this divergence. It was not the ancestral CTXφ that infected an ancestral vibrio, but rather it was its two lineages, which infected separately the classical and El Tor vibrios. Phage CTXφ of *Vibrio cholerae* could infect *Vibrio mimicus* and transfer horizontally the cluster of these genes with their regulatory sequences into its new host. *V. cholerae* and *V. mimicus* diverged from a common ancestor, and their acquisition of CTXφ occurred after their divergence. First *V. cholerae* might have been infected, and from there *ctxAB* operon was horizontally transferred by CTXφ into *V. mimicus* [28].

The highly epidemic new strain of *V. cholerae,* O139 Bengal, emerged in 1992–1993. It replaced the pre-existing O1 serogroup El Tor strain. In 1994, the O1 serogroup El Tor strain recurred and re-occupied its territory. Later in 1996, O139 re-emerged and thereafter co-existed with O1 El Tor *V. cholerae* [34]. The new O139 *V. cholerae* strain derived from an ancestral El Tor vibrio with preservation of its virulence factors, but with new and different serotype. *V. cholerae* O139 exhibits the insertion of a large new genomic region foreign to the pre-existing O1 El Tor strains, while the O139 vibrio suffered a deletion all of its O1 antigen-specific gene cluster. The O-antigen biosynthesis gene cluster occupies the *wbf* (wild-type biofilm) region in the genome of the vibrio O139. The O139 LPS is antigenically different from that of the O1 El Tor vibrio. Patients recovering from O1 El Tor vibrio-caused cholera remain susceptible to infection with the vibrio O139. A large portion (22-kb) of DNA strands of the regulator of biofilm region (*wbf; rfb*) was deleted in the O139 vibrio. This deleted region is replaced by a new fully sequenced 35-kb *wbf* region encoding the O139 antigen. It is also possible that the new DNA segment was not transposed from an outside source, but that it originated by homologous recombination events within the ancestral El Tor vibrio residing in biofilm colonies on chitin surfaces (chitin skeletons of crustaceans) in aquatic reservoirs, where resistance acquired against bacteriophages were the driving force. The emergence of O139 vibrios in the intestinal tract of individuals who survived prior exposure to O1 El Tor *V. cholerae* indicates that ineffective host immune reactions might be another driving force of the transformation [35–37]. Chitin-induced natural transformation resulting in O1 conversion to a different serogroup (non-139 and O139) occurred experimentally. It is the O1 recipient (the El Tor vibrio) that acquires a new O139 LPS-antigen-encoding cassette. These cassettes are incorporated into the recipient genomes by homologous recombination. The exact mechanism of such large gene cluster (operons) transfers from the transformed vibrios into not yet transformed vibrios remains unclear. Under consideration are conjugative plasmids as vectors, transducing but as yet undiscovered phages, and other as yet unrecognized mechanisms.

In addition to phage-mediated CTXAB, cholera vibrios express the protein synthesis inhibitor cholix toxin [38] and hemolysins, which render bilayered plasma membranes of eukaryotic cells non-selectively and indiscriminately permeable [39]. The multifunctional autoprocessing repeats-in toxin (MARTX) destroys the actin cytoskeleton in eukaryotic cells [40, 41]. Some of the hemolysin genes (*hly* :Hly) encoding these toxins may reside in the virulence islands of the vibrios, but they appear to be genuine vertically transferred bacterial genes.

Enterotoxigenic *Escherichia coli* (ETEC) produces an enzymatically active A subunit toxin and a receptor-binding pentamer B subunit toxin. Its Longus pilus induces self-aggregation and adherence of the bacteria to intestinal epithelial cells. The ETEC lytic phage enters the bacterium through a colonization factor pilus [42–45]. Phages lytic to ETEC cells are well known, but toxin-encoding

phages remain elusive. The C57 pilus is the entry site of most ETEC phages. The porcine ETEC phage phiEcoM-GJ1 is a recombinant of a Myoviridae, a Podoviridae and a Siphoviridae bacteriophage with Myoviridae outside morphology (icosahedral head, contractile tail with fibers). There are no toxin-encoding genes in the genome of this lytic phage [46, 47]. Lambdoid bacteriophages possess and spread Shiga toxin (*stx*) genes in populations of *E. coli* bacteria: for example, Shiga toxin- (Stx-) producing *E. coli* (STEC), such as *E. coli* strain O157:H7 of cattle origin [48]. From the point of view of the bacterium, the toxin promotes its colonization in the gut of cattle (or human patients) and protects it from bactivorous protozoa, like *Tetrahymena pyriformis* in the colon of the cattle [49]. Through unknown ancient mechanisms, Shiga toxin-encoding bacteriophage 933W acquired and operates a eukaryotic-like ATP-binding and phosphotransfering serine/threonine tyrosine protein kinase-encoding gene (*stk*) [50].

The High Pathogenicity Islands (HPI) may travel with excessive speed and long distances in between human communities and from hospitals to hospitals infecting strains of *Enterobacteriaceae* through horizontal routes of transfer. The *Enterobacter hormaechei* outbreak in the Netherlands occurred by transfer of a new variant HPI to *E. coli* and *K. pneumoniae.* Both the genomic islands (GI) containing the virulence genes (named in the article) and the genomic modules (GM), five GMs listed one by one in the article, were transferred. The new HPI contained integration sites to a mobile DNA element. The mobile DNA element was able to excise, circularize and insert the HPI at multiple sites: multiple combinatorial transfers of both GI and GM occurred [51]. In the USA, uropathogenic *E. coli*, and in France, *Salmonella typhimurium*, enterohemorrhagic and uropathogenic *E. coli* are suspect to have acquired through horizontal transfer HPIs. This subject matter will be returned to later in the discussion of horizontal gene transfers across prokaryotic lineages through rapid pathway evolution against the background of the evolution of prokaryotic genomes (*vide infra*, in The Darwinian threshold).

2.1.3 The Phage and Its Genome

One of the filamentous phages of *V. cholerae* is CTXphi (CTXφ). This phage carries the cholera toxin (CT) genes *ctxAB*. The 6.9-kb genome of CTXφ integrates into the genome of its host, the vibrio (*V. cholerae*). Replication sequences (RS) encode the enzymes needed for the integration of the viral genome into the host genome. The genome of the classical El Tor vibrio does not offer an integration site to the phage. In this host, the CTXφ phage exists as an extrachromosomal circular DNA plasmid. Several genuine phage genes encode the structural proteins of the virus and their assembly into a particle (among them open reading frame U, *orfU*, and zona occludens toxin, *zot*). The non-integrated plasmid is the replicative form (RF) of the phage and produces an abundance of viral particles. These plasmids lack ctxAB and ToxR binding sites, but possess a *zot* sequence, which is different from the *zot* gene of the pathogenic *ctxAB*-containing integrated phage. These plasmids must have derived from the ancestral preCTXφ phages. Not the host cell, but *ctxA* and *ctxB* gene-carrier phages encode the cholera toxins CTXAB. The GC content (34–37%) of the three *ctxAB* genes ($CTX^{ET\varphi}$, $CTX^{class\varphi}$, $CTX^{calc\varphi}$) significantly differ from that of the genuine phage genes. The *ctxAB* phage genes must have evolved differently from the genuine phage genes. There is no similarity, indeed there is a lack of congruence, between the genuine vibrio gene *mdh* (encoding malate dehydrogenase) and the two phage genes *orfU* and *zot* (required for coat proteins and their assembly). The *mdh* genes are identical in classical and El Tor epidemic *V. cholerae* isolates, whereas the CTXφ genes are widely divergent. Thus, CTXφ genes behave like mobile genetic elements [28]. Indeed, *ctxB* contains a Mariner-based transposon [52]. The pathogenicity genes of *V. cholerae* operate from pathogenicity islands of the genome. Vibrio pathogenicity island-2 (VPI-2) encodes integrase, recombinase, a restriction modification system, Mu phage-like proteins, neuraminidase and glycosylhydrolase, and other sialic acid

metabolizing enzymes; these enzymes expose GM1 gangliosides, which serve as receptors for cholera toxin. El Tor and O139 cholera vibrios operate the vibrio seventh pandemic island (VSP). These islands can excise and re-insert themselves from and to the vibrios' genome [53]: ready for horizontal transfer and insertion. These horizontally transferred and genomically inserted virulence genes have been acquired "recently" and repeatedly, are clustered in several chromosomal regions and derived from an unknown original source [54].

The core region of the CTXφ prophage encodes CT, structural proteins for its morphogenesis and the repeat sequence region-2 (RS2) for regulation of its replication and integration. Another RS element (RS1) is inserted next to and flanking the integrated phage genome. This RS1 segment contains all open reading frames of RS2 and the truncated gene *rstC*. The RS1 element can exist in a single-stranded circularized form and in an excised double-stranded replicative form (RF) and as such it may enter host cell genomes horizontally [55–57]. *V. cholerae* Mozambique 2004 strains carry a tandem repeat of the CTXφ prophage integrated both into its small and large chromosomes; RS1 element *cla, env*, and CTX elements *env* and *rstR* (env) are integrated in the vibrio's large chromosome [58]. It was unexpected that the Mozambique 2004 cholera epidemic was caused by the O1 serotype El Tor vibrio infected not with the $CTX^{ET\varphi}$, but with the $CTX^{class\varphi}$ phage; another Indian (Kolkota, India) El Tor vibrio strain is infected with the $CTX^{class\varphi}$ phage. The difference was the absence of the El Tor-specific free RS1 element of the prophage in the Mozambique vibrio, while the Indian vibrio strain possessed this element [59].

The O139 strains isolated in 1992–1993 harbored two copies $CTX^{ET\varphi}$ connected to an RS1 element (*vide supra*). The O139 strains spreading upward from Bangladesh through the Ganges delta and to the Indian continent held three copies of the CTX prophage in tandem arrangement. One of these prophages is different from the $CTX^{ET\varphi}$ in its *rstR* gene, which encodes the repressor protein of CTXφ; it is referred to as the $CTX^{cal\varphi}$ (Calcutta) prophage. Thereafter O139 isolates form different ribotypes and undergo further genetic diversity and genetic reassortments [36, 60, 61] The antibiotic resistance (SXT, *vide supra*) genes in O139 were acquired by 62-kb self-transmissible transposon-like elements; these genes are also transmissible by conjugation (*vide supra*). Indeed, by the transfers selective advantage (phage; host immunity and antibiotic resistance) was conferred to the recipients [37]. Vibriophage-mediated CT gene horizontal transfers between donor O1 El Tor to recipient non-O1/O139 vibrios occurred recently in California coastal waters [62].

Repeat sequences (RS1, 2) flank the integrated CTXφ gene (*vide supra*). The ds replicative form (RF) of the RS1 element was marked with a kanamycin resistance (Kmr) marker (pRS1-Km). The pRS1-KM construct in O1 vibrios acted like the filamentous phage RS1-Kmφ. All classical, El Tor and O139 vibrios are susceptible to this phage; nontoxigenic (CTX^-) vibrios encoding TCP are the most susceptible. Using the integration sequence *attRS* (attachment), the RS1φ genomes also integrate into vibrio chromosomes. Only fused genomes of RS1-KmφCTXφ generated extracellular phage particles. Thus, these phages are transmitted vertically in the host vibrios, but are able to propagate horizontally as well. The cholera toxin-encoding, *ctxAB* gene-carrier CTXφ integrates into host cell chromosome at attachment site *attRS* due to the RS2 region within the CTXφ genome. The RS region encodes regulatory, replicative, and integrative functions of the CTXφ phage. The integrated toxigenic CTXφ genomes are flanked by the RS1 element possessing ORFs *rstA, B, R*; RS1 expresses one additional ORF, *rstC*. The RS1 excised from the chromosome may act as a filamentous phage particle, but without containing CTXφ genomic elements. In contrast, in CTXφ virions the RS1 gene *rstC* is replaced by the core genes of CTXφ. The RS1 genome encodes the RstR repressor protein. The CTXφ genome carries the gene for the RS2-encoded repressor protein. Phage-induced repressor proteins in many phage-carrier bacterial genera maintain the lysogenic state. By the acquisition of its own new core genes, CTXφ separated from its ancestor, the RS1 element, and became a new phage. Its RS1 ancestry is further evidenced by the ability of CTXφ to integrate into its host cells' genome [63a].

The RS1φ phage remains a satellite phage in the genome of the vibrio. CTX-negative vibrios do not replicate the RS1φ phage. The exception is the non-toxigenic *V. cholerae* strain 55V71. Thus, the 55V71 genes are essential for the replication of the RS1φ phage. *V. cholerae* strain55V71 harbors another filamentous phage. The 7.5-kb ssDNA of the 55V71 gene cluster acts as if it were the genome of another filamentous phage, KSF-1φ. This phage enters the vibrio through its mannose-sensitive hemagglutinin pilus. The presence of this phage genome in the vibrio promotes full maturation and horizontal transfer from vibrio-to-vibrio of RS-1φ phage particles. Thus, there is a close cooperation of at least three phages (CTXφ, RS-1φ, KSF-1φ) in the transformation of non-toxigenic to toxigenic strains of *V. cholerae* [56, 57, 63a].

Not one or two, but several filamentous phages cooperate to encode the cholera toxin: the well known filamentous lysogenic phage CTXφ, the replicase-encoding toxin-linked cryptic element, TLC, whose genome consists of the morphogenesis-encoding satellite filamentous phage, fs2φ, and the phage genomes encoding infectious TLC-Knφ phage particles. The recombination sequence encoded by the TLC-Knφ phage is used by phage CTXφ to integrate its genome into the vibrio's genome. The ssDNA genome of phage fs2 is the circularized variant of the TLC genome. In order to achieve the acquisition of toxigenicity by a pandemic *V. cholerae*, two satellite filamentous phages (TLCφ, RS1φ), three helper filamentous phages (fs2φ, CTXφ, KSFφ) and two type IV pilus-based phage receptors (MSHA, TCP) have to establish molecular interactions. (KnR = kanamycin resistance; MSHA = mannose-sensitive hemagglutinin; RS = repeat sequence region; KSF = Kamruzzaman, Sack, Faruque; TCP = toxin-coregulated pilus; type IV cholera vibrio pilus) [63b]. It is now well understood how filamentous cholera vibrio phages insert the toxin gene(s) into the bacterial host's genome and how they encode the toxin from the genome of the host bacterium. It remains to achieve a grasp of the origin of the toxin genes that were horizontally transferred originally into the phages' genome from an unknown source (a vibrio ancestor that acquired the toxin gene(s) from a mysterious eukaryotic host still in hiding).

2.1.4 The Hiding Place of the Original Cholera Virulence Genes

The planktonic marine and estuarine aquatic environments vibrios inhabit, is close to be saturated with archaea, prokaryotes and protozoa and their viruses. Just the tailed phage particles in the environment are estimated to number 10^7/ml, all phage particles 2.5×10^8/ml, and in total on the planet 10^{31} particles. The estimate was offered that 10^{25} phage infections occur/sec worldwide; counting retroactively for 3 billion years, the number of phage infections that had taken place on Earth is incalculable (cited in [64]). Metagenomic characterization of the viral flora is discovering innumerable new members of viral genera. Metagenomic analysis (pan-viral microarrays, polymerase gene sequencing, high throughput sequencing) of seawater and reclaimed water samples for "marine viromes," "marine phage genomics" and "global virospheres" revealed an abundance of free bacterial genes, bacteriophages, pathogenic plant viruses, eukaryotic nucleocytoplasmic large dsDNA viruses and picorna-like eukaryotic RNA viruses [65–71]. This is the environment in which *V. cholerae* and its phage CTXφ acquired the CT genes *ctxAB* from an unknown external source. What is the biological function of the gene product proteins, the CTAB, in the vibrio? It is in the virulence island where the genes *tcp* encoding the pilus TCP reside. It was by sequential acquisition that the predecessor of *V. cholerae* acquired then the *tcp* and *ctxAB* genes [64]. However, the biochemical function of the CTAB in the human small intestine (*vide supra*) indicates that these molecules fit best into eukaryotic biological systems, thus must have originated from a eukaryote host.

The non-choleragenic serogroup vibrios can cause watery diarrhoea and enterocolitis (*vide supra*). Were the non-choleragenic vibrios exposed to CTXφ and related phages, but successfully defended

themselves by the prokaryotic RNA interference-mediated defense system? This system consists of the clustered regularly interspaced short palindromic repeats with closely associated genes (CRISPR; CASS). If this antiviral mechanism worked faultlessly, phages and viruses would have been extinguished from the rest of evolution. However, viruses are able to circumvent the CRISPR/CASS barrier [72]. The system was found to be installed in *V. cholerae* O395: this vibrio is capable of mediating an RNAi interference pathway [73]. The a-virulent *V. cholerae* strain ATCC14033 (American Type Culture Collection) isolated in 1910 is considered to be the predecessor of the highly pathogenic El Tor vibrio [74]. Its pathogenicity island is devoid of the *toxP* and *tcpP/tcpH* (hemolytic) genes, yet it could not protect itself from transformation to the El Tor vibrio class as it has accepted the phage-mediated horizontal insertion of the *ctxAB* operon. Where did CTXφ and related vibrio phages acquire the *ctxAB* operon from?

Vibrios populate the intestinal tracts of sea birds, sea mammals [75], fishes, crustaceans (*vide infra*), sea horses [76], mollusks, oysters and coral [77]. These bacteria- and virus-infected (*V. alginolyticus, V. parahaemolyticus, Listeria monocytogenes*, hemorrhagic septicemia virus) hosts mobilize innate and adaptive immune reactions. The title of this report does not reflect to its deep penetration into basic immunology [78]. Crab hemocytes and some parenchymal cells express antibacterial peptides (crustin; anti-lipopolysaccharide factor) in response to *V. alginolyticus* infection [79]; through these responses, some of the afflicted hosts survive as vibrio-carriers. Halophilic vibrios are rich in substances (neuraminidase, hemolysin, permeability factor, lethal toxin) which kill mammalian cells [80]. The toxic *gyr* (gyrase) gene was present, while the CT *ctx* genes were so far not identified in halophilic vibrios [81] The vibrios notoriously carry phages and release plasmids. For example, the halophilic vibrio, *V. alginolyticus* possesses covalently closed circular plasmids 6,075 bp in length with 42% GC content and seven ORFs encoding over one hundred amino acid length sequences. These encoded proteins are relaxases, replicases and mobilization proteins (MobC) [82]. The vibrios deriving from these hosts may go through the human food chain. The vibrios are not intracellular bacteria, but they adhere to cell surfaces. Could the vibrios pick up eukaryotic genes? Could the phages or plasmids of the vibrios incorporate such eukaryotic genes deriving from their hosts? Of these possibilities, the last step is the most likely: that is, vibrios co-express their phage receptors and exchange their phages and plasmids. This imaginary chain of events presumes the acquisition of eukaryotic genes by a vibrio and its phage in their natural habitat (*vide infra*).

The attachment of vibrios to crustaceans, dead or alive, is mediated by the bacterial chitinases. Vibrios form biofilms on the dead crustaceans' chitin shells. Numerous viruses infect crustacean colonies (baculovirus, hypodermal and hematopoietic necrosis virus, hepatopancreatic parvovirus, white spot syndrome virus, myonecrosis virus, yellow head nidovirus, Taura syndrome virus, nodavirus, gill-associated virus, loose shell syndrome virus, spawner-isolated mortality virus) [83, 84], and others. In a small segment of the oceanic viral flora, shrimp and prawn viruses abound. A brief list of these viruses is provided with references. No implications are offered that any of these viruses could be picked up by vibrios and from them by vibrio phages. Human pathogenicity, if any, would come through the food chain (the List). Such viral infections are devastating to crustacean colonies, however crustacean hosts defend themselves by all means of innate immunity (anti-lipopolysaccharide factor, lectins, RNAi, CRISPR/CASS) and some manage to survive [85–87]. Molting copepods and nauplii of the zooplankton are able to spread some of these viruses [88]. Vibrios colonize the chitinous exoskeletons of certain copepods [89]. However, no vibrios were as yet isolated that would have picked up a crustacean virus; copepods transfer some of these viruses from crustacean to crustacean hosts, but not to vibrios. The so far sequenced genomes of crustacean viruses were devoid of *ctxAB*-like segments. Thus, the derivation of the *ctxAB* eukaryotic genes and the mode of their transfer to the CTXφ and related vibrio phages remains an unresolved problem.

The common ancestor of extant vibrios emerged in the sea about 600 million years ago [90]. Crustaceans and fish harbor pathogenic and non-pathogenic vibrio species [91–94]. Vibriosis with *V. splendidus* of larval turbots carries significant mortality. *Vibrio harveyi* causes "luminous vibriosis"

of shrimp and expresses hemolysin gene(s) (*vhh*). Shrimps or lobsters (*Homarus* sp.) with hemorrhagic and ulcerative enteritis carry vibrio sp. without proven etiologic relationship to the disease in their intestinal tract [95–97]. Is it far-fetched to consider the acquisition of the disease-causing genes from these hosts by the vibrios and from the vibrios by their phages, which promiscuously infect other species of vibrios including vibrio species with human pathogenicity?

Zooplankton blooms precede the outbreaks of cholera epidemics [98]. Is there a so far hidden niche for the *ctxAB* operon in zooplanktons or crustaceans (*vide infra*)? An imaginary sequence of events is envisioned, in which a toxin-producer marine/aquatic-estuarian eukaryotic host is infected by a phage-carrier bacterium: a vibrio. The bacterium acquires the toxin gene from its host and gains invasiveness and proliferative advantage. The lysogenic phage integrated its genome into the genome of its host bacterium, the vibrio. The phage excises its genome from the bacterial genome. The genuine phage genes in the excised genome include full or close to full sequences of the toxin gene(s). At burst, the liberated phage particles find another host bacterium, which they invade and into whose genome they integrate their phage genome. That newly infected bacterium has pathogenicity (virulence) islands towards mammalian (including human) hosts. The integrated toxin gene-carrier phage genome encodes the toxin. The bacterium gains invasiveness and proliferative advantages in his infected host due to the expression of the toxin proteins. The infected host dies due to the pathophysiological effects of the toxin (*vide infra*).

An abbreviated listing of Shrimp and Prawn viruses: Baculovirus [99a]; Dicistroviruses [100]; Hepatopancreatic parvovirus [99b, 101]; Taura syndrome virus [102–104]; Yellow head nidovirus [105]; Gill-associated and yellow head okavirus [106]; Whispovirus. White spot syndrome virus [107–110]; Loose shell syndrome virus [111]; Crustacean antiviral immunity [112].

2.2 Mimivirus and Its Companions

2.2.1 Viruses and Toxins of Blooming Dinoflagellates

Chloroplasts derive from cyanobacteria; the gene orders preserved in chloroplasts prove their cyanobacterial origin [113]. Extreme intraphylum diversity characterizes cyanobacterial aminoacyl-tRNA synthetases. Horizontal gene insertions, deletions and gene duplications created inconsistencies in the evolutionary course of these enzymes, the intraphylum diversity of aminoacyl-tRNA synthetases [114]. Filamentous freshwater cyanobacteria and the dinoflagellates *Alexandrium* spp. produce paralytic shellfish poisoning toxins, but encoded from different gene clusters [115a]. The 26 putative saxitoxin genes (stxA to stxZ : STX) were identified in toxic cyanobacteria (not to be mistaken for the STX antibiotic resistance gene-product proteins). Some of these genes (17 of them) are of cyanobacterial origin. Other genes in the cluster originate from different other cyanobacteria species, a halodurans archaea, the delta-proteobacterium *Myxococcus xanthus*, and the actinobacterium, *Frankia.* A methyltransferase may be of the dinoflagellate *Alexandrium tamarense* derivation. The non-cyanobacterial genes were acquired through horizontal transfers. Did the dinoflagellate *Alexandrium* receive cyanobacterial toxin genes in exchange? The abundant cyanobacterial phages (myo-, podo-, and siphoviruses) are diligent vectors of host cell genes. For example, the bacterial photosystem-I and II genes are propagated by cyanophages. A unique, not T4-like myovirus cyanophage infects the blooming and toxic cyanobacterium *Microcystis aeruginosa*. The *M. aeruginosa* toxin induces hepatocellular carcinoma in experimental animals. This phage possesses a large 162, 109 bp genome containing 184 protein-coding genes. While several host bacterial genes have been incorporated into the genome of this phage, the bacterial toxin-encoding genes could not be found among these horizontally trsnsferred genes [115b]. Were the cyanobacterium a human pathogen, and the phage a carrier of the toxin-encoding genes, a system analogous to that of *V. cholerae* would have been identified.

The chromalveolate diatoms possess genes of red algal origin; endosymbiotic red algae delivered these genes into the nuclei of diatoms [116].

Bacteria attached to the surface of dinoflagellate cells (*dinos*, Greek: rotation, eddy) were not transformed into toxin-producers [117] The blooming *Raphidophyceae* family member microalga *Heterosigma akashiwo* causes red tides. Chinook salmons and amberjacks die in the red tides. The large DNA *H. akashiwo* virus (HaV) kills the microalga and as a lytic algicidal virus is able to clear red tides, not in natural, but at least in experimental conditions [118]. The hemolytic and cytotoxic karlotoxin and ichthyotoxin from the marine dinoflagellate *Karlodinium veneficum* kill fish [119]. Other dinoflagellate toxins (pectenotoxin, yessotoxin, okadaic acid toxins) are hepatotoxic and cardiotoxic in experimentally exposed mice [120]. Yessotoxin poisons bivalve mollusks; when injected intraperitoneally into mice, it inhibits phagocytosis of *Candida albicans* by macrophages [121]. *Gambierdiscus* spp. dinoflagellate microalgae produce ciguatoxins (abbreviated as CTX: not to be mistaken for cholera toxin) [122]. The alga *Karenia brevis* in the Gulf of Mexico, produces brevetoxin and its antagonist brevenal; lower water salinity favors more toxin and less antitoxin production [123].

Blooming phyto- and zooplanktons release the toxins that are frequently lethal to crustaceans, shellfish, and higher metazoans, including human patients, if these toxins enter the food chain. The same dinoflagellates, algae, amoebas and diatomes carry a large number of bacterial, fungal and protozoal symbionts (including vibrios, like *V. alginolyticus*), and viruses, both lytic and symbiotic, either attached extracellularly, or invading intracellularly [124].

Juvenile coral cells take up for intracellular endosymbiosis zooxanthellae *Symbiodinium* algae, and thus gain photosynthetically produced carbon-rich nutrients. Free-living symbiodinium cells do not, but these cells in symbiosis with coral cells do activate their H^+-ATPase. This 105 kDa protein works as a proton pump, dehydrates bicarbonates by carbonic anhydrase and liberates inorganic carbon. The chloroplast-encoded ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisCO) enzymes provide the fixed carbon both for the symbiont and its host [125]. Both the nuclear and mitochondrial genomes of the coral cell and the genome of the symbiont were sequenced. The symbiont resides in the gastrodermal cells of the coral and provides fixed carbon to these cells. The symbiotic relationship depends on the acceptance of symbiodinium sub-clade by the haplotype coral [126–130]. Entry of the symbiont in a stealth manner leaves the host cell's transcriptome undisturbed. Juvenile corals are rapidly dominated by the symbiont, whereas adult or parental coral cells are less tolerant toward the symbiont. Compatible symbionts are accepted without a reaction, but incompatible symbionts massively excite the recipient's transcriptosomes [131]; The expression of metabolic genes (carbohydrate and lipid metabolism, transmembrane ion transport) is intensified in corals possessing symbiont algae [132]. Possession of the symbiont keeps the coral from senescence in that its telomere length is preserved by continuous telomerase activity [133]. Rejection of the unacceptable symbiont is carried out by caspase-activated apoptosis of the algal cells [134]. Mature corals may lose the symbionts during "bleaching" events. Loss of the symbionts frequently leads to the demise of coral colonies. Corals retaining the symbionts during the bleaching event survive [135]. Coral colonies attacked by *Vibrio coralliilyticus* undergo bleaching and die. The target of the vibrio protease is more the intracellular symbiont, than the coral tissue [136]. It is most peculiar that a coral-pathogenic vibrio (*V. coralliilyticus*) in the Mediterranean sea would be multiple antibiotics-resistant [137]. Antibiotic resistance genes must have evolved under natural circumstances predating with millions of years the discovery and use of penicillin: "the soil antibiotic resistomes." A transposon-induced mutant of *V. coralliilyticus* lost its flagellum, and with it its pathogenicity [138]. It was not reported if this vibrio harbored phages. A 81 pages, 451 references outstanding article on the biodiversity of vibrios managed not even to mention vibrio phages [139]. *V. coralliilyticus* infections of corals induce mass mortality in the warm Mediterranean sea [140]. Corals defend themselves against bacterial and fungal pathogens by mobilizing amoebocytes to the sites of infection [141]; the vibrio apparently bypasses these defense reactions. This mass mortality of corals was recently matched by *V. cholerae* in human mortality in Zimbabwe [142].

Zooxanthellae-carrier and heat-shocked corals release diverse virus-like particles of varying morphologies: tail-less hexagonal particles of 40–50 nm diameters, droplet-shaped particles, and filamentous particles, all unidentified. It was not determined if these viral particles were of coral- or alga-derivation [143]. Ultraviolet-irradiated symbiodinium cells released filamentous virus particles resembling *Closteroviridae* RNA plant viruses [144]. In terrestrial plants (like citrus trees in Florida), these ssRNA tristeza citrus viruses are transmitted by toxoptera aphids [145]. The integrated and activated genomes of the algal filamentous viruses are similar to lysogenic bacteriophages, thus these hosts are subjected to genes horizontally inserted.

Diatoms and dinoflagellates maintain ancient host-virus relationships. The photosynthetic diatoms (*Bacillariophyceae*), contributors to the Earth's oxygen level, foodstuffs for zooplanktons and for various larvae, are harboring tail-less icosahedral ssRNA viruses. The RsRNAV (*Rhisosolenia setigera*) icosahedral ssRNA virus and the Cten virus (*Chaetoceros tenuissimus*) infect diatoms. The Cten virus in its lytic cycle, appears as a very large progeny of new viral particles released: 10^{10} infectious units/ml. These viruses form a new family, BacillarioRNAviridae/*Bacillariophyceae*. In contrast, the CsNIV (*C. salsugineum* nuclear inclusion virus) has a ssDNA genome [146, 147], thus representing a different, but non-interfering class if viruses. Viral interference is often replaced by viral coexistence.

The blooming and toxic photosynthetic dinoflagellate member of *Dinophyceae*, the *Heterocapsa circularisquama*, harbors two ancient viruses: a positive sense ssRNA virus and a dsDNA virus. In this host, the RNA and the DNA viruses tolerate each other. In the human host, DNA herpes- and RNA retroviruses synergize with one another (*vide infra*). The HcRNAV encounters sensitive, resistant and delayed lysis-udergoing hosts. The initiation codon for viral replication is the universal AUG. Sensitive hosts undergo rapid lysis and release viral particles. Resistant cells inhibit viral replication. Viral ORF-1 encodes the RdRp (RNA-dependent RNA-polymerase). The phylogenetic derivation of some land viruses (mushroom bacilliform virus, and others) from marine viruses is supported by the similarities of their RdRp to that of HcRNAV. Viral ORF-2 encodes the viral coat protein [148–150].

A giant dsDNA virus, HcDNAV, infects and lyses *H. circularisquama* cells and thus it can control this blooming toxic dinoflagellate. The viral capsid is icosahedral up to 210 nm in diameter; the DNA genome consists of 356 kbp. Viral replication takes place in the cytoplasmic viroplasms. By its looks, the virus was made a member of the PhycoDNAviridae, but by its biochemistry it does not fit into that group of large marine dsDNA viruses (*vide infra*). The amino acid sequence of its PolB (type B DNA polymerase) gene differed from that of the phycodnaviruses. This gene sequence was related closest to that of the Asfarviridae member, African swine fever virus (ASFV). Thus, this ancient marine virus and the much later evolved terrestrial mammalian virus have preserved their phylogenetical relationship [151].

Algae are generous gene-donors: stress-related algal genes (ascorbate peroxidases, metacaspases) operate in choanoflagellata [152]. The green alga *Paramecium bursaria* chlorella virus, and the coccolithovirus of *Emiliania huxleyi* (EhV) fit into the group of PhycoDNAviridae, the nucleocytoplasmic large dsDNA viruses. The haptophyta *E. huxleyi* represents one of the deepest branching lineages of the eukaryotic tree of life. Its exoskeleton displays carbonate scales, the coccoliths. The icosahedral 1,900 Å diameter chlorella virus (PBCV-1) genome encodes 365 proteins and is capable of glycosylating its major capsid proteins (without any help from the host cell's endoplasmic reticulum and Golgi apparatus). Beneath the glycoprotein capsid, lipid bilayer membrane surrounds the dsDNA core [153, 154]. The coccolithovirus EhV enters its host cell of the diploid calcified phytoplankton microalga *E. huxleyi* by envelope fusion and endocytosis [155]. The haploid phase of the *E. huxleyi* life cycle disallows viral entry and survives uninfected [156]. EhV encodes cytotoxic glycosphingolipids, which induce apoptotic death in infected cells [157]. There are seven sphingolipid biosynthetic genes (named in the article), one of them is longevity assurance factor, LAG1: wise little creature, *E. huxleyi*! EhV acquired these host genes *via* horizontal transfer; or *vice versa*, was it the virus that transferred these genes to its host? The virus needs lipid metabolism to construct its lipid membrane. Since lipid metabolism was present in eukaryotic cells ancestral to *E. huxleyi*, the

direction of gene transfer was very likely from host cell to its virus [158]. The picoeukaryotic photosynthetic green alga, *Ostreococcus tauri* harbors a large linear dsDNA virus, OtV5, a phycodnavirus. The host cell is so small, and a virus particle is so large, that at burst only 25 virus particles can be released. One of the viral coding sequences (CDS, flanked by start and stop codons) encodes praline dehydrogenase. This stress-reactive enzyme is coming from a horizontally transferred gene, probably bacterial-to-algal-to-viral derivation [159].

While the algal viruses may pick up host cell genes, being eukaryotic viruses, they could not integrate their genomes into bacteria; thus, these algal viruses can not be depicted as vectors of the dinoflagellate toxin genes to higher mammalian hosts, which they may not even be able to infect. The *Asfarviridae* hemorrhagic swine fever virus (ASFV) acquired its PolB gene on a long distance phylogenetic scale (*vide supra*). However, dinoflagellates may associate with bacteria (among them phage-carrier vibrios), which may acquire dinoflagellate toxin genes by horizontal transfers. These toxin genes may flank of, or fuse with, integrated phage genomes and thus may be spread by promiscuous phages to new bacterial hosts pathogenic to mammalian, including human, hosts.

2.2.2 Genes of the Mimivirus Shared with Its Phage and with Its Host Amoeba

The large nuclear-cytoplasmic dsDNA viruses (poxviruses, asfarviruses, iridoviruses, phycodnaviruses) show monophyletic origin. Poxviruses are grouped with asfarviruses and mimiviruses are grouped with iridoviruses and phycodnaviruses. In a most peculiar way, divergence of these viral families appears to have predated the divergence of the major eukaryotic lineages. After the divergence of the host cell lineages, further evolution of the viral genomes occurred due to horizontal receipt and incorporation of genes from their host cells and co-infecting bacteria and viruses. These large dsDNA viruses co-evolving not only with dinoflagellates, but with animal hosts (asfarviruses, iridoviruses, mimiviruses, mimicking microbes) acquired anti-apoptotic and immunosuppressive genes. The genes acquired from bacteria include bacteriophage genes [160, 161]. The viral B family DNA polymerases (PolB) are conserved in the large dsDNA viruses, herpes- and baculoviruses and show low frequency of recent horizontal transfers. Comparable PolB genes occur in archaea viruses and in the three archaeal lineages (*Nanoarchaeota, Crenarchaeota, Euryarchaeota*). In the mimiviridae group, the 16 PolB fragments underwent substantial segment variations. Viral PolBs show much higher diversity than bacterial PolBs [161].

Acanthamoeba polyphaga harbors mimivirus; with its 750 nm size and 1.2 Mbp genome, it is the largest known dsDNA virus. Mimivirus is closely related to the large dsDNA algal viruses. The ancestral mimivirus infected microalgae of the phytoplankton. The mimiviral genome containing both DNA and RNA stands on the boundary between viruses and bacteria (or even eukaryotic protocells). Of the four mimivirus aminoacyl-tRNA synthetase genes, two (TyrRS; MetRS) are archaea/eukaryota-related, except for their anticodon binding sites [162]. Mimivirus-like PolB and amino acid sequences occur in algal phycodnaviruses of the Norwegian coastal waters: CeV01 (*Chrisochromulina ericina*), PpVo1 (*Phaeocystis pouchetii*) and PoV01 (*Pyramimonas orientalis*). The mimivirus PolB sequence exhibits 45, 41, and 31% identity with the PolB sequences of PoV01, CeV01 and PpV01. These icosahedral viruses are smaller than the mimivirus, being of 160–220 nm in diameter with genome sizes of 485–560 kb. For comparison, the genome of the phycodnavirus EhV-86 is 407 kb [155, 163]. In the host amoeba, the mimivirus genome is allowed to grow rather than retract. Instead of genome reduction it gains genome size by the acquisition of horizontally transferred genes and mobilomes. The viral genome enlarges within the amoeba by lineage-specific gene duplications, lateral gene transfers from the host and accretion of mobile genetic elements (transposons and retrotransposons) [164]. It was proposed that poxviruses accept host cell mRNAs reversely transcribed into cDNA, ready for integration. Among horizontally acquired poxvirus genes are those of IL-10, thymidine kinase,

ribonucleotide reductase, glutathione peroxidase (for oxidative damage protection), deoxyribopyrimidine photolysase (for repair of ultraviolet light damage). The IL-10 gene in the poxvirus genome (yatapox, canarypox viruses) is the result of horizontal transfers from eukaryotic hosts [165]. Ancient poxviruses vectored retrotransposons from reptiles to mammals [166]. The amoeba is a "melting pot of genes and evolution" in reference to mimivirus and the 368 kb genomic Marseillevirus residing in it [167, 168].

The arrangement of major capsid proteins of large dsDNA viruses (chlorella virus, PBCV1; *Sulfolobus* turreted icosahedral virus, isolated from the acidic hot springs (90°C) of Yellowstone park, where it infects the hyperthermophilic crenarchaea *Sulfolobus solfataricus* (*vide supra*), the photosynthetic marine cyanobacteriophage PM2, and coliphage Period A, PRD1, show by X-ray crystallography close identity. Even though these viruses infect host cells of wide variety (archaea, prokaryota, eukaryota), they derive from a common precursor [169, 170]. The crenarchaeota viruses, however, display unique features suggesting an evolutionary path with gene gains from both their hosts and also from prokaryota [171]. The enveloped dsDNA acidianus filamentous virus-1 (AFV-1) of the *Lipothrixviridae* class infects a crenarchaeal host in the acidic hot springs of Yellowstone Park and its gene repertoire is quite unique [172].

The organization of the mimivirus capsid, the starfish-shaped arrangement of its capsomers and fiber components, indicates that its encoding genes are of prokaryotic and eukaryotic derivation [173]. Viruses (coronavirus; human immunodeficiency virus-1 (HIV-1)) usually utilize host cell cyclophilins; mimivirus encodes its own cyclophilin [174]. The mimivirus genome can encode 911 proteins; of these, 298 have recognized functions (translation enzymes, DNA repair pathways, topoisomerases). This virus resides also in corals and sponges [175, 176]. The two cytochrome p450 genes of the mimivirus show 23–26% identity with bacterial (*Proteus mirabilis*), fungal (*Aspergillus*), streptomyces (*S. peucetius*) and caenorhabditis cytochromes. The short region of mimivirus dysferlin (Ca^{2+}-binding protein) has 35% homology with that of the sea urchin (*Stongylocentrotus purpuratus*). The ADP-ribosyltransferase pierisin-1 shows 24% identity with that of *Pieris rapae* (the lepidopteran pest of cruciferous vegetable crops) [177, 178].

If large dsDNA viruses existed before cells were formed, then viral genes were donated to protocells, and not *vice-versa*. The transcriptional gene silencing Tgs eukaryal enzymes methylate the N2 atom of-7-CH_3 guanosine nucleotides. The *Giardia* Tgs protein and the mimivirus Tgs protein are unique and similar in their capacity to methylate guanine-N2 in the absence of prior N7 methylation [179].

In addition to amoebae, corals and sponges may host as yet unidentified mimivirus relatives [175, 176]. Of the new proteins encoded by the mimivirus genome there are four aminoacyl-tRNA synthetases. Several non-coding RNAs are prominently expressed. Gene expression late promoters of the mimivirus were shared with its phage, the sputnik. Polyadenylated transcripts derived from new gene (previously unknown: no database homolog) with gene product proteins of unknown function. Mimiviral tRNA methyltransferases and aminoacyl-tRNA synthetases (new for a virus to encode it) are activated early. In response, a burst in the transcription of mitochondrial genes of the amoeba takes place. This event coincides with the viral "eclipse phase", during which neither formed, nor functional viral elements are present in the viroplasms. The eclipse phase is followed by the upsurge of mimiviral, and the decrease of amoebal gene transcripts. For comparison, Fig. 2.1 shows the eclipse phase of influenza A virus adapted to chicken embryo, or to mouse lung (Fig. 2.1) [180]. The synthesis of the LPS-like outer layer of the viral particle is a late event. Mimiviral genes encoding capsid proteins and collagen-domain proteins are among the last to be activated [181]. The cellular nucleoside diphosphate kinases are small 150 aa proteins highly conserved within archaea, bacteria and eukaryota (>40% identity). The cellular nucleoside diphosphate kinases (NDKs) do not distinguish ribonucleotides from deoxyribonucleotides, acting equally in both, transferring phosphate groups from nucleoside triphosphates (NTP, other than adenosine triphosphates, ATP) to nucleoside diphosphates (NTP to NDP). In contrast to the cellular enzymes, mimiviral NDKs display distinct affinity for deoxypyrimidine

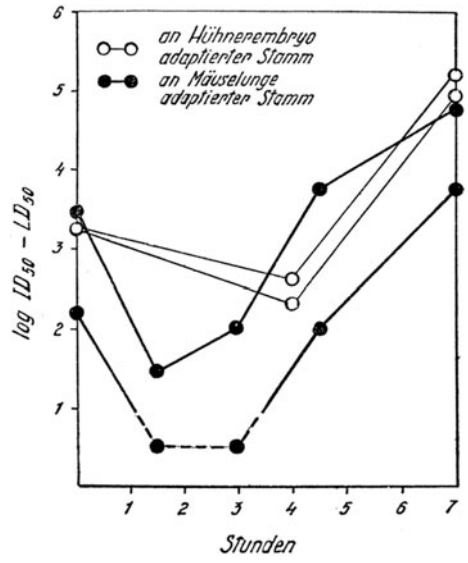

Fig. 2.1 Graph from "Die Grundlagen der Virusforschung (1956)" showing the disappearance ("eclipse") of all detectable viral activity ("die Dauer des Vermehrungszyklus die Infektionsfähigkeit verliert") after the inoculation of influenza A virus into the allantois cavity of a chicken embryo, or into a mouse lung, and the reappearance of viral structural proteins and then mature extracellular infectious virions. The work started in 1950 and was published by Sinkovics and Molnár in 1954 [818, cited in 180]. Permission to re-publish is from Akadémiai Kiadó, Budapest

nucleotides. The mimiviral enzyme is ancestral to the cellular (including that of the amoeba) NDK enzymes. The mimiviral enzyme clusters with euryarchaeal, crenarchaeal and bacterial sequences. The Acantamoeba (*A. castellani*) enzyme clusters with fungal, and metazoan sequences. The viral enzyme was not acquired from a eukaryotic organism by horizontal gene transfer [182].

An icosahedral dsDNA virus 50 nm in size packing a 18 kb circular genome replicates only in the viroplasm of the mimivirus. In the presence of the replicating "sputnik", the mimivirus can not mature into complete particles; its capsid assembly becomes distorted. It is not lysed; yet the sputnik acts like a phage to the mimivirus: a "virophage". Three sputnik genes encode proteins of mimivirus derivation; other sputnik genes encoding integrase, helicase, ATPase and transposase are of bacteriophage and eukaryotic viral derivations. The sputnik displays a double jelly-roll capsid. Sputnik lacks RNA- and DNA polymerases and borrows mimiviral transciptosome. The palindromic signal characteristics of mRNA polyadenylation sites in many sputnik genes are those of the mimivirus; so is its late protein element [181, 183, 184].

Intraamoebal bacteria, *Legionella drancourtii* and *Coxiella burnetii*, possess a gene of aquatic viridiplantae origin. This gene encodes the enzyme sterol delta-7 reductase; the intraamoebal mimivirus also transcribes this gene. The host amoeba generously donates its genes to its parasites, the intraamoebal viruses and bacteria. The intraamoebal parasites exhibit genomes larger than that of their extraamoebal relatives. However, in the case of the sterol delta-7 reductase and another eukaryotic enzyme, the ATP/ADP translocase, the gene donor was not the amoeba, but a chlamydia. The chlamydia acquired the gene from a parasitized aquatic plant. Upon transferring itself into the amoeba, there the chlamydia encountered other guests of the amoeba. Through another horizontal transfer, these genes were implanted from the chlamydia to the bacteria (and to the mimivirus) [185].

The *Acanthameba polyphaga mimivirus* (APMV) has the potential to infect the lungs of intubated patients in the intensive care units. Macrophages of the respiratory tract take up the mimivirus by phagocytosis. Patients with ventilator-associated pneumonia develop antibodies to mimivirales [186–188].

2.3 Interviral (Virus-to-Virus) Gene Transfers

2.3.1 Marek's Disease Herpesvirus

"Multiple Nervenentzündung (Polyneuritis) bei Hühnern" described in 1907 in the *Deutsche tierärztliche Wochenschrift* by the Hungarian veterinarian József Marek was a new disease entity characterized by heavy lymphocytic infiltrates in multiple organs, but especially in nerve sheaths and in the meninges. The causative agent of the "Mareksche Geflügellähmung", which was recognized to be a neoplastic entity, was transferable by filtrates. Thus, the viral etiology of the lymphomatous tumors was declared [189]. The replication of the Marek virus in tissue cultures was shown first in B. R. Burmesters's laboratory [190, 191]. The first Marek's herpesvirus strains were isolated in chicken kidney cell cultures by J. L. Spencer [192]. The co-operative interactions in malignant lymphoma induction between Marek's herpesvirus and chicken leukosis and chicken sarcoma (Rous) retroviruses were first documented in tissue cultures at Rutgers, the State University of New Jersey, by J. W. Frankel and Vincent Groupé [193] and confirmed and elaborated on at Columbia University in New York and in the germ-free animal laboratories of Life Sciences in St. Petersburg, Florida [194].

2.3.2 Reticuloendotheliosis Virus Genomic Sequences in the Marek's Virus and in the Fowlpox Virus Genomes

Leuko-, lympho- and sarcomagenic retroviruses were recently shown in a condensed tabulated form [195]. The T strain of chicken reticuloendotheliosis virus (REV-T; REL) transforms chicken lymphocytes, but not chicken embryonic fibroblasts. This virus infects both CD4 and CD8 T lymphocytes [196]. The length of its sequenced proviral genome is in the range of 8,284 nucleotides [197]. The *v-rel* oncogene and its cellular homologue *c-rel* encode the p59v-Rel phospho-oncoprotein. The oncoprotein remains in the cytoplasm of transformed spleen cells, but in transformed E26 myeloid cells the p59v-Rel oncoprotein translocates from cytoplasm into the nucleus [198]. The natural protein Rel/NF-κB is an inducer of inflammatory and immune responses, promotes cell-proliferation and it exerts anti-apoptotic effects. The viral oncoprotein transforms lymphoid cells and induces malignant tumors resembling human mediastinal B cell lymphomas and Hodgkin's disease. In the human counterparts of such tumors, overexpressed or mutated c-Rel oncoprotein activates the expression of anti-apoptotic and pro-proliferative genes in the nucleus. The vRel oncoprotein promotes the expression of telomerase reverse transcriptase (TERT), thus preventing the shortening of telomeres at cell divisions.

The viral transcription activation domain (vTAD) interacts with CAPERα, which synergistically modulates the transactivation by vTAD. The co-activator of activating protein-1 (AP-1) and estrogen receptors (CAPERα) is expressed in liver cirrhosis and in hepatocellular carcinoma. In vRel-mediated lymphomagenesis, CAPERα acts as a transcriptional co-regulator and antagonist of the transforming activity of vRel oncoprotein. Neutralizing CAPERα mRNA by siRNA in vRel-transformed lymphoma cells increased the cells malignancy, as expressed by their enhanced colony formation. Tumor suppressor CAPERα has to be silenced by siRNA or by disabling mutation for the cRel oncoprotein to act uninhibited in human lymphoma cells. In human Reed-Sternberg cells of Hodgkin's disease, cRel is an active oncogene. In avian malignant lymphomas transcriptional co-activation of vTAD by CAPERα is promotional to its antagonism for vRel's transforming activity [199]. Three oncogenic viruses, avian leukosis virus (ALV), reticuloendotheliosis virus (REV) and Marek's disease virus (MDV) down-regulate the expression of the non-coding tumor-suppressive gga-microRNA-26a (*Gallus gallus*), the regulator of tumor suppressor PTEN gene (phosphatase and tensin homologue deleted on chromosome ten). One of the tumor-suppressive effects of gga-miRNA-26a is antagonism to the "T cell growth factor" IL-2 expression in these avian tumors [200].

The JARID2 gene product histone lysine demethylizing proteins (Jmj, jumonji, cruciform in Japanese) bind to cyclin D1 promoter and repress the transcription of cyclin D1. Jmj forms complexes with histone methyltransferases and reacts with the cyclin D1 promoter, thus increasing histone methylation, while cyclin D1 is repressed. Jmj family proteins regulate both methylation and demethylation of histones (J. jumonji in Japan; ARID, AT-rich interaction domain) [201]. In chicken B cell lymphomas, REV-T induces the oncogenic miRNA-155 for targeting JARID2. Part of the histone methyltransferase complex, JARID2, rapidly loses its pro-apoptotic activity under the effect of the antagonistic miRNA-155. The pro-survival function of miRNA-155 promotes lymphoma cell growth in the absence of JARID2 activity [202].

Phylogenetic relationship of gallid herpesvirus-2, MDV, did not reveal how its oncogenes *meq* and *pp38* evolved [203]. The MDV encodes oncoprotein Meq, a homologue of cellular proto-oncogenes *fos* and *jun* (*fos*, Finkel osteosarcoma murine retrovirus oncogene; *jun*, ju-nana Japanese for seventeen, after avian sarcoma virus ASV-17). MDV also encodes the immediate-early transactivator protein, ICP4, to which a small antisense RNA is expressed in lymphoma cells [204]. MDV-related abbreviations: the lytic antigen pp38 (Meq, Marek's EcoRI-Q DNA restriction one minifragment probe, Eco from *Escherichia coli*; pp38, phosphoprotein-38). The MDV-encoded RNA telomerase subunit (vTR) shows 88% sequence identity with the chicken gene cTR. The MDV vTR maintains telomere length in transformed cells [205]. The virus releases a number of miRNAs (similarly to Epstein-Barr virus, EBV, and HHV-8, Kaposi sarcoma-associated herpesvirus, KSHV). The MDV miRNAs interact with viral oncogene *meq* and with the viral latency associated transcript (LAT) [206]. MDV may either lay latent in avian CD4 T cells, or it malignantly transforms them. In a REV-transformed lymphoid cell line, latent MDV expressed its Meq oncoprotein antigen and contributed to the cells' apoptosis resistance. Treatment with bromodeoxyuridine induced the expression of MDV lytic antigens [207]. The Meq oncoprotein due to its Pro-Leu-Asp-Leu-Ser motif binds C-terminal binding protein (CtBP), a transcriptional co-repressor. The Meq-CtBP complex is essential for oncogenesis: cells with mutated CtBP are exempted from MEq-induced oncogenesis. The EBV oncoprotein EBNA3A/3C (nuclear antigen) also interact with CtBP. MDV vaccine strains do not encode the Meq oncoprotein and do not interact with CtBP [208].

Syngeneic and allogeneic cell-mediated cytotoxicity against Marek's disease virus- (MDV-) transformed lymphoblastoid tumor cell lines revealed brisk alloantigen-directed (allogeneic) reactions, and rare syngeneic reactions by host lymphocytes [209]. Targeting lymphocytes against specific oncoproteins (phosphoprotein pp38 of MDV), or harvesting them from REV-sensitized donors, yielded virus-specific syngeneic cytotoxic lymphocytes [210]. Splenic lymphocytes of MDV-sensitized donors killed transformed syngeneic lymphocytes expressing MDV oncoprotein antigens pp38 and Meq [211].

These types of lymphocyte-mediated immune reactions directed at human sarcoma cells were observed already in the late 1960s and early 1970s in the author's laboratory at M. D. Anderson Hospital, Houston, TX. The autologous (or syngeneic) reactions were mediated by immune T cells, whereas the allogeneic reactions were mediated by large granular lymphocytes that were later designated to be natural killer (NK) cells [212–214]. These observations are documented and illustrated in Sinkovics' monograph "Cytolytic Immune Lymphocytes. . ." (Fig. 2.2a,b) [213].

REV-transformed avian T-lymphoblastoid cells accept co-infection with MDV [215, 216]. The two avian T cell lymphoma viruses (MDV and REV) synergized their pathogenicity, as the MDV genome accepted the insertion of the REV genome. Tumor cells co-infected with these two viruses either in vitro or in vivo, yielded MDV isolates that accepted the insertions of retroviral genomic segments in their genomes. It was the long terminal repeat (LTR) of REV that penetrated the MDV genome; both attenuated and virulent strains of MDV exhibited such insertions [217]. Herpes- and retroviruses were known to synergize their infectious and cell-transforming processes by augmenting each other's efficacy in malignant transformation [193, 218]. The Rous sarcoma virus LTR was transactivated by MDV [219]. The LTR of HIV-1 yielded to transactivation by human herpesvirus-1 (HHV-1) and other

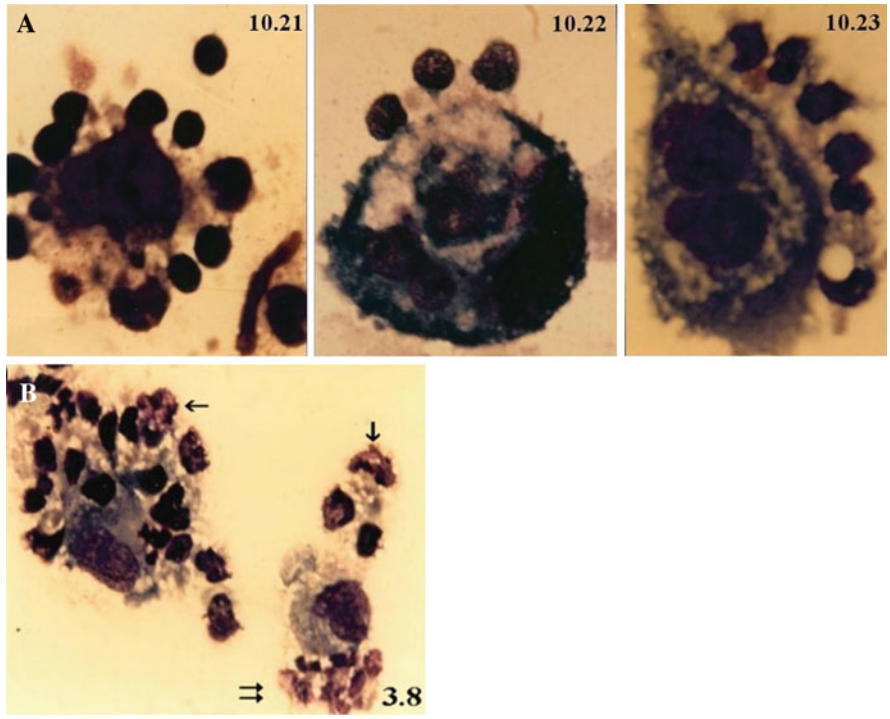

Fig. 2.2 (a) Human sarcoma cells attacked and lysed by autologous lymphocytes. The compact small round cells are immune T cells. It appears as if the lymphocytes injected "cytolysins" into the cytoplasms. The large granular lymphoid cells in 10.21 are NK cells. (**b**) In 3.8, human sarcoma cells withstand attack by a mixed population of lymphocytes; some lymphocytes die apoptotic death (*arrows*) next to the attacked tumor cell. From the Section of Clinical Tumor Virology & Immunology, M. D. Anderson Hospital, Houston, TX, in the early 1970s [213]. Permission to re-publish is from Schenk Buchverlag, Passau and Budapest

DNA viruses [220]. *Vice-versa*, MDV transactivated the promoters of avian leukemia and sarcoma (Rous) viruses [221, 222].

The phenomena of retroviral genomic segmental insertions into the genome of DNA viruses were observed naturally and induced artificially. The two avian T cell lymphoma viruses, MDV and REV, synergized their pathogenicity as the MDV genome accepts inserted segments of the REV genome [217] Transcripts of the REV's LTR promoters enhanced the expression of MDV US (unique short) genes [223, 224]. Insertions of the retroviral genome occur within one or two passages in MDV-infected cells. The retroviral insertion sites are two, 1 kb region each at the junction of the short unique and short repeat regions of the MDV genome. To the malignancy of a MDV-transformed lymphoma cell line, REV contributed the activation of the *c-myb* (myeloblast) proto-oncogene [225]. A clone of MDV derived through REV genomic insertion exhibited attenuated oncogenicity, but remained infectious by contact, caused thymic and bursal atrophy and induced severe immunosuppression [226]. In MDV- and REV-co-infected cells, chimeric molecules were formed from REV-LTR and MDV flanking proteins [227]. In comparing the horizontal transmissibility of MDV-GX-0101 field strain harboring the LTR of REV, LTR-deleted viral clones were more immunosuppressive and less transmissible through horizontal routes, than the original REV LTR-positive strain [228].

Fowlpox virus (*Avipoxvirus*) vaccines were frequently contaminated with REV. Fowlpox vaccine virus isolates in the chorioallanois membranes of chicken embryos grew in lesions yielding both fowlpox virus and REV. It could not be distinguished if proviral REV DNA was integrated in the

cells' DNA and/or into fowlpox virus DNA. The REV-contaminated fowlpox virus vaccine in inoculated chickens caused fowlpox lesions and feathering defects and proventriculitis [229]. In fowlpox virus field isolates, the integrated genomic segments of REV *env* gene were identified. Fowlpox virus vaccines carrying integrated REV genomic sequences induce weak protection against fowlpox [230]. In some fowlpox vaccine viruses, the full genome of REV was found integrated. These integrated REV full length genomic sequences (*gag, pol, env* and LTR) are replication competent [231], *gag,* group-associated antigens are the virions' structural proteins, *pol*, polymerase, encodes the viral enzymes: protease, integrase and reverse transcriptase; *env*, envelope proteins. REV-free field isolates of fowlpox virus (both chicken and turkey) exist. Some fowlpox virus isolates from wild birds or from poultry harbor none, or only remnants of REV LTR, and no REV *env* genes [232] The production of REV-free fowlpox virus vaccines is possible. A refined quantitative multiplex real time polymerase chain reaction (PCR) is available to select out REV-free fowlpox virus strains [233]. It is with the MDV vaccines and the fowlpox virus vaccines, where the biological importance of horizontal viral gene transfers and recombinations between unrelated viruses are surpassed by the practical urgency of the problem.

2.3.3 Herpesviruses Activate Latent Retroviruses

Over ten million years of coexistence in sharing the same hosts in Africa and co-evolving with each other and with their simian and hominid hosts, created a special relationship between herpes- and retroviruses. Instead of interference with each other, herpes- and retroviruses co-operate. Their cooperation consists of either suppression of lethal infections by the partner virus in the interest of keeping the host alive; or of the activation of the other virus to gain growth factors (cytokines and chemokines), have access to transformed host cells protected against apoptotic deaths, into which to integrate, and benefit from immunosuppression of the host by creating a Th2-type environment, and thus eliminating IFN-γ and TNF-α production in the host. From the human point of view, the cooperation between herpes- and retroviruses is a form of "criminal collusion" [213]. The MDV increases the rate of transcription of the avian leukemia virus, Rous-sarcoma-associated retrovirus (RAV-2 ALV). Five- to tenfold RAV-2 AVL RNA and viral structural proteins are produced in the presence of MDV [221]. The phenomena of latent retrovirus activation in herpesvirally co-infected human tumor cells have been well recognized [234–241]. The widely spread leukemogenic-sarcomagenic retrovirales of vertebrates from the fish up to Old World simians are not expressed in human leukemia and sarcoma cells [195, 213]. However, in herpesvirally (HHV-8; KSHV) induced human Kaposi sarcoma cells there appears an activated endogenous retrovirus (Fig. 2.3a,b) [213]. If there is a latent human sarcoma retrovirus not lost during human evolution and still is in hidden existence, it may be found in EBV-infected leiomyosarcoma cells of children [213].

2.3.4 Avian Herpesviruses Descend from Theropod Dinosaurs?

The ancestors of alligators, crocodiles and turtles coexisted with dinosaurs (*deinos,* Greek, terrible; *sauros*, Greek, lizard) and avian genera descended from feathered "bird-footed" "beast-footed" theropod (*therio,* Greek, wild carnivorous beast) dinosaurs. The ancestors and the "missing links" are preserved in the Gobi desert and elsewhere (Liaoning, China) possessing melanosomes for the spectacular coloring of their skins and plumage [242–247].

There is a gap between extant herpes- and retroviruses of crocodilians, reptilians, amphibians and *Aves* represented by the viral flora of the extinct dinosaurs (*Archosauria)*. Turtles are at the base

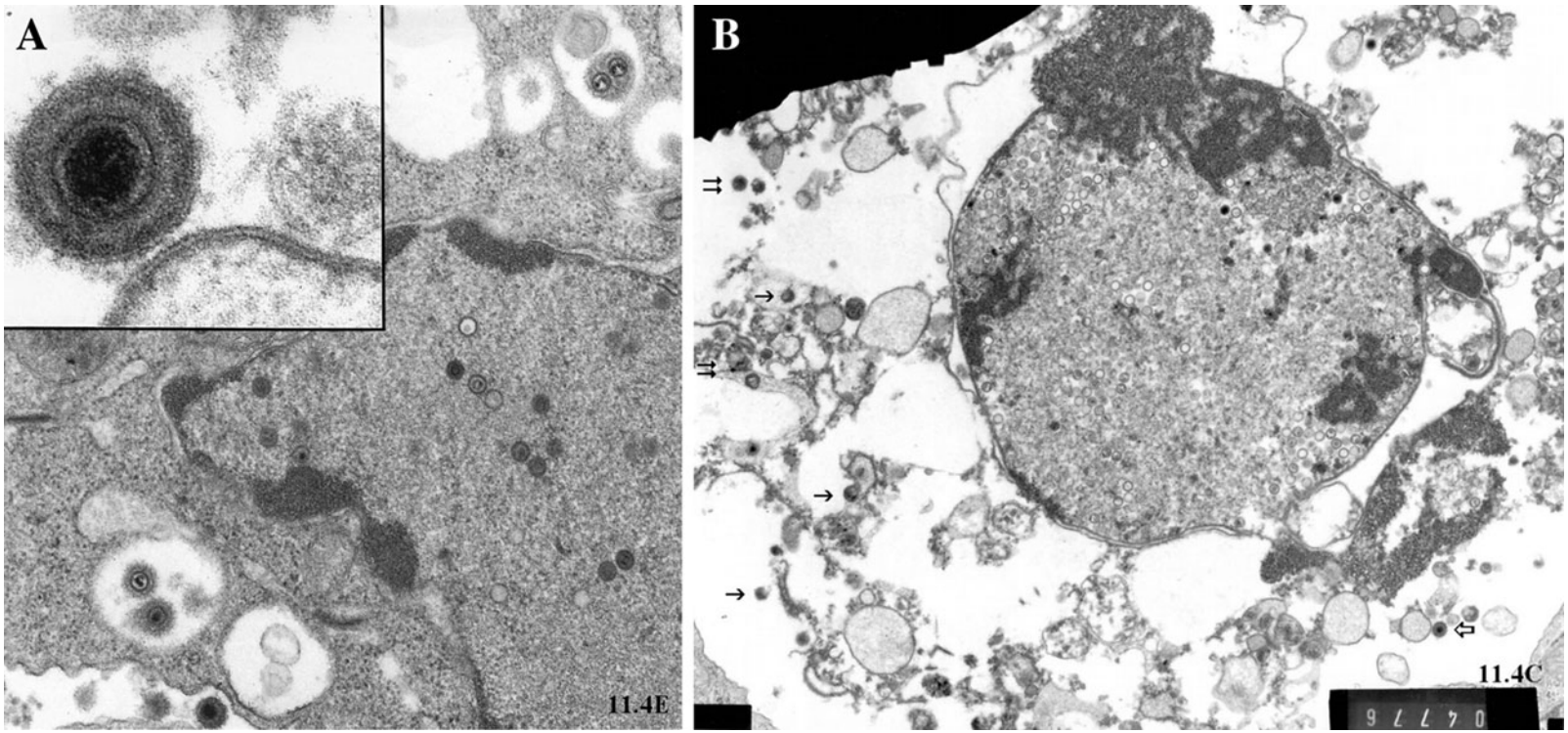

Fig. 2.3 (a) Classical Mediterranean Kaposi's sarcoma cells from the pre-AIDS era, in the early 1970s. Herpesvirus particles are those of HHV-8 (KSHV) (not known at that time). (**b**) Unidentified budding retrovirus particles (different in morphology from HIV-1) are those of an activated endogenous retrovirus (*four arrows*) in the disintegrating cytoplasm of a Kaposi's sarcoma cell. Next to the nucleus (*single arrow*) a mature herpesvirus-like particle is present (HHV-8, unidentified). The cell nucleus contains immature herpes-like virus particles. From the Department of Pathology (chief, Prof. Ferenc Györkey†), Veterans' Administration Hospital Medical Center, Houston, TX [213]. Permission to re-publish is from Schenk Buchverlag, Passau and Budapest

of the crocodile-bird branch [248, 249]. Reptilian α-herpesviruses cause the chelonid fibropapillomatosis in marine turtles (*Chelonia* sp.). Marine leeches (*Ozobranchus* sp.) vector huge loads of the fibropapilloma-associated turtle herpesvirus [250–256]. The herpesviruses infecting loggerhead turtles (*Caretta caretta*) cause fibropapillomatosis, tissue ulceration and necrosis and cell syncytia formation with intranuclear inclusion bodies [257]. Thus, new families of Herpesviridae emerge to include the bivalve (oysters), fish, reptile, turtle and tortoise, lizard, snake, crocodilian and amphibian herpesviruses [258, 259]. The herpesviruses form three distinct groups: (1) reptilian, avian and mammalian (reflecting to "ancient coevolution of these virus lines with the development of birds and mammals from reptilian progenitors"); (2) fish and amphibian; and (3) invertebrate bivalvian herpesviruses [260, 261].

Tortoises (*Testudinidae*) carry pathogenic herpesviruses causing glossitis and gastritis. Multifocal hyperemic-hemorrhagic nodules and plaques of the cloacal and phallic mucosa of juvenile alligators (*A, mississippiensis*) yielded isolates of tortoise herpesvirus-1; the lesions were infiltrated by monomorphic round cells resembling monoclonal lymphocyte populations. The isolates fitted into a phylogenetic tree of α-herpesviruses in comparison with turtle, tortoise, crocodylid and varanid herpesviruses; other herpesviruses in this phylogenetic tree were the gallid HV-2 (Marek's virus), bovine, equine, feline and human herpes simplex viruses-1, 2 and psittacid HV-1 [262]. The psittacid HV-1 causes cloacal papillomatosis in the Amazonian parrots (*A. aestiva*). These birds eventually succumbed to bile duct and pancreatic adenocarcinomas. The principle of Koch's postulates for herpesviral etiology for the adenocarcinomas has not been satisfied [263].

In the evolution of *Aves* in the families of *Archosauria*, there were gene preservations and gene losses. Mitochondrial DNA sequences identify *Archosauria* descendants (crocodilians and birds) and *Lepidosauria* (lizards and snakes) [264]. Conserved nuclear genomic sequences are reptilian olfactory receptor genes in birds [265]; melanosomal matrix genes [266]; the interspersed repetitive elements of chicken repeats (CR1) [267]; alligator isochors as GC-rich bird isochors [268]; the glycine-proline-tyrosine rich beta-keratin protein similarities in crocodilians and birds; reptilian beta-keratins as glycine-rich feather keratins and cornifications in feather morphogenesis [269–271]. Tuatara (*Sphenodon* sp.) reptilian chromosomal segmental regions show homology and orthology with chicken chromosomal segments, among them some tuatara autosomal regions are homologous with the sex chromosomes of birds and mammals [272].

Sex chromosomes remain highly conserved: chicken Z chromosome corresponds to turtle chromosome 6q, snake chromosome 2p and crocodile chromosome 3 with the order of genes preserved. The absence of homology between bird Z chromosome and snake and turtle Z sex chromosome indicates that these sex chromosome genes have had different origins [273]. Nucleotide and aa sequence alignment of saltwater crocodiles' oocyte maturation factor (C-mos) display strong similarities with that of birds (chicken and zebra finch) [274]. Ancient syntenies are conserved in fish, reptilian, avian and mammalian microchromosomes [275]. The chemokine IL-8 (CXCL8) shows up first in the reptilian turtle genome [276] and remains conserved through birds [277–279] and mammals up to *Homo* [280].

Genomic sequences "lost in translation" are IgD of the gecko missing in birds [281]; the Na^{+}-Ca^{2+} exchanger NCX family regulators of teleosts, amphibians and reptilians missing in birds and mammals [282]; of the tooth-forming genes, the dentin matrix protein-1 gene lost in birds [283].

The abundant presence of retroviral sequences in vertebrate genomes so far failed to detect the evolutionary connections between reptilian progenitors of birds and mammals [284]. In extant descendants of *Archosauria*, the crocodiles and birds, the CR1-like retrotransposons are active and the encoded C-mos (*vide supra*) between crocodiles and birds reveal significant sequence similarity [274]. Retrovirally (avian sarcoma retrovirus) mediated gene insertions into young chickens revealed those genes that are essential to feather morphogenesis. These genes are those of the bone morphogenetic protein-4 (BMP) for interacting with noggin (BMP antagonists noggin and dickkopf) to induce rachis formation, barb fusion and barb branching; and for the sonic hedgehog protein (Shh) to remove by apoptosis induction marginal plate epithelial cells in between barbs [285]. Was it retrotransposons that

horizontally inserted such genes into theropod dinosaurs? Were the first flights of the pterosaurs 250 million years ago bat-like or fathered bird-like? Pterosaurs were flying 150 million years before bats, and 70 million years before birds, whereas the first placental mammals appeared just over 100 million years ago. In the Cretaceous-Tertiary boundary 65 million years ago, the first primates diverged from ungulates and a cataclysmic extinction of the dinosaurs occurred, but the crocodilian ancestors survived [286]. The *Pan* and *Homo* lineages separated about 7 million years ago, and *Australopithecus afarensis* lived 3.7 million years ago in what is Hadar, Ethiopia, today. Both birds and flying bats constricted the sizes of their genomes; retrospectively calculated (Markov chain Monte Carlo approach), pterosaurs operated with a much constricted genome [287]. Conserved genomic segmental overlaps occur between alligators, turtles, emu and chicken, despite a drastic reduction of the chickens genome size in comparison with the sizes of the reptilian genomes [288]. Even though a scrutiny of herpes- and retroviral agents active in birds, especially the Marek's virus (*vide supra*), so far failed to identify with exact precision the reptilian or amphibian ancestors of these avian viruses (*vide infra*), the descent of *Aves* from theropod dinosaurs is strongly supported by genetic evidence. Whether it was exclusively through a vertical line of evolution how birds emerged from reptilian ancestry, and/or was it with the help of horizontally inserted genes, it will be determined by renewed further research. May be, the answers will come from the International Chicken Genome Sequencing Consortium (Washington University School of Medicine, Campus Box 8501, 4444 Forest Park Avenue, St. Louis, Missouri 63108, USA).

Is there a chain of herpesviral viral evolution in which ancestral reptilian and amphibian herpesviruses (the ancestor of the fibropapillomavirus of extant turtles) (*vide supra*) infected the dinosaurs? Before their extinction, the dinosaurs passed these viruses to the ancestors of *Aves*. Extant species of *Aves* are infected with the descendants of these ancestral herpesviruses (gallic herpesvirus 2, the MDV; psittacine cloacal papillomatosis herpesvirus). After the divergence of the reptilian/amphibian and the mammalian lineages, the mammalian species continued to harbor the descendants of the ancient reptilian-amphibian herpesviruses (the ancestor of the fibropapillomavirus of extant turtles) and expressed them up to the simian lineages. These herpesviruses are known as the baboon, green monkey, mandril and rhesus rhadinoviruses, the retroperitoneal fibromatosis herpesviruses. These ancient rhadino-herpesviruses (herpes, erpein, creepeing-creeper; rhadino, fragile viral DNA); already encode an IL-6 homolog and their LANA (latent nuclear antigen) is an ortholog of the HHV-8/Kaposi sarcoma HV ORF73 product protein (reviewed in [213]). The anti-apoptotic LANA proteins promote cells survival and "immortalization" [289]. The polymerase enzyme of the chimpanzee rhadinovirus shows 82% nucleotide sequence homology and 93% aa identity with the HHV-8/KSHV (but chimpanzees do not develop Kaposi sarcoma-like tumors) (reviewed in [213]). In KS cells HHV-8/KSHV activates the MAPK pathway (mitogen-activated kinase) [290]. The ORF (open reading frame) K12-product kaposin [291, 292], and other oncogenes of HHV-8 (v-Bcl-2; the ORF72 product v-cyclin D; the ORF74 product G-protein-coupled receptor, long unique region, cyclooxygenase-2) [293] are new acquisitions of KSHV (HHV-8), which are not yet present in the chimpanzee rhadinovirus (reviewed in [213]). In the latent form of HHV-8/KSHV, the viral genome is circularized in an epigenetic extrachromosomal location. Hypomethylation of the promoter of replication and transcription activator, and histone acetylation trigger the "lytic switch" and thus the active replication of the virus leading to cytolysis [294]. It is not only HIV-1-induced immunosuppression that activates the latent HHV-8/KSHV The HIV-1 *tat* gene product transactivator protein Tat activates the MAPK pathway in the host and the kaposin gene in the latent HHV-8/KSHV [295]. Kaposi sarcoma cells through human leukocyte antigen HLA-A2 express epitopes that attract cytotoxic lymphocytes. These are *aa* 16–25 in latent antigen kaposin and aa 59–68 in lytic antigen glycoprotein H. The lymphocyte donors were healthy volunteers [296, 297]. This author presented microphotographs of the phenomena of cytotoxicity by autologous lymphocytes to KS sarcoma cells in the pre-AIDS era [298–300]. The coexistence of the herpesviral pathogens (HHV-8/KSHV) and an as yet unidentified endogenous retrovirus in pre-AIDS era "classical" KS cells is shown in Fig. 2.3a,b. The pathogenicity-

and proto-oncogenes of HHV-8/KSHV very likely are host cell gene derivatives expropriated through horizontal transfers by the ancestral rhadinovirus as it was following the hominid lineage after the divergence from the *Pan* lineage some 7 million years ago. These horizontal new gene acquisitions by the ancestral rhadinovirus must have taken place during its residence in the ancestors of *Homo* (the *Australopithecus afarensis* and upward).

The ancestry of the other human "lymphocryptovirus," the Epstein-Barr virus (HHV4/EBV) can be traced back to New World marmoset (owl and squirrel) monkeys diverging from the Old World simian lineages some 33 million years ago and represented by the oncogenic *Herpesvirus saimiri* in its host the squirrel monkey, *Saimiri sciureus* [301]. In New World (South American) monkeys, in contrast to retroviruses dominating in Old World (African) monkeys, the lymphogenic viruses are herpesviruses [302]. Further down, in the Cambrian sea and at the time of the emergence of the ancestral sharks (Placoderms; *carcharhine* sharks, *chondrichthyes*, *gnathostomata*), an ancestral lymphocryptovirus might have been instrumental in inserting genes to encode the basic elements of adaptive immunity (*vide infra*).

2.4 Horizontal Gene Transfers in Archaea and Prokaryota

2.4.1 The Darwinian Threshold (Woese)

Many new viral genomes emerge from the sea (through metagenomics, *vide supra*), that encode proteins so far unknown in multicellular organisms ("viral hallmark genes") [303]. Thus, a "primordial virus world scenario" has been envisioned [304–306].

Some of the large dsDNA viruses of dinoflagellates replicate exclusively in the host cells' cytoplasm, where they create "virus factories" or "viroplasms" (*vide supra*). These events reflect back to the most ancient times at the origin of precellular and protocellular life, and to the forms in which predecessors of the protocells in the "virus world" existed ("Abiogenese der Virusarten. Eine weitere Möglichkeit der Entstehung der Virusarten ist, daß sie, aus leblosen Stoffen stammend, als erste Lebewesen auf der Erde erschienen") [180]. There, RNA viruses, then retroid elements (RNA → DNA), and then DNA viruses formed sequentially. A network of interacting nucleic acids segments existed before protocells were formed [303, 304]. Viruses pre-dating the origin of and not fitting into the tree of life of the cellular living formations [307], entered the first archaea, prokaryota and eukaryota cells from the outside. Or was it ancestral cellular genomes from which the first viral nucleic acid segments excised themselves ("Endogene Abstammung der Virusarten") [180]. Or was it intracellular bacterial symbionts, that through gene losses became what is now known as the *lymphogranuloma inguinale* virus, *molluscum contagiosum* virus, psittacosisvirus, trachomavirus ("die Abstammung der Viren von den Mikroben der Urzeit"), whose replication by fission of large "initial bodies" resembles that of pleuropneumonia bacteria, or L-forms ("in Elementarkörperchen zerfallende große Gebilde", "Plaquebildung", "Matrixmaterial"?) [180]. This is how the trachoma virus became *Chlamydia trachomatis.* As the large nucleocytoplasmic dsDNA viruses are replicating in "virus factories" or "viroplasms" exclusively in the cytoplasm (*vide supra*), they reactivate the idea that the first nuclei were formed in the large dsDNA virus-infected cells [308a,b, 309].

Protocells needed thymidylate synthetase enzymes (ThyA/ThyX) for the production of deoxythymidylate to build DNA. Rampant lateral transfers of these enzymes occurred between the three domains of ancient life and phages/viruses were enlisted as vectors [310]. It is well accepted and reviewed that fused archaea and prokaryota cells might have formed the first eukaryotic cells [311–313]. Ancient fusogenic viruses, like the ancestor of the extant mycoplasma phage MV-L3 (from *Acheloplasma laidlawiii*) might have mediated the first such unisons of *Crenarchaeota* and prokaryota spheroplasts [213, 314–316], since *Crenarchaeota* and eukaryota are evolutionarily related due to

similarities of their cell divisional machinery [317, 318]. This seminal experiment of Nature can be repeated in the laboratory today by fusing extant crenarchaeal and prokaryotic proto-spheroplasts with fusogenic mycoplasma viruses for the production of some primordial eukaryota-like cells [315, 316].

The rRNA studies of C. Woese aimed at the phylogenetic evolution of prokaryota lead to the discovery of archaea. Ribosomal proteins reflect backwards to the phylogenesis of the species. The universal ribosomal proteins functioned in coalesced protoplastic-spheroplastic cells prior to the separation of the phyla archaea and prokaryota. These cells exchanged large portions of their genomes by massive horizontal transfers. Speciation ("the origin of species") has taken its beginning when the first domain-specific ribosomal proteins appeared. At that point, the Darwinian threshold was established and the uncontrolled exchanges of genetic material became replaced by the rules and regulations of vertical inheritance. The 16S and 23S rRNAs established their sequence identity. Structural signatures of 16S and 23S rRNAs clearly distinguish archaea and prokaryota (bacteria), while the universal rRNA genes and proteins remain conserved and recognizable. The relative ordering of the universal r-protein genes within the rRNA gene cluster in archaea and prokaryota (bacteria) preserve their very extensive similarities, Hyperthermophilic archaea survive at 90°C. Archaea preserved their genomes by vertical inheritance, but were gene donors to prokaryota and eukaryota beneath the Darwinian threshold. The hyperthermophilic bacteria (*Aquifex aeolicus; T. maritima*) expropriated the archaeal genes of thermophily. The *Thermus thermophilus* megaplasmid is operational in *Deinococcus radiodurans*; if these two entities shared a common ancestor, it was through vertical inheritance, otherwise by horizontal gene transfer that they possess this megaplasmid. The archaeal stem diverged into the three lineages (*Crenarchaeota, Euryarchaeota, Nanoarchaea*) forming the first domain of life on the primordial Earth (prokaryota, second, and eukaryota, third). Euryarchaeota are methanogenic. The ribosomal superoperon consisting of some 50 cotranscribed and coregulated genes encoding ribosomal proteins is operational in both archaea and prokaryota. *N. equitans* lives as a parasite of the archaeon *Ignicoccus hospitalis* [319–323]. In the genus of Neisseria, widely dispersed genomic clusters identical with those of other Neisseria species and other bacterial genera, suggested to Maynard Smith that the excess of horizontal gene transfers obliterated speciation and that "there are no such entities as species in these pathogenic bacteria" [324, 325].

Horizontal gene exchanges between ancestral archaea and prokaryota were so pervasive that the early evolution of cells did not follow the vertical outbranchings of a Darwinian phylogenetic tree. Thereafter the vertical outbranchings of the Tree of Life gained dominance and continuing horizontal gene transfers failed to significantly alter the course of evolution. The central trend prevailed undisturbed by random horizontal gene transfers. At and after the level of the radiation of archaeal and prokaryotic phyla, the central trend representing vertical inheritance remained quite discernible [326].

There are examples of horizontally (laterally) occurred gene acquisitions for practically all bacterial functions (photosynthesis, aerobic respiration, nitrogen fixation, sulfate reduction, methylotrophy, isoprenoid biosynthesis, quorum sensing, flotation on gas bubbles, thermophily, and halophily) [327]. The EMBO Conference on Molecular Microbiology, Heidelberg, 2006, discussed noncoding regulatory RNA, RNases and gene expression, genomics, evolution and bacteriophages, signal transductions, protein interactions and networks, pathogenicity, virulence and endosymbiosis, chromosome dynamics, DNA uptake, and other subject matters. These presentations were published with extensive literature quotations [328]. Here is the background and the environment in which horizontally transferred genes are released and accepted. The PLoS Genetics review specializes on those horizontal gene transfers in prokaryotes that accelerated the evolution of the recipients [329] (*vide supra*). One example is the lack of lysine biogenesis pathway in "the last common ancestor of life" and the acquisition of this pathway by ancestors of *Crenarchaeota, Deinococcus-Thermus* and *Pyrococcus* occurred through horizontal gene transfer from prokaryota. The prokaryotic genomes evolved rapidly due to horizontally transferred genes of whatever means of transfer, other than vertical [323].

Reverse gyrase of the hyperthermophilic archaeon, *Sulfolobus acidocaldarius* (*vide supra*) is shared between the archaeal and the bacterial (prokaryota) ancestors of hyperthermophiles,

Thermotogales and *Aquificales*. These genes have been widely dispersed between archaea and bacteria through routes of horizontal transfers [322]. The *Thermotogales* genomes reflect well to events predating the Darwinian threshold. The composition of the genomes of *Thermotoga maritima* (Ttm) and *Aquifex aeolicus* (Aa) (*vide supra*) places them together at the base of the bacterial tree, in the era when the construction of a phylogenetic tree based on strictly vertical transmission of genes was invalidated by pervasive horizontal gene transfers. So far no prophages were found in the Ttm and Aa genomes. Some remnants of phage gene sequences in the thermotogales genome were discovered when sequences related to an *E. coli* phage showed up within two thermotogales genomes. *Thermotogales* bacteria were already armed with the antiviral related CRISPR elements (*vide supra*). Hyperthermophilic archaea (*Thermococcales* and *Pyrococcus furiosus*) horizontally exchanged their CRISPR elements [330]. In the absence of phages, horizontal gene transfers in thermotogales were not likely to be virally mediated. The genes securing life at high temperatures are shared with hyperthermophilic archaea. All thermotogales share closely related rRNA genes; rRNA analysis proves monophyly for Ttm and Aa. The operon for the membrane-associated proton-pump ferredoxin oxidoreductase is an archaeal feature; a derivative of it shows up in *Pyrococcus furiosus*. A derivative of the thermotogales ruBisCO gene, or rubisco-like proteins (*vide supra*) are operational in *Bacillus subtilis*. Derivatives of the thermotogales genes for the methionine salvage pathway are present in some deep sea bacteria. The first protocells might have been thermophilic and later life forms lost thermophily, even in some thermotogales with a change from ancestral to later 16S rRNA variations [331]. Thermotogales genomes show incongruent evolutionary history dating back to the pre-Darwinian threshold era.

The aminoacyl-tRNA synthetases secure the fidelity of protein synthesis as specified by the mRNAs. The aminoacetylated amino acids are attached to the 3′-ends of cognate tRNAs. The anticodons of the aminoacyl-tRNAs specifically react with the trinucleotide codons of the mRNA. The recognition of amino acids (aa) is quite specific. The anticodon GUC for aspartate is different from anticodon GUU for asparagine in the crenarchaeon *Sulfolobus* [332]. These ancient enzymes converted the RNA world into the protein world. These sequences of the aminoacyl-tRNA synthetases (aaRS) evolved by gene duplications, horizontal gene transfers and genetic recombinations. It appears that some aaRS genes readily crossed between Archaea, Eubacteria (Bacteria) and Eucarya (Eukaryota). Other aaRSs are individually analyzed and their phylogeny reconstructed. Duplications, fusions, recombinations and horizontally executed exchanges of the aaRS genes exhibit great diversity. While the majority of aaRS genes can be fitted into the phylogenetic pattern of vertical transmission within the three domains of life, some individual aaRS genes defy the rules of vertical transfers. For example, the yeast mitochondrial PheRS is related to that of *H. influenzae* and *Synecoccus* [333]. Crystallographic studies of the *Pyrococcus horikoshi* tyrosyl-tRNA (TyrRS) and tryptophanyl-tRNA synthetase (TrpRD) indicate that the original TyrRS was the ancestor of TrpRS in archaea, and that from archaea the TrpRS was horizontally transferred to bacteria [334].

A phototrophic filamentous microbial community existed in the Buck Reef Chert along what is now the South African coast 3.4 billion years ago. This population fixed CO_2 in the Calvin cycle and received electrons from atmospheric hydrogen [335]. These microorganisms were the ancestors of cyanobacteria already practicing anoxygenic photosynthesis. Their reaction center 1 (RS1) reduced nicotinamid adenine dinucleotide phosphorylated $(NAD(P)^+$ to NAD(P)H. In starvation these cells fixed nitrogen. Upon acquisition of photosystem II (PSII) and RS2 (able to oxidize water) over the pre-existing PSI and RS1, these procyanobacteria transgressed the Darwinian threshold and evolved into oxygenic photosynthesis conducting extant cyanobacteria. Cyanobacterial photosynthetic genes (gene clusters and gene product proteins, the core cyanobacterial clusters of orthologous groups of proteins, core CyOGs) spread either by horizontal transfers in the contemporaneous living world: into the green sulfur bacterium *Chlorobium tepidum*, the green nonsulfur bacterium *Chloroflexus aurantiacus*, the Gram-positive phototrophic bacterium firmicute *Heliobacillus mobilis*, and the purple α-proteobacteriun *Rhodopseudomonas palustris*. Endosymbiotic cyanobacteria in plastid-carrying

eukaryotes, the apicomplexans, the vestigial plastid-carriers (*Plasmodium falciparum*), diatoms (*Thalassiosira psudonana*), and algae (*Cyanidioschyzon merolae*), and from algae transferring eventually into plants occupying dry land (*Arabidopsis thaliana, Oryza sativa*, etc), propagated further the chlorophyll- and phycobilin-based photosynthetic genome. Protective chlorophyll-binding proteins dissipate excess light energy and thus secured the survival of cyanobacteria [336, 337].

Beneath the Darwinian threshold, and later transgressing it, pro-cyanobacteria and their phages coexisted. Phages form the bacterial "mobilomes." The oceanic prototroph, *Prochlorococcus,* carries myo-, podo- and siphoviruses (named after their morphology; for example the member of tailed phages, *Caudovirales, Siphoviridae* possess long noncontractile tails). The myo- and podoviruses propagated their structural genes to T4/T7 coliphages. In addition, these *Prochlorococcus* phages carry cyanobacterial photosynthesis genes. In contrast, the siphovirus lacks the cyanobacterial photosynthetic genes, but exhibits 14 other cyanobacterial homologue genes. Lytic cyanobacterial phages replicate in the bacterium and egress by bursting it. Temperate phages insert their DNA genome into the host bacterial chromosome and as prophages replicate with the host genome. Temperate phages carry out horizontal gene transfers, including those genes that encode pathogenicity islands and toxin genes (*vide infra*). However, most cyanobacterial isolates are devoid of prophages (and pathogenicity islands and toxins) [338, 339]. Cyanobacteria of the genus *Synecoccus* are infected by the phage, cyanomyovirus S-PM32. This phage shares some of its structural proteins with coliphage T4, its other structural proteins are unique and unrelated to other phages [340]. Phage homing endonucleases encoded by the endonuclease genes perform site-specific DNA cleavage. The cyanobacteriophage S-PM2 endonuclease is homologous to the resolvase of coliphage T4. Group I introns (inteins) can disrupt the DNA recognition site of the endonuclease. Otherwise the endonuclease makes a specific double strand cut of the DNA. The endonuclease can not cleave the introns-containing core photosynthesis reaction center gene (*psb*A). Mobile introns in the same target sequence that the endonuclease attacks, protects the host cell genome. Collaborative homing introns protected the photosynthetic genes [341].

Ancient bacteriophages are the ancestors of all viruses [342]. Some archaeal prophages (*Sulfolobus* turreted icosahedral virus in a crenarchaeal host; and others integrated into the tRNA of euryarchaeal hosts) are the ancestors of eukaryotic adenoviruses [343]. While temperate phages are accepted, even welcome for the useful genes they might insert, lytic phages are opposed for the life of the host. In this most ancestral virus-host relationship, the host genomes arranged the clustered regularly interspersed short palindromic repeats (CRISPR), the small RNA-guided defense system in archaea and prokaryota. The system prevails in eukaryotic genomes as well. Halophilic archaea defended themselves against lytic phages by a highly conserved CRISPR mechanism [344]. The first use of this defensive system must have occurred beneath the Darwinian threshold. In prokaryota the use of the system transgressed the threshold [345–348]. Bypassing vertical inheritance, the valuable gene clusters of the CRISPR system were acquired by horizontal transfers mediated by phages and plasmids [349]. Beyond the Darwinian threshold, prokaryotic genera widely accepted the horizontal transfer of, and applied the CRISPR defensive system against phages and plasmids, including the universal *cas* 1 gene. Same in eukaryotic cells, the system targets the nucleic acid of the invaders in a sequence-specific manner. In response, point-mutated viral genomic sequences escape recognition by the CRISPR system; a massive viral invasion of a cell could overwhelm the CRISPR system [350].

2.4.2 The Ancient Origin of "Virulence Genes"

Salmonella typhimurium and *E. coli* acquired multiple virulence genes through horizontal transfer mediated by phages and plasmids [351]. These gene product proteins enabled ancient bacteria to thrive under "adverse circumstances," The sites of residence for these genes are the "pathogenicity islands."

When the bacteria infect a host and thus encounters "adverse circumstances," it is the activation or acquisition of "pathogenicity islands" that secures the new life style of the invader. These complex gene cluster transfers appear to have developed after speciation occurred above the "Darwinian threshold." These gene clusters are transferred by conjugation or by phage transduction. However, intruding phages and plasmids encounter the CRISPR/CAS defensive system (*vide supra*). The CRISPR/CAS system can eliminate horizontal gene transfers conducted either by conjugation or phage transduction. While it protects bacteria from lytic phages, it may deprive them from the acceptance of genes beneficial to them in "adverse circumstances." A CRISPR system prohibits the acceptance of staphylococcal conjugative plasmids in clinical isolates of *Staphylococcus epidermidis* [352].

Most of the virulence genes of *E. coli* were acquired during the last 100 million years through horizontal transfers [353]. The uropathogenic *E. coli* acquires its pathogenicity island genes (fimbria, adhesins, LPS, toxins, hemolysins, siderophores) through horizontal transfer and in its biofilms and within bacteria-loaded epithelial cells it commonly overcomes both innate and adaptive immune reactions of the host [354, 355]. In the Netherlands, Enterobacter strains (*E. cloacae, E. hormaechei)* may be one of the sources of the pathogenicity island genes of uropathogenic *E. coli* strains [51] (*vide supra*). The high pathogenicity island (HPI) gene cluster may be transferred also to *Klebsiella pneumoniae* strains. Yersiniabactin (from *Y. pseudotuberculosis*) is such a HPI. The HPIs contain integrases, an iron uptake system (a catecholate siderophore) and an integrative and a conjugative element (ICE) with genes producing enzymes for excision and integration for DNA conjugative transfer. Some ICEs do, others do not contain helicase encoding DNA [51]. In Hungary, *Klebsiella pneumoniae* strains acquired plasmids up to 230 kb in size. The ciprofloxacin-resistant VTX-M-15-producing *K. pneumoniae* strains (VTX, from verocytotoxigenic *E. coli*) spread in an epidemic fashion in six nosocomial outbreaks [356]. In Galveston, TX, fluoroquinolin-resistant uropathogen *E. coli* emerged to infect renal transplant patients; these "virulent appearing" *E. coli* strains remained susceptible to 3rd generation cephalosporins [357]. The EMBO conference on prokaryotic genomic evolution and gene expressions [328], dealt with the noncoding regulatory RNAs of *E. coli*, one of which, a sRNA, suppresses the synthesis of toxic peptides [358]. Could this mechanism neutralize toxin production in a HPI?

Acquisition of HPIs is usually combined with antibiotic resistance. The genes for antibiotics production in streptomyces fungi, for antibiotics resistance and for pathogenicity island acquisition in bacteria are received through horizontal transfer by plasmids or by bacteriophages. *Enterococcus faecium* existed as an avirulent commersal until after it developed a new surface antigen Esp, and acquired a collagen adhesion gene and gene product protin, It has become a multidrug-resistant pathogen [359, 360]. Some strains of *E. faecium* received a large pathogenicity island containing the virulence genes *esp* (enterococcal surface protein), *hyl* (hyaluronidase), *acm* (collagen adherence) and genes for cytolysin and exotoxin. *E. faecium* and *E. faecalis* acquire these genes from "another common source." The CRISPR-CAS system (clustered regularly interspaced short palindromic repeats, C; C-associated) is non-functional in these enterococci. *Siphoviridae* bacteriophages enter the bacterial genome uninhibited [361]. Thus, these genes circulate now in colonies of streptomyces fungi and bacteria. Just when the original genes were generated in the ancient communities shared by the ancestors of prokaryotes and streptomyces, or after these taxa and genera diversified, remains to be resolved.

The mobile genetic elements (DNA transposons), plasmids and phages serve as vectors of the virulence genes of pathogenicity islands; repeated insertions may unite individually transferred genes into the whole of a pathogenicity island. The virulence gene-product proteins (*rpsl* genes, ribosomal protein subunits L) reside in the donors' ribosomes. Among others, *Streptococcus agalactiae* and *Serratia marcescens* possess such *rpsl* (*rpl*S) genes. At the dawn of combination chemotherapy, patients with acute leukemia succumbed to *Pseudomonas aeruginosa* septicemia in 8–24 h. The pseudomonas pathogenicity island (PAP) of 115 genes is first excised from the donor's chromosome. Then a 10 gene cluster (related to an enterobacterial plasmid) with the help of a prepillin peptidase conjugation system, transfers through a type IV pilus to another *P. aeruginosa* strain [362]. The antibiotics

resistance-encoding genes are transferred either by conjugation (STX, *vide supra*), or by means of horizontal transfer by plasmids or phages.

Conjugative plasmids transfer bacteriocins. Circular bacteriocins are linked at their N- and C-terminal ends. Could the *Enterococcus faecalis* circular bacteriocin, enterocin [363–365] be used to kill HPI-expressing enterococci (*vide supra*)? All these transfers, especially those of bacteriocins, show species-specificity. Thus, the genes encoding these systems (antibiotics production; antibiotics resistance; HPI acquisition; bacteriocin production) must have evolved above the Darwinian threshold, after speciation, when rigidly controlled vertical inheritance established itself[2].

2.4.3 A Selected Example of Speciation

Mycobacterium marinum is a close relative of *M. tuberculosis* in that they derive from a common ancestor. *M. marinum* possesses a 6,636,827-bp circular chromosome with 5,426 coding sequences of which 23 are nonribosomal peptide synthases and 18 are of unknown function and without orthologous genes in other mycobacteria. The early secreted exported antigenic targets (ESX1-6; SX-A/B; ESAT) ATP-dependent system is encoded from 29 *esx* genes; this gene number is reduced to 23 in *M. tuberculosis*. The ESX expressions relate to virulence, intercellular spread, induction of granuloma formation and to the ability to grow in vitro. The ESX proteins activate inflammasomes through induction of IL-1β and IL-18 secretion. The ESX-5 protein suppresses the production of IL-12, TNF-α, and IL-6. The *mel*2 locus (*mel* loci confer enhanced infection; *mrl* loci confer repressed infection) encodes proteins protecting *M. marinum* in infected macrophages from reactive oxygen and reactive nitrogen species (ROS; RNS). Bacterial cell wall lipooligosaccharides suppress TNFα production in infected macrophages. These data derive from *M. marinum*-infected mouse and human macrophages, not from infected fish [367–370]. *M. marinum* harbors the genomes of 10 prophages, and a 23-kb mercury-resistance plasmid. Some of the large repertoire of non-ribosomal peptid synthase genes of *M. marinum* were acquired horizontally. *M. marinum* and *M. tuberculosis* share 3,000 orthologs with amino acids identity of 85%. The large genome of *M. marinum* provides for its extensive host range and its ability to survive in its aquatic environment. The downsized genome of *M. tuberculosis* restricts its host range; it is adequate for its intracellular life style; the acceptance of horizontally acquired genes provides for its survival in an immunologically active host [371]. There are 695 coding sequences (CDS) present in *M. marium* and missing from *M. tuberculosis.* Of the remaining CDS,

[2]This author at the end of December, 1956, upon his introduction as a Rockefeller fellow to Professor Selman Waksman, the Nobel-laureate discoverer of streptomycin, and director of the Waksman Institute, at Rutgers, the State University of New Jersey, New Brunswick, N. J., was immediately asked to elaborate on the mechanisms how streptomyces fungi in the soil acquire the potency of antibiotic production. He answered that antibiotic production must have occurred as a response to challenge, as bacterial and fungal species competed for space ("niche") and nutrients. Dr. Waksman said: "but you failed to show this in an experiment you have published." This astonished fellow was speechless: "will the professor cancel my fellowship"? Dr. Waksman was referring to a paper published the year before in German (J. Sinkovics: "Untersuchungen über die Wechselwirkung nicht-antibiotischer Pilze und Bakterien." [366]), in which no antibiotics appeared in the fluids of common cultures of fungi and bacteria. It was amazing that he knew about it. Most generously Professor Waksman did not cancel the fellowship for this fellow's failure to generate antibiotics production at will. If asked the same question today by his students, this author would answer: "In my cultures there were no antibiotics-encoding gene donors for the fungi; these ancient genes are not generated anew, in extant fungi and bacteria they are acquired from donors by horizontal gene transfers mediated by mobile DNA elements (transposons), plasmids and phages. The same principles apply to antibiotic resistance genes and pathogenicity island genes. These latter genes serve bacteria to survive under 'adverse circumstances.' Amoeba, unicellular protists and some nematodes (Caenorhabditis) feed on bacteria and win. It is a great misfortune to advanced multicellular organisms that they present themselves to bacteria as 'adverse circumstances.' The bacteria must acquire and activate their pathogenicity island and antibiotic resistance genes to survive and thrive in these hosts. No offense meant." The students would be disappointed with this answer and would look up better answers in Internet/PubMed.

80% are orthologs between *M. marinum* and *M. tuberculosis*. Native *M. marinum*, the causative agent of fish tank granuloma in the human host, can be eliminated by healing and can be effectively treated with antibiotics (rifampin, ethambutol, clarythromycin and others). This author wonders if patients with healed fish tank granulomas acquire some immunity against *M. tuberculosis*?

The virulence operon Rv0986-8 of *M. tuberculosis* was transferred by a plasmid of gamma-proteobacterium derivation [372–374]. Laterally (horizontally) acquired genes in *M. tuberculosis* are in loci encoding sulfolipid metabolism, lipid glycosylation, adhesins, pilin development, fumare reductase synthesis in anaerobiosis, molibdopterin synthesis for nitrate respiration in reduced oxygen tension, such as within granulomas. *M. tuberculosis* engages 250 of its genes in fatty acid metabolism. Some of these genes (gene families) originated from actinobacteria [375]. The direct repeat region (DR) of *M. tuberculosis* contains 30-bp repetitive sequences and spacers with genes encoding proteins of unknown function. These sequences are absent in micobacteria (*M. smegmatis*; *M. avium*) that are philogenetically older than *M. tuberculosis*, therefore these segments were not vertically inherited, but horizontally acquired from an unknown source [376]. Thus, the DR including the CRISPR locus for providing resistance to bacteriophages, is another horizontally acquired operon. The original CRISPR in prokaryota were synthesized by the bacteria as modified phage genomic sequences placed as "spacers" in the bacterial genome [377].

The PE/PPE (PE = Pro-Glu; PPE = Pro-Pro-Glu) acidic glycine-rich subgroups of recombinant proteins (PGRS) are encoded by a family of genes to occupy positions on the bacterial surface. There, they induce host B cell- (the ORF Rv2430c derivative) and T cell- (the Rv2608 gene product) mediated immune reactions [378–380]. The PPE gene regions are hypervariable [381, 382], that is able to alter surface antigens under the pressure of the immunoreactive host.

The *M. bovis*-derived Bacille Calmette Guérin (Pasteur Institute BCG; BCG Denmark; BCG Tice & Glaxo USA) has been distributed around the world as far as to Russia and to the Orient (BCG Russia; BCG Beijing; BCG Tokyo). BCG Tokyo retained its full size of 4,371,711 bp containing 4,033 genes, of which 3,950 encode proteins. Retention *versus* losses of the original genes varies in the foreign passage lines of BCG strains. For example, BCG Tokyo/Japan retained its trehalose 6,6′dimycolate (TDM) production, and thus induces strong IL-12, IFN-γ, TNF-α response, whereas the BCG Connaught lost parts of its TDM production and therefore is a weak inducer of Th1-type immune reactivity. In India, weak cytokine-inducer BCG treatment of bladder cancer resulted in higher rates of relapses. Some BCG strains induce more the tolerizing cytokine IL-10, than the Th1-type cytokine IFN-γ [383–387]. A mutated narK2X promoter in its 110 region deprives *M. bovis* of the nitrate reductase enzyme. This enzyme in *M. tuberculosis* is vital for bacterial survival under hypoxic/anaerobic conditions, which prevail within granulomata [388], thus reducing BCG's pathogenicity. Horizontal gene implants (perfringolysin; MUC1 mucin and granulocyte-macrophage colony stimulating factor, GM-CSF) into the genome of BCG strains intensifies the vaccines immunogenicity [389, 390]. The multiple drug-resistant Beijing *M.* tuberculosis strain emerged as "an evolutionary response to BCG vaccination against, and antibiotic therapy for" tuberculosis [391]. Ancient records of paleopathology reveal that human tuberculosis predated that of domesticated animals (bovine tuberculosis) [392] Tuberculosis decimated mankind 35,000 years ago (or much longer).

The strictly intracellular parasite, *M. leprae* enters Schwann cells through α-dystroglycan-laminin cell surface complex. The same entry site is used by some arena viruses (Lassa virus, lymphocytic choriomeningitis virus). The Schwann cell invaded by *M. leprae* either dies in apoptosis, or proliferates. Cell proliferation is induced by the activated p56Lck (lymphoid cell kinase), a Rous sarcoma virus-related kinase (*src*) gene-product proteins. In surviving Schwann cells, NFκB translocates into the nucleus. These cells release solubilized receptors of TNF-α, but infected myelinating Schwann cell can not produce myelin. If myelin protein P0 (Pzero) is produced, *M. leprae* binds to it [393]. Activated macrophages surround the lesions and phagocytose, but fail to kill the bacteria. In Lucio's phenomenon, extensive vascular endothelial cell necrosis occurs, numerous skin ulcers develop and contain large foamy macrophages loaded with bacteria (*M. leprae*). Leprosy becomes a

chronic ailment in patients with Th2-type immune reactions (producing IL-10 and TGF-β). Patients with lepromatous leprosy generate regulatory T cells (T_{regs}) suppressing Th1-type immune reactions (reviewed in [213]). The intracellular NOD system (nucleotide-binding oligomerization domain) activates the first innate immune reaction in the host; single nucleotide polymorphisms render the NOD system deficient, increase the susceptibility upon exposure and direct the disease to advance into the multibacillary stage instead of the paucibacillary stage [394]. Lectins of *M. leprae* and *M. tuberculosis* activate in DCs the signaling pathway of proto-oncogene *raf*-1 (rat fibrosarcoma) and the Raf-1 protein acetylates NFκB subunit p65 after NFκB was already induced IL-10-mediated Th-2 type environment [395]. Thus, *M. leprae* activates proto-oncogenes (*src-p56lck, raf*-1) to induce host cell proliferation, or host immunosuppression, but without malignant transformation of the invaded cells.

Mycobacteria (*M. tuberculosis,* BCG, *M. leprae*) induce immune T cell- ($CD4^+$/$CD8^+$-) mediated reactions in the human host. The immune T cells are polyfunctional secreting more than one lymphokines/cytokines (IFN-γ, TNF-α, CD107a, macrophage inflammatory protein-1β). Patients with sarcoidosis produce similar immune lymphocytes responding to *M. tuberculosis* antigens ESAT-6 and katG (Elispot-associated antigens; catalase-peroxidase) [396, 397].

In contrast to the variability of BCG strains maintained in laboratories worldwide, the naturally dispersed strains of *M. leprae* originally penetrating Europe through the Silk Road from the Orient and remained stable. Brazilian, Indian, USA (Mexican) and Thailand strains of *M. leprae* share 99.995% sequence identity [398]. Single nucleotide polymorphisms were interpreted as resistance to gene mutations and to horizontal gene insertions. Variable number of tandem repeats (VNTR) distinguishes four genotypes of *M leprae* based on single nucleotide polymorphism (SNP) [399]. While *M. leprae* lacks the ability to grow in laboratory media, it can infect the nine-banded armadillos in the Southeastern USA from Texas to Florida [400]. In the laboratory *M. leprae* can be grown in the foot pads of nude rats. *M. leprae* DNA/RNA extracted from such tissues so far did not reveal the acquisition of host cell genes; instead, *M. leprae* eliminated many of its resident genes by silencing them, and converting them to nonprotein encoding pseudogenes. Patients with leprosy yield large numbers of bacteria from their nasal smears. The *M leprosy* genome contains 1,514 ORFs and 1,133 pseudogenes; the *M. tuberculosis* genome contains 278 pseudogenes [401, 402].

A new isolate from a patient with lepromatous leprosy significantly differs from *M. leprae;* it is a new species by detailed genetic analysis. *M. lepromatosis is* closely related to *M. leprae.* The divergence of *M. leprae* and *M. lepromatosis* is estimated to have occurred 10 million years ago. *M. lepromatosis* was being isolated and studied at M. D. Anderson Hospital, Houston, TX [403]. In this hospital, patients with leprosy usually coming from Mexico [404, 405] are periodically encountered and attended, either granulomatous diseases were mistakenly considered to be malignant tumors, or Lucio's phenomena erupted when a patient with a *bona fide* malignant tumor (a sarcoma) and latent leprosy received chemotherapy [406].

In the course of the mycobacterial speciation, the strictly intracellular parasites (*M. leprae; M. tuberculosis*) constrict their genomes rather than expanding them by horizontally acquired new genes. *M. ulcerans*, the causative agent of the devastating Buruli ulcer in Africa, is responding to rifampin and streptomycin therapy [407]. *M. ulcerans* diverged recently (in evolutionary terms) from the *M. marinum* lineage to become a "niche-adapted specialist;" as such, it is undergoing continuous genomic reduction [408]. By variable number tandem repeats (VNTR) typing. *M. ulcerans* strains diverged further into substrains [409]. *M. ulcerans* now exists in two distinct lineages. The ancestral lineage emerged and spread from South East Asia (China and Japan) to South America including Mexico. The classical lineage emerged and spread in South East Asia, Australia and Africa. Genome reduction is more advanced in the classical lineage [410]. In Australia, Aedes mosquitoes and other carnivorous insects (*Naucouris* sp.) harbor *M. ulcerans* bacteria [411–413].

A mycobacterium isolated from frogs (MU128F) produces mycolactone toxin slightly different from the toxin produced by human-pathogen *M. ulcerans* (MUAgy99) [414]. The *M. ulcerans* 5,632 kb chromosome contains 771 pseudogenes. The 174 kb virulence plasmid produces the

polyketide toxin mycolactone [415]. Mycobacteria producing the ulcerogenic and immunosuppressive mycolactone (MPMs) originally deriving from laterally moving virulence plasmids (from *M. marinum* to *M. ulcerans*), by now due to shared common ancestry, preserve the gene by vertical inheritance [416]. The plasmids, the 174 kb pMUM001 and the megaplasmid of the 190 kb pMUM002 of *M. ulcerans* contain three *mls* genes. Non-mycolactone-producer *M. marinum* strains accept artificially created shuttle vector-mediated transfer of these genes, which encode polyketide synthetases and the toxin [417, 418]. *M. marinum*-derivatives *M. ulcerans* strains producing mycolactones (MPMs) infect fish and frogs in marshlands of the USA, and in the Red and Mediterranean seas. It is highly possible that the ancestral *M. marinum* acquired the original mycolactone genes from one of its ancient hosts (an amphibian, a frog). The genes encoding the toxins reside in horizontally spreading plasmids [419].

Natural horizontal gene gains occurred in *M. avium paratuberculosis* from soil dwelling proteobacteria and actinobacteria. Three mycobacterial genes, one of them the sigma factor regulator, show sequence similarity with some eukaryota genes [420]. *M. abscessus* gained horizontally transferred genes (phospholipase C; ABC Fe^{3+} transporter) from actinobacteria (*Rhodococcus*) and streptomyces sp. Non-mycobacterial genes of *M. abscessus* were acquired from *Pseudomonas aeruginosa* and *Burkholderia cepacia* in co-infected patients with cystic fibrosis [421].

2.5 The Insertion of Adaptive Immunity Genes

2.5.1 Retrotransposons

The innate immune system is based on antiviral (iRNA, siRNA, CRISPR, *vide supra*) and antibacterial defensive mechanisms, to which protection against invasion by foreign cells was added (like the first NK cells in the *Botryllus*) [422, 423]. The system consists of Toll-like cell surface, and NOD-like (nucleotide-binding oligomerization domain) intracellular receptors; phagocytes, monocytes and macrophages, the ancestors of dendritic cells, residing in coeloma cavities, or circulating in the hemolymph. Chemokines and cytokines work with the Toll-like signaling receptors, while it is not clear just exactly when the first alpha-beta interferons (IFN-αβ) were produced. Humoral immunity was, and still is, practiced in the lamprey and hagfish by the generation of antigen-reactive leucine-rich repeats in the variable lymphocyte receptors. The variable lymphocyte receptors (VLR) are hypervariable and occupy the concave surface of the structure. The secreted hypervariable receptors appear in dimers, tetramers and pentamers to bind highly specifically the targeted antigens in the extracellular spaces [424–427]. In cyclostomata fish (hagfish and lamprey), the VLR gene-carrier lymphoid cells undergo clonal expansion in response to antigenic stimulation, and encode a great diversity of leucine-rich repeats (LRR), which react with different antigens. The LRRs are not immunoglobulins.

The second system of adaptive immunity emerged in ancestral sharks hundreds of million years ago. One of the basic doctrines of the adaptive immune system is the preservation of the faculties of the entire innate immune system and cooperation with them in a mutually dependent fashion. This principle manifests itself best in the fundamental functions of the innate dendritic cells and natural killer (NK) cells in the adaptive immune system. Inhibitory NK cell receptors recognize the self major histocompatibility complex (MHC) molecules and the NK cell remains silent. Cells with down-regulated MHC molecules (virally infected cells; malignantly transformed cells) are recognized by the killer receptors (KIR) of NK cells and are attacked. In mammals, inhibitory and killer NK cell receptors are encoded from different chromosomes. Human KIRs are encoded from the leukocyte receptor complex (LRC) of genes [428]. In the adaptive immune system, histocompatibility antigens are recognized; antigen-presenting cells educate T lymphocytes for the induction of Th1-, Th2-, Th17-type inner immune environment in the host. The most intricate specific antibody production

(immunoglobulins, Igs) by B (bursal) lymphocytes maturing into plasma cells is the exclusive property of the adaptive immune system. T lymphocytes release most of the interleukins and cytokines; B lymphocytes through processes of somatic hypermutations construct the antigen-specific light chains of the immunoglobulin molecules. See original research [429–431] and reviews [431, 213].

Ancestral elements of the adaptive immune system existed singly, the V (variable), J (joining) and C (constant) regions in the protochordates, and an ancestral complement and both RAG1,2 elements (recombination activating genes) in the amphioxus (*Branchiostoma floridae*) but certainly in the sea urchin (*Strongylocentrotus purpuratus*) [432–434]. The VJ elements are the distant precursors of the B- and T-Lymphocyte receptors. These dispersed elements found each other in the placoderm sharks and in their descendants and started to work for the first time in a strongly regulated unison (reviewed in [213]).

The genetic recombinations of the V(D)J (variable, diversity, joining) elements and the single constant (C) region lead to specific antibody production. The system is activated by the *rag*1 and *rag*2 → RAG1,2 gene product proteins and regulated by the 9 bp (nonamer), 7 bp (heptamer) RSS recombination signal sequences. Recombinations of 12 and 23 bp take place between the heptamer and nonamer subsequences of RSSs; spacers separate the heptamer and nonamer RSS subsequences. The V and J segments recombine into a light chain coding region. The rearranged VJ segment is transcribed into a mRNA. Splicing of RNA removes the introns and the native J region. The light chain protein is arrived at by translation of the mRNA [435, 436].

Before the appearance of jawed sharks (chondrichthyes, gnathostomata), no creatures of the sea possessed this system working in unison. The sea urchin (*Strongylocentrotus*), sea anemone (*Nematostella*), lancelet (*Branchiostoma*), the mollusks, sea slug (*Aplysia*) and the hydra (*Hydra*) all possess and operate *rag*1 genes, but not for V(D)J somatic hypermutation activation. However, in the sea urchin, *rag*1 and *rag*2 already co-exist with the zink finger domain in *rag*1 fully operational. Some innate immunological function is assigned to RAG1,2 in the sea urchin, inasmuch as these gene product proteins are expressed in coelemocytes, the sea urchin's defensive cells. Coelomocytes respond to LPSs. The responding coelomocyte receptors 185/333 are capable of nucleotide sequence variations as the 185/333 genes encode similar proteins, but with a high level of sequence diversity [437]. These are the first immunological diversity (D) responses so far recorded (but these proteins are not immunoglobulins). Sea urchin coelomocytes also express the vertebrate complement components B and C3; these opsonize targets for phagocytic engulfment. In contrast, all other elements of the adaptive immune system are absent in sea urchins: there are no lymphocytes, no immunoglobulins and no B or T cell receptors. Crystallographic studies show structure similarities between sea urchin and vertebrate RAG1, 2. These findings may be interpreted so, that the *rag*1, 2 sequences were not newly inserted into the ancestors of he sharks, they might have been acquired at the sea urchin level. However, evolutionary inheritance lines in one direction toward echinodermata and the other line toward cartilaginous fish (chondrichthyes) diverged 400 million years ago.

Insect (drosophila, anopheles) transposases (transib transposases) are also similar in structure to the Rag1 proteins [438]. Mobile DNA elements have inverted terminal repeats (TIRs) similar to RSS and encode DNA-reactive enzymes [439]. The transposon N-RAG-TP of the sea slug (mollusk) *Aplysia californica* encodes a protein with its N terminal part being similar to that of the vertebral RAG protein. Transposon N-RAG is distinct from the transposons transib of other invertebrate species. The other transposon, similar to *rag1* is transposon Chapaev [437]. Some bacterial integrases are related to *rag* gene product proteins. The inverted repeat structure and the left/right asymmetry of the RSS elements are like the end structures of terminal repeats of the insertion sequences in bacterial genomes, when mobile DNAs encode transposases. A *rag* gene passage from *Bacteroides* to *Porphyromonas* has just recently been recognized [440]. The recombination of RSS-flanked DNA sequences is brought about by the *rag* gene pair encoding recombinases. These recombinases excise the RSS-flanked DNA and catalyze its transposition in a "cut and paste" manner. The *rag*1 and *rag*2 genes have a tight genomic linkage. Probably they had to travel together. The structure of the *rag*2 gene differs from that

of the *rag*1 gene. The *rag*2 is a eukaryotic gene. RAG2 may be an activator of RAG1. Indeed, RAG2 switches the catalytic center of RAG1 into its active conformation [441, 442].

The somatic hypermutations create millions of new genes encoding the antigen-specific immunoglobulin light chain molecules, or the configurations of the T cell receptors. Since there is no straightforward vertical inheritance lineage recognized between the lower sea animal-carriers of the RAG/RSS elements and the sharks, unless their common deuterostome ancestor already possessed the *rag*1,2 genes, these events of specific immunoglobulin synthesis and T cell receptor conformation in the sharks and above suggest that retrotransposons inserted through a horizontal route the V(D)J/RAG/RSS elements in unison or sequentially into the genome of ancient sharks. The aplysia (*vide supra*) *rag*1 gene resembles the N-terminal part of the vertebrate *rag*1 gene. Therefore, the transposon this gene is associated with is a N-RAG-TP. It unites the transib and N-RAG-TP elements. It may be an ancient recombinant from which all *rag*1 genes derived. These elements might have traveled a long way from prokaryota to primitive and very complex eukaryota phyla. While the sea urchin RAG1,2 fail to work in the human V(D)J system, its RAG2 binds histone tails as the vertebrate RAG2 does. The distance shortens between the shark and human RAG1: the shark and human RAG1 aa identity is 65% and their similarity is 77% [443, 442].

Immunoglobulin M appears first in jawed cartilaginous fish. B cell receptors and immunoglobulin light (L) chain molecules were synthesized first in cartilaginous fish (chondrichthyes, gnathostomata) [444]. IgG developed from IgY that appeared first in amphibians (frogs). IgA appeared first in reptiles. The original Ig gene cluster in cartilaginous fish includes the single V, D, J and C genes. The heavy (H) chain class switch gene appeared first in amphibians and preserved its basic structure up to its mammalian gene. The αβ T cell receptors (TCR) recognize the peptides presented to them in class I or II major histocompatibility complex (MHC) molecules with restrictions. The β loci contain the D and J segments. The γδ T cell receptors are encoded from the γ gene for the J segments and from the δ gene for the two D segments. This T cell receptor interacts with free antigens without restriction. BCRs, TCRs and MHC appear first in cartilaginous fish. Some innate natural killer (NK) cell receptors are connected with some MHC genes and this connection is preserved up to mammals [445]. The whole genome duplications one and two of the vertebrate genomes occurred in the common ancestor of all vertebrates after the appearance of urochordates and before the out-branching radiations of the jawed vertebrates [446]. The amphioxus (*Branchiostoma floridae*) contains only one MHC-like region and no class I and II genes (the proto-MHC), whereas from the cartilaginous fish upward to mammals, MHCs are represented by large clusters of gene families [447, 448]. The predecessor NK cell receptor and leukocyte receptor genes were already represented in the proto-MHC. Endogenous intronic retroviruses persist within the gene clusters of human MHC class II, as remnants of prior proviral DNA insertions. In multigenic regions (as in MHC gene clusters) these retroviral DNA insertions promote the generation of diversity [449].

The evolution of the immune system in deuterostomes extends from echinoderms (sea urchins) through hemichordates (acorn worms), cephalochordates (amphioxus) and urochordates (sea squirts) to cyclostomata (hagfish and lamprey), where it culminates. Then, from the placoderms to cartilaginous fish (sharks), bony fish, amphibians, reptiles, the extinct dinosaurs, birds and mammals, not in a straight line, but in several divergences, another second adaptive immune system emerges. The first adaptive immune system culminated its course in cyclostomata fish (agnatha), but without B and T lymphocytes, no immunoglobulins and without antigen presentation in the grooves of MHC molecules. If genetically re-arranged antigen receptors are the first signs of adaptive immunity, these appear firmly installed in cyclostomata. If the sea urchin coelomocytes posses such a receptor (*vide supra*), then echinodermata exhibited the first sign of direction of development from innate toward adaptive immunity. *Echinodermata* appear to have possessed in their germ line the *rag*2 element to which through horizontal gene insertion the *rag*1 element joined. The donor of the *rag*1 element might have been a prokaryota, and its vector a transposon [450, 451]. Indeed, the inverted RSS repeats are like those of a transposon. From sea urchins to sharks, from fish to mammals, the span of evolution is

estimated to be close to 900 million years. The span from first cartilaginous fish to mammals is placed at approximately 500 million years. If the distance in time is some 3 billion years from prokaryotes to mammals, then the *rag*1 gene is a champion biological space traveler.

2.5.2 An Ancient Herpesvirus

The end product of the long evolutionary line of HHV-4, the Epstein-Barr virus (EBV), is an inducer of autoimmunity (suspect in systemic lupus erythematosus, SLE; in myelolytic encephalopathies represented by multiple sclerosis), and in lymphomagenesis (suspect in Reed-Sternberg cells of Hodgkin's disease; African Burkitt's lymphoma; B-lineage brain lymphomas in patients with acquired immunodeficiency syndrome, AIDS; NK cell lymphomas of the facial sinuses and mediastinum; in body cavity lymphomas with effusions). As to solid tumors, EBV is active in Chinese lymphoepitheliomas, co-infects with human papillomaviruses nasopharyngeal squamous cell carcinomas, detectable in Japanese stomach adenocarcinomas, and probably co-pathogenic in childhood leiomyosarcomas. In many pathological entities (SLE, multiple sclerosis, malignant lymphomas), EBV co-exists with reactivated latent endogenous retroviruses, or with the pathogenic retrolentivirus, human immunodeficiency virus-1 (HIV-1). Association of EBV with these pathological entities has been repeatedly reviewed, biochemically documented [452, 453] and illustrated [213]. One of the most significant biochemical documentations consists of the interactions of EBV gene product proteins BZLF-1 with crucial pro- and anti-apoptotic cellular elements, p53 and NFκB (and the common, but by now diverged evolutionary lineage of p53 and NFκB is also well documented) [454–458].

Recombinases of diverse derivation are Mg^{2+}-dependent enzymes expressing a magnesium ion-binding site, DDE (D, aspartic acid; E glutamic acid). The enzymes with DDE sites are the transposases, retroviral integrases, innate antiviral-response enzymes RNase H and RNA-induced silencing complexes (RISC), and the RAG recombinases. It was pointed out that paradoxically the pathogenesis of HIV-1 depends on its DDE enzymes (which can be inhibited therapeutically), whereas mutations or inhibitions of the *rag* gene-product proteins, RAG, result is immunodeficiencies and severe illnesses (Omenn syndrome) [459].

Dreyfus found similarities of the structures and functions of the DDE proteins and the EBV DNA-binding protein (DBP), the product of the BALF-2 gene. Thus EBV BALF-2 gene product proteins could interact with the V(D)J recombination process [460] The presence of a *rag*-1-like sequence in EBV genome may be the remnant of an ancient acquisition. A scenario can be envisioned in which the ancestor deuterostomes at an early stage of the line that led to sea urchins, acquired a *rag*1-like sequence from prokaryotes and carried that gene in their germ lines inserted next to their genuine eukaryotic *rag*2 gene. That RAG1/RAG2 complex did not interact with the V(D)J gene cluster, which is non-existent in the echinodermata genomes. As sea urchins and placoderms coexisted in the post-cambrian sea, a herpesvirus infecting the echinoderm sea urchins might have excised the *rag*1 gene from its host's genome and incorporated it into its own viral genome. Host gene acquisition by herpes (and other) viruses has been and remains a common practice referred to as gene drain, usurping and expropriating host cell genes. The deuterostome lines leading to echinoderms (sea urchins) and sharks (placoderms, carcharine sharks; gnathostomata chondrichthyes) diverged into these two directions hundreds of million years earlier, but preserved the germline *rag*2 gene. The herpesvirus, a probable ancestor of extant EBV and carrier of the *rag*1 gene, infected the placoderms and/or carcharine sharks and inserted into their germ line the *rag*1 sequences. There the *rag*1*rag*2 genes encoded their gene product proteins the RAG1/RAG2 recombinase-mediating enzymes interacting with the V(D)J and RSS complexes. Were the V(D)J RSS complexes arriving into the same hosts from other sources (as transposons)? The G/C content of the termini (terminal repeats, TR) of the EBV genome is enriched up to 70%, like the V(D)J RSS A/T rich nonamer region. One of the EBV TR nonamer sequences

is adjacent 5′ to a sequence with V(D)J RSS similarity. In this aspect EBV termini resemble transposon termini. The intracellular herpesviral genomes are either circular in latent episomal position, or replicative linear form inserted in the host cell's genome. The transition of the herpesviral genome from circular to linear form is initiated by transcription factor BZLF-1 gene product protein. In EBV's replication cycle sequences resembling V(D)J RSS are produced [460].

Adjacent to EBV's DNA-binding protein, the product of EBV gene BALF-2, lie response elements AP-1 and SP-1 (activating protein-1; specificity protein). AP-1 is involved in cell proliferation, differentiation and migration and it interacts with the *jun* proto-oncogene (*vide supra*) [461]. The *jun* proto-oncogene disallows cell survival in the autophagic state under distress; it induces apoptotic death of autophagic cells [462]. The SP transcription factors regulate those genes that encode neoangiogenesis factors, and invasiveness of malignantly transformed cells. When the SP factors active in tumor cells (ovarian carcinoma; pancreatic carcinoma) are targeted with antibiotics (mithramycin and its derivatives) or with chemotherapeuticals, overexpressed SP-1 becomes downregulated with cessation of tumor growth resulting [463, 464]. Both AP-1 and SP-1 interact with the RAG proteins, probably indirectly by elevating cyclic AMP. The binding sites of the EBV BZLF-1 protein are similar to those of AP1; these binding sites are located within 2 kb of the BALF-2 ORF. EBV infection of T lymphocytes results in a robust stimulation of RAG activity. The structures of the RAG1 and EBV's DBP show similarity in their N-terminal regulatory domain and in their C-terminal DNA-binding domain. Both proteins display Mg^{2+}-dependent DDE residues (*vide supra*). Both proteins express a zink finger in similar regions. This suggests that these two proteins descended from a common ancestor proto-RAG recombinase, imitating a transib insertion (but for proof more primary sequence similarity would be required). Dreyfus concludes that insertions of DDE recombinase of ancient herpesviral origin occurred adjacent to primordial *rag*2 genes in ancestors of sea urchins or in ancient sharks and the remnants of this RAG1 protein are still expressed in the BALF-2 protein and its promoter in extant EBV particles [460].

What could be the value of *rag* sequences in the genome for a herpesvirus? Since structural and functional similarity is evident between the RAG and DDE/RNase H family nucleases and the dimethyl arginine argonaute protein component of RNA-induced silencing complex (RISC), such sequences may serve within the defensive mechanisms of the virus within its host cell. Both RAG1 and RISC enzymes utilize Mg^{2+} ions at their DDE site, as another herpesviral DNA-binding protein, infected cell protein-8 (ICP-8). Argonaute and RISC are RNA-silencing elements; argonaute was operational in archaea (*Thermus thermophilus*). The argonaute gene family members underwent extensive horizontal transfers from *Aquifex aeolicus* through archaea and eukaryota [465]. The targets of RISC and argonaute are double-stranded mRNAs. Both EBV and HHV-8 (Kaposi sarcoma-associated herpesvirus) produce large numbers of micro-RNAs (miRNA). The targets of viral miRNAs and interfering RNAs (iRNA; RNAi) are the ds mRNAs translating host cell proteins that are encoded for anti-viral defense. Viral miRNAs form complexes with RISC and argonaute in order to effectively attack targeted ds mRNAs. The small regulatory RNAs (sRNA) are small interfering siRNAs, miRNAs, and piRNAs (pi, piwi, P-element-induced wimpy testis discovered in infertile males of drosophila and mouse colonies). PIWI-RNAs protect the germline genome by eliminating alien retrotransposons of invasive intent [466–471].

Dreyfus writes: "Obviously, it will not be possible to revisit the origins of the acquired immune system, except through empirically testable hypotheses" [458]. Maybe it is possible to simulate the ancient events. Can an experiment be designed in which a herpesvirus devoid of the *rag*1 sequence infects sea urchin caelomocytes to see if it can pick up the sea urchin *rag*1 sequence? If the herpesvirus becomes a carrier of the *rag*1 sequence and it infects deuterostome-derived cells, preferably cells from a primitive cartilaginous fish in lack of *rag*1 sequences (but carrying germline *rag*2 sequences), can the herpesvirus insert its *rag*1 sequence into the genome of the fish cell, and if so, next to the germline *rag*2 sequence [213]?

In its May 1, 2009 issue volume 324, pages 580–581 *Science* published a science writer's assessment "On the origin of the immune system." Of the letters the editors received in reply to this article, they published only one [472], and well deservedly so. It was D. H. Dreyfus' letter entitled "Immune system: success owed to a virus?" One of the other letters submitted to *Science*, but not published, is printed here[3].

2.6 Horizontal Gene Gains in Eukaryota

2.6.1 Viral Genes

2.6.1.1 Bornavirus in Human Brain Cells

In Borna, Germany, a strange neurological disease ("die Bornasche Krankheit im Bereich Borna/Leipzig, der Kreisstadt Borna in Sachsen, die bereits seit über 200 Jahren bekannt") of horses was observed over 200 years ago ("Gehirn und Rückenmarkentzündung der Bornaschen Krankheit beim Pferd"). In 1885 in Saxony, Germany, next to the township Borna, the German cavalry lost many horses to a disease of unknown causation ("als Verursacher einer tödlichen Hirnerkrankung bei Pferden"). Intranuclear eosinophilic Joest-Degen inclusion bodies in brain cells of horses afflicted with the disease [473], lymphocytic infiltrates of involved brain tissue, and transferability of the disease with cell free extracts to rabbits, rats and mice strongly suggested a viral causative agent for Borna disease. The causative agent was replicated in tissue cultures including human cells. The Borna disease virus (BDV) was identified as a ssRNA virus consisting of 8,910 bp; it is a unique member of the *Mononegavirales* group, which possess an RNA genome with a sequence opposite to a mRNA. The viral nucleoprotein gene encodes a viral structural protein for the packaging of the viral RNA genome in the nucleocapsid. BDV is not explicitly cytopathic as infected cell may survive, but the immune reaction thus elicited may kill infected cells ("BDV verursacht die Bornasche Krankheit, eine virus-induzierte, immunvermittelte Entzündungsreaktion des zentralen Nervensystems"). In addition to horses, the pathogenicity of BDV is quite broad, both by experimental transfer of the disease to rabbits, rats, mice (rat brain virus transferred by intracerebral injection into newborn mice) [474], tree shrews, cats, rhesus monkeys, and by its natural infections through unknown routes to sheep, birds

[3]THE FABULOUS TOPIC OF JOHN TRAVIS' ESSAY continues to excite biologists, geneticists, immunologists, retro- and herpesviral virologists and even oncologists. It was in the belly of the chondrichthyes gnathostomata sharks, where the elements of the adaptive immune system, previously existing in primordial forms dispersed in the amphioxus, ascidian tunicates (the Botryllus), mollusks, anemones and sea urchins, united to work together for the first time. The system withstood the challenge of placentation in mammals, but fails to promptly control epidemics. The malignant cell masquerading as "self" manages to recruit both the innate and the adaptive immune faculties of its host for its own advantage. The innate natural killer (NK), and the adaptive immune T cells can recognize and kill cancer cells. However, the subverted cancer-bearing host mobilizes $CD4^+CD25^+FoxP3^+$ regulatory T cells and induces an armada of innate chemokines to efficiently antagonize NK and immune T cells within the tumor. In a desperate effort, clones of interferon-γ-producer $ICOS^{hi}$-expressor (inducible costimulator) $CD4^+$ T cells rise under the effect of CTLA4 blockade (cytotoxic lymphocyte antigen) to react to cancer antigens and to outnumber the regulatory T cells. Another overstimulated TGFβ-producer and $ILEI^+$ (interleukin-like ETM-inducer) $CD8^+$ T cell clones emerge to initiate the process of ETM (epithelial to mesenchymal transition) in parenchymal stem cells, and in enlisting the ras proto-oncogenes, promote the malignant transformation of these misled stem cells. These T cells commit high treason against their host. This author proposes that these clones be referred to as those of traitor/transforming T (T/T T) cells. However, ras-transformed tumor cells can not produce interferons. Thus, these transformed stem cells succumb to infection with oncolytic viruses. The purpose of this brief note is to direct the attention to a recent lavishly illustrated and referenced monograph and to articles elaborating on these issues in great detail. The title of the last article is "Horizontal gene transfers and cell fusions in microbiology, immunology and oncology." Joseph Sinkovics.

(from psittacine birds to ostriches) and to human patients ("vom Tier auf den Menschen übertragbare Erkrankung"). The first proposal that BDV infects human beings and that it causes neuro-psychiatric disorders was entirely unacceptable to learned retrovirologists of the highest rank. The laboratory documents that anti-BDV antibodies and antibody-virus antigen immune complexes have been identified in the blood of patients with neuro-psychiatric disorders ("dass Menschen Antikörper haben können") were rejected as experimentally erroneous and thus unacceptable (unconfirmable). The authors of such publications were silenced (ordered to refrain from such publications and lectures) by executive orders from the institute director. However, the laboratory documentation of antibody production in human patients with neuro-psychiatric disorders was repeatedly confirmed by independent investigators and on occasions BDV isolates from human patients were reported. The German Institute of Laboratory Medicine held a conference in Berlin in January 2008 on BDV infections in animals and in human patients and published the material of the entire conference in the Acta Pathologica Microbiologica et Immunologica Scandinavica in 2008 [473]. In this volume, B. Norrild, the author of the Introduction, and H. Ludwig, the author of the Epilog refer to the authoritativeness of the institute director declaring that such dictatorial orders are peremptory to the freedom of medical research (cited in [473]). The author (L. Bode), who was ordered to refrain from presenting data from her laboratory and clinics concerning the human pathogenicity of BDV, spoke at the congress under the protection of a disclaimer: "this article reflects the author's but not the institutions opinion" (cited in [473]). This valuable volume discusses the biology of bornavirus (H. Ludwig, pp. 14–20), the neuropathology and pathogenesis of bornavirus diseases (G. Gosztonyi, pp. 53–57); other authors' presentations concern the worldwide distribution of bornavirus diseases from Scandinavia to Australia and Japan, and very extensively the human pathogenicity of the disease in children and adults (cited in [473]).

Even in the era when inadequate laboratory tests could not equivocally prove the validity of "Koch's postulates" as to the etiological role of BDV in human neuro-psychological diseases, the freedom of publication of new ideas prevailed in several editorial offices [475–485].

The classical publications on persistent infection by Borna virus in the central nervous system, including the human brain, are those of Bode, Gosztonyi and Ludwig [484, 486–488]. Borna virus-exposure, as documented by antiviral antibodies is the serum, and its connection with psychiatric (cyclic affective) disorders was first claimed by Rott et al. in 1985 [475].

It has now been firmly established that the non-segmented, negative sense ssRNA BDV establishes a persistent infection in brain cells preferring for its host cells those residing in the limbic systems of the brain. In mammalian brain cell nuclei even without active viral replication, sequences of the viral nucleoprotein (N) gene may persist. These are endogenous borna-like N elements (EBLN) inscribed in the germ line genome of the cells. Some EBLN elements display ORFs and their mRNAs; thus, the sequences may encode proteins of unknown physiological function in their hosts. The human EBLNs express ORFs suggestive of protein encoding capacity. Could this activity be psychogenic-pathopsychogenic in the human brain? Several ancient insertional events for EBLNs can be detected in the genomes of mammalian cells beginning in primates, dating back to pre-human hosts (40 million years ago), and in squirrels with more recent (10 million years ago) acquisition. Some EBLNs lost sequences and exist as inert pseudogenes. The in vivo events of natural insertion of EBLN elements into the genome of brain cell nuclei could be duplicated in vitro in tissue cultures of human cells infected with BDV [489]. These authors state: "Our results provide the first evidence for endogenization of non-retroviral virus-derived elements in mammalian genomes…" [489]. However, the genome of the exogenous negative sense ssRNA virus, the lymphocytic choriomeningitis virus of the Arenavirus class, performed an illegitimate recombination event with the genome of the endogenous intracisternal A-type retrotransposon, which reverse-transcribed the entire recombined genome of both viruses into a cDNA and integrated it into the host cell's genome [490]. Thus, most anything can happen between viruses co-infecting a host cell. The BDV intragenomic sequences are retropositioned probably by LINEs (long interspersed nucleotide element); LINEs are mobile DNA elements (their RNA retrotranscribed into DNA) that copy themselves and excise and reinsert themselves in

host cell genomes. Promiscuous LINEs can act upon non-self LINE templates [491]. and being especially hyperactive in the human brain [492], possibly can retrotranscribe BDV RNA into a DNA strand [493]. Further, non-retroviral RNA viruses, the dsRNA totiviridae, are able to horizontally transfer and integrate their genomes into fungi (candida, penicillium, uromyces) [494] (*vide infra*).

2.6.1.2 Human Herpesvirus-6 in Human Telomers

The phylogenetic comparison of herpesviral genomes show overlap between alpha (equine HV-1, human HSV-1, 2, varicella-zoster VZV, Aujeszky HV) and beta (human CMV, HHV-6) herpesviruses, while gammaherpesviruses (HHV-4 EBV, marmoset HV saimiri, bovine HV-4) are diverse, but without overlap with the αβ-classes. HHV-6 is an ancient human herpesvirus; its genome is the closest to the human progenitor herpesviruses [495]. HHV-6AB are closely related to human CMV and HHV-7 [496]. The overall nucleotide sequence identity of HHV-6A and HHV-6B is 90%. HHV-6AB, especially HHV-6A, are opportunistic pathogens in immunocompromised patients. While HHV-6B causes exanthem subitum (*Roseolavirus*) in children, HHV-6AB association with Stevens-Johnson's syndrome or with Langerhans cell histiocytosis [497, 498] does not prove etiological role of these viruses in these pathological entities. HHV-6AB act as co-factors in lymphomagenesis (including Hodgkin's disease) and in the etiology of multiple sclerosis. Indeed, HHV-6 co-infects CD4 T lymphocytes and promotes HIV-1 replication in AIDS [499]. A HHV-6 genomic sequence encodes a 490 aa polypeptide REP protein), which is homologous to the human adeno-associated virus type-2 (AAV-2) *rep* (replication) gene product protein. This gene in HHV-6 probably was horizontally acquired in a eukaryotic host cell co-infected by both HHV-6 and AAV-2. In such an association, HHV-6 mediates the replication of the helper virus-dependent parvovirus AAV-2 [500, 501]. A 1,473 bp genomic sequence of HHV-6A encodes the transformation suppressor protein (ts → TS). The TS displays 24% identity and 51% similarity to the Rep protein of AAV-2. Both HHV-6A TS and AAV-2 REP suppressed Harvey-*ras* gene-induced transformation of NIH 3T3 cells, but not when *ras*-mutated Finkel murine osteosarcoma retrovirus induced sarcomagenesis. When tested against HIV-1 LTR promoter, REP and TS inhibited it, but the retrolentiviral transactivator response (TAR) element reversed the inhibition [502].

The first proof for the integration of HHV-6 genomic sequences into the genome of a human cell was provided in Japan by the hematology team of Professor Isao Miyoshi[4]. By FISH technique and PCR, the HHV-6 genome was shown to be integrated in the long arm of chromosome 22 (22q13) of an EBV^- Burkitt's lymphoma cell line. The integrated viral genome could be activated to replicate by phorbol acetate and calcium ionophore [503, 504].

That genomic segments of HHV-6 integrate into human chromosomes other than chromosome 22, the chromosome 17 (17q13.3), was immediately confirmed [505]. Transmission of integrated HHV-6 genomic segments from parent to child by vertical inheritance was reported [506]. The chromosomally integrated and vertically transmitted HHV-6 genome is present in every cell of the body. In a most peculiar way, the HHV-6 genomic segments integrate most frequently within the telomere region of the targeted chromosome. These ribonucleoprotein (reverse transcriptase) holoenzymes maintain the telomere lengths of chromosomes. HHV-6 integration sites were 9q34.3, 10q26.3, 11p15.5, 17p13.3 and 19q13.4. Integration actually within chromosome was best documented for the 9q34.3 site [507].

[4]Resident fellows Dr. Isao Miyoshi and this author served together in 1959 at the Department of Medicine of the University of Texas M. D. Anderson Hospital, Houston, TX [213]. This author had the privilege to personally meet and know Dr. Dennis Burkitt in Kenya and Uganda, Africa, in 1966. He succeeded in visiting with Professor Isao Miyoshi on the occasion of the 9th International Cancer Congress held in Tokyo in 1966.

In sequencing the HHV-6A genomic integration sites in patients with families of integrated and inherited HHV-6, at chromosomal sites 17p13.3, 18q23, and 22q13.3, the telomeric repeats TTAGGG were found to be the integration loci. In chromosomes with integrated HHV-6A genomic sequences, no circular episomal viral genomes were found and the integrated viral genomes could be chemically activated to replicate [508]. Patients harboring integrated HHV-6 genomic segments become immunosuppressed, when latent viral genomes switch to replicative state. HHV-6 not only attacks lymphocytes representing adaptive immunity, it also blocks signaling from TLRs. In HHV-6-infected DCs, LPS-stimulated TLR4 do not generate immune reactivity [509].

Telomerases are regulated by transcription factors acting on their promoters. Herpesviruses express attractions to telomeres. The HHV-8/KSHV evolved in Africa from the rhesus retroperitoneal fibromatosis herpesvirus (RFHV) to be a human pathogen, Both RFHV and KSHV use identical strategies to parasitize their target cells, to induce their proliferation without killing them, and to reduce the expression of their MHC surface antigens to render them invisible to host T cells [510]. In Kaposi sarcoma cells, it is the latency-associated nuclear antigen of the HHV-8 Kaposi sarcoma-associated herpesvirus (KSHV) that transactivates the telomerase promoter [511]. The product protein of ORF12 of HHV-8 is kaposin, the KSHV's transforming "oncogene-oncoprotein," the unique property of HHV-8/KSHV [291]. HHV-8 is not known to integrate into its host cell genome in its tumor, the Kaposi's sarcoma, which is a "breeding ground of herpesviridae." This tumor harbors in lymphocytes infiltrating it HHV-4 (EBV) and HHV-6, and in the tumor cells CMV, and HHV-8 and an activated endogenous retrovirus (Fig. 2.3a,b) [213, 512].

2.6.2 Horizontal Gene Transfers in Eukaryota

The first eukaryotes constructed their genomes by adding to their vertically preserved gene repertoire genes horizontally transferred from cells with which the "last eukaryotic common ancestor" (LECA) fused. The first chimeric eukaryotes built their nucleus, cytoskeleton and locomotion (cilia) from horizontally acquiesced donated genes [513–516]. However, it might not have been acquiescence from the part of LECA in tacitly complying with the invasion of its genome by exogenous genes. It might have been a voracious engulfment of alien genes and genomes for the achievement of superiority over other cells in competition for niche and nutrients. LECA was a "conscious cell" with microtubules that were evolving into neurotubules [517, 518]. Beneath the Darwinian threshold (*vide supra*) widespread distributions and exchanges of archaeal and prokaryotic/eubacterial genes occurred admixed with vertical inheritance. The repeated lateral transfers of the archaeal reverse gyrase into thermophilic bacteria (*Thermotogales; Aquificales*) by plasmids and transposases [519] exemplifies the promiscuous life style of the early proto-spheroplasts. The era of speciation above the Darwinian threshold favored the events of organelle acquisition by endosymbiosis. Excised and re-inserted "genomic islands" carrying virulence and antibiotic resistance gene clusters traveled between related species [520].

The photosynthetic protist, *Euglena gracilis*, acquired its transketolase nuclear genes and other genes from multiple endosymbiotic events, most prominent being the one with cyanobacterial plastids [521–523]. The eukaryotic organelles, plastids, derived from cyanobacteria through endosymbiosis, as protists captured and engulfed green and red algae. The early eukaryota experienced primary, secondary and tertiary endosymbiotic events resulting in the horizontal movements of the plastids. Most of the plastid genes ended up in the nuclei of the host cells [524, 525]. The apicoplasts, the remnants of engulfed red algae, remain either as relics, or as functional chloroplast-like organelles in some apicomlexan unicellular eukaryotic parasites (*Eimeria, Plasmodia, Theileria, Toxoplasma* sp.). Ciliates diverged into dinoflagellates and apicomplexan parasites. Plasmodia at one time possessed both chloroplasts and mitochondria, both much reduced in size and function in the extant host. In *P. falciparum* proteins encoded in the nucleus (endoplasmic reticulum-associated protein degrading

enzymes) communicate with the apicoplast [526–528]. Both plasmodia and toxoplasma express special nuclear genes (*pfprex*) to encode proteins (DNA helicase, polymerase and primase) with affinity to the apicoplast. In response, the apicoplast contributes encoded proteins to host cell metabolism (the pyruvate dehydrogenase complex). The plasmodium apicoplast gene *tuf*A encodes a translation elongation factor (EF-Tu), which, however, renders the red cell-parasite host susceptible to the chemotherapeutic drug thiostrepton [529–531]. The presence of horizontally transferred genes of mitochondrial origin within apicoplasts strongly suggests that these two organelles of the same host cell co-operate in the interest of their host [532].

First, parasitic chlamydiae gained genes of important enzymes from free-living actinobacteria [533–535]. Then, the transferosomes of chlamydiae transferred genes through the routes of "endosymbiotic gene transfer" to their unicellular eukaryotic hosts [536]. This paper gives a spectacular view of the details of this ancient association. Among the laterally acquired gene winners are human parasites (entameba, trichomonas) and the free-living amoeba, *Dictyostelium* [537–539]. Some horizontally transferred genes travel via transposons, "the most abundant, most ubiquitous genes in nature" [540]. Marine invertebrate crustaceans yield some of the most ancient mariner-like elements (transposons); for example the Bytmar1 transposon in the hydrothermal crab *Bythograea* [541]. Some transposons find their way to the nucleus with the help of (attachment to) DNA-binding proteins [542]; some are transferred by viruses or plasmids (by phages in bacteria and by their descendants, all the eukaryotic viruses). Cyanophages (cyanomyovirus related to T4 coliphage) operate with 64 genes. Of these, there is a highly conserved hyperplastic region containing inserted genes of host cell-like sequences encoding enzymes (plastoquinol, plastocyanin, 6-phosphogluconate and glucose 6-phosphate dehydrogenase) [543]. The *Rhodothermus marinus* RM378 phage encodes a polynucleotide kinase with some sequence similarities to the same enzyme of coliphage T4. These enzymes work to counter the anti-phage miRNA defense of the bacterial hosts [544]. The T4 coliphage (phi1) encapsidates small circular host cell DNAs for horizontal transfer to new host cells [545]. Even rickettsiae release plasmids to communicate with their host cell, ranging from those of arthropod vectors to that of mammalian hosts [546].

Genes and gene product proteins now recognized as proto-oncogenes and oncoproteins appeared first to perform physiological functions, that they preserved (reviewed in [213]). The origin of the Ras family of widely multifunctional proteins (Kirsten and Harvey rat sarcoma oncogenes, *ras,* in multicellular eukaryotic hosts) could be traced back to prokaryotes. The prokaryotic MgIA proteins of eubacteria, and one archaea (after *E. coli* methylgalactoside transport operon) appear as analogues of the Ras proteins showing the five characteristic motifs of their guanosine GDP/GTP-binding pattern [547].

Genes not part of the hosts' vertically maintained ancestry are those of prolyl-tRNA and alanyl-tRNA in diplomonads and parabasalia. The origin of these genes could be traced back to the hyperthermophile *Nanoarchaeum equitans.* It is unsettled if there is a common archaeal ancestor for *Nanoarchaea* (diverging from *Crenarchaeota* and *Euryarchaeota*) and the diplomonads and parabasalia, the first independent eukaryota to appear after the divergence of the archaeal lineages [538]. Protochordate ascidian larvae utilize cellulose synthetases (CesA) of prokaryotic origin for the formation of the long cellulose fibrils along the larval tail. In CesA knockout larvae, the notochord cells were misaligned and the tail failed to elongate. The CesA gene was inserted from a prokaryotic donor into the early lineage of the tunicates. Extracellular cellulose microfibrils started the morphogenesis of the notochord and tail in the larvae of *Oikopleura dioica* [548].

The yeast cells *Candida parapsilosis* and *C. tropicalis* use the CTG codon to translate leucine, as if it were serine. These yeast cells might have acquired this anomaly from an ancient proteobacterium. *C. parapsilosis* lost its phenazine superfamily (PhzF) gene cluster, but re-acquired it through horizontal transfer from proteobacteria [549]. Bacterial genes encoding arsenite reductase, catalase, racemases and peptidoglycan metabolism enzymes show up in fungi [550].

Horizontal exchange of genes between plants and fungi occurs in both directions [551]. Fungal pathogens of plants include the basidiomycetes *Moniliophthora perniciosa* attacking cacao plants causing the "witches' broom" and "frosty pod rot" diseases. These fungal species horizontally acquired genes from oomycetes (the necrosis inducing proteins), from actinobacteria (the metallo-dependent hydrolase) and from firmicutes/bacteriodetes (the mannitol phosphate dehydrogenase) rendering them pathogenic [552]. Interspecies horizontal gene transfers render saprophytic fungi pathogenetic [553]. In the genome of *Aspergillus fumigatus* segments containing 214 alien genes were detected. These genes were of bacterial (40%), fungal (25%) and viral (22%) origin [554]. A tobacco plant plastid inserted its DNA into the soil of Acinetobacter sp. The inserted sequence consisted of a leucyl-tRNA encoded by tobacco gene *trnL*, and an anchor sequence of an antibiotic-resistance (to spectinomycin and streptomycin) gene cluster [555]. Angiosperms resist horizontal gene insertions from fungi, but in rice plant (*Oryza sativa*) genomes five fungi-to-plant and 4 plant-to-fungi horizontal gene transfers are documented. Such gene exchanges are very rare (14 in 3,177 gene families examined), but may result in phenotypic changes of the recipients [551]. Plant pathogenic *Fusarium* spp. possess pathogenicity-related chromosomes and by horizontal transfer render non-pathogenic fungal strains pathogenic [556].

Flowering plants may increase their beauty by the acquisition of horizontally transferred mitochondrial genes encoding ribosomal and respiratory proteins [557]. In rice, maize and sorghum, a LTR-retrotransposon (Route66) transfers horizontally genomic sequences [558]. However, from transgenic rice, the trehalose phosphate synthase and phosphatase genes were not (as yet) transferred to soil microorganisms in paddy rice fields [559].

Crown gall disease and hairy root disease are caused by the phytopathogenic bacteria *Agrobacterium tumefaciens* and *A. rhizogenes* [560]. Interkingdom horizontal ssDNA transfers between plants are mediated by agrobacteria. The T-DNA of plant-transforming *A. tumefaciens* travels to the targeted plant cell nucleus via the ssDNA-binding VirE2 protein; the phosphorylated VirE2 interacting protein VIP1 accomplishes the nuclear targeting of the agrobacterial T-DNA [561]. Plasmids and their vectors (*A. tumefaciens*) for expression of heterologous genes in transgenic plants by horizontal transfer are commercially available [562–565]. The alpha- and beta-proteobacteria, *Rhizobia*, form nodules on the roots of legumes, within which they enter plant cells to fix atmospheric nitrogen. Symbiotic plasmids of rhizobia horizontally transferred to the pathogenic *Ralstonia solanacearum* converted its pathogenicity to mutualism (symbiosis). It was the inactivation of the *hrc*V structural gene that allowed nodulation and the inactivation of the *hrp*G master virulence regulator gene product protein (HrpG) and hypersensitive response and pathogenicity *(hrc = hrp* conserved). This process allowed the intracellular entry of the bacteria, which then accomplished nitrogen fixation, thus modulating the transformation from pathogenicity to symbiosis [566]. Extensive horizontal exchange of genetic material occurs between plant cells in the process of grafting [567].

The plant-parasitic "root-knot nematode" *Meloidogyne* sp. acquired genes (L-threonine aldolase; glutamine synthetase; N-acetyltransferase) from sympatric rhizobia, *A. tumefaciens* (also called *Rhizobium radiobacter*) and *R. leguminosarum* [568].

Some diplomonad protists do not have mitochondria, but very likely, after transferring mitochondrial genes from a symbiont proteobacterium, got rid of the original structure. Two anaerobic intestinal parasites, one in the salmon (*Spironucleus salmonicida),* one in the human gut (*Giardia lamblia, G. intestinalis, G. duodenalis*) diligently collected (84 of them) horizontally transferred genes. Most of the horizontally transferred genes originated from prokaryotes, but some of them were recognized as of eukaryotic derivation. A glucose-6-phosphate isomerase (G6PI) gene was donated by the cyanobacterium Nostoc [569, 570]. The common ancestor of these diplomonads was aerobic; the acquired genes helped the development of anaerobiosis in the intestinal tracts. Cystein-rich surface proteins serve as virulence factors; nineteen lineage-specific gene acquisitions distinguish the two lines of the diplomonads, as to their host selection [539].

Bacteria acquired alpha2-macroglobulins for colonization factors by horizontal transfers from metazoa. The *yfh*M/*pbpCV* tightly linked genes (named in *E. coli*) encode α2-macroglobulins and peptidoglycan transglycosylase (for abbreviations of *yfh*M/P76578 and *yfa*S/P76464, and further explanations as to donor metazoan: toby.gibson@embl.de [571].

The non-coding RNA-1 for heat shock response (HSR1), the stimulation of heat shock factor-1 (HSF1) in eukaryota (mammalians) derives from bacteria. The mammalian HSR1 consists of 604 nt; there is only 4 nt difference between hamster and human HSR1. However, the amino terminal regions of the bacterial chloride channel proteins (in *Burkholderiales*) and the ORF of HSR1 are close to identical. This suggests the horizontal acquisition of the original eukaryotic HSR1 sequence [572].

The sponge species *Reniera*, the sea anemone species *Nematostella*, the amoeba species *Dictyostelium discoides* possess very similar α-amylases. Does this mean horizontal gene transfers between these eukarya? [573].

The small spore-forming obligately intracellular pathogenic eukaryota microsporidium, *Encephalitozoon cuniculi,* lacks protein kinases (MAP kinase cascades, AMP-activated protein kinase, stress-response, ion homeostasis, nutrient signaling protein kinases) in comparison to those in *Saccharomyces* yeasts. *E. cuniculi* and the yeasts shared a common ancestor that lived 800 million years ago. The *E. cuniculi* genome lost its old ancestral yeast meiosis kinases, but retained core cell machinery kinases (Aurora, Polo, etc); its kinome consists of only 32 protein kinases [574].

E. cuniculi depends on its host cell's metabolism, yet it is not a symbiont, but a lethal pathogen. Tandem repeat DNA regions in different isolates of *E. cuniculi* indicate that sex by recombination occurs between *E. cuniculi* individuals. Further, *E. cuniculi* managed to receive genes by horizontal transfer from co-intracellular resident Chlamydia. The zygomycete *Rhizopus oryzae* shares germ line genes with *E. cuniculi*. These two microsporidia might have shared a common ancestor [575].

The eukaryotic phylum, Apicomplexa, includes among others plasmodia, toxoplasma, and cryptosporidia (*vide supra*). The intracellular endosymbiont and pathogen, *C. parvum,* is the recipient of horizontally transferred genes. Deprived of its plastid (apicoplast), it depends on genes coming from other apicomplexan parasites, or from bacteria. The gene for leucine aminopeptidase from cyanobacteria is present in the genomes of cryptosporidia, plasmodia and toxoplasma. Other enzyme-encoding genes derive from proteobacteria. Description of the mode of acquisition does not mention viral transfer. However, algal or cyanobacterial endosymbionts of cryptosporidia are possible sources. There is no explanation for a leucine aminopeptidase plant-like gene in the cryptosporidium (and plasmodium). The "plethora of prokaryotic genes" might have derived from the mitochondria, but their acquisition time is much more recent, than that of the acquisition of mitochondria [569]. The *Entamoeba histolytica* genome encodes an endonuclease that was transferred from a bacterial source (EhLINE1) [576]. In reverse, the intra-amoebal *Legionella drancourtii (vide supra)* acquired its sterol reductase gene from its eukaryotic host [577]. Prokaryotic enzyme-coding gene donations show up in *E. histolytica* and *Trichomonas vaginalis* [578].

The sea slug, *Elysia chlorotica,* feeds on the alga *Vaucheria litorea*. The algal cells are digested, but the algal plastid (chloroplast) remains intact and continues its photosynthetic activity in the sea slug. There may be a virus involved in the incident by transferring to the sea slug the algal gene(s) that encode(s) photosystem complex substances needed to keep the chloroplast functional [579].

Prominent intracellular bacteria are *Anaplasma, Ehrlichia* and *Wolbachia.* Wolbachia are alpha proteobacteria. Wolbachia A infects drosophila, WO-B infects insects other than drosophila, including mosquitos, and WO-D infects nematodes. The horizontal transfers range from less than 500 bp to the entire wolbachia genome (>1 Mb) and involve 4 insect and 4 nematode species as recipients [580]. The α-proteobacterium, in the order of Rickettsiales, *Wolbachia pipientis*, interferes with reproduction, kills male insects, or feminizes them. Wolbachia genomes express up to 1,386 coding sequences [581]. The one-way crossing incompatibility between infected males and uninfected females is the event of "cytoplasmic incompatibility". The male testicles are infected, but the sperm cells do not carry live wolbachia; female egg cells are infected with wolbachia. The infected paternal

chromosomes are lost, while the uninfected female chromosomes segregate properly yielding haploid male progeny. When an infected male fertilizes an infected egg cell, and the infecting wolbachia strains are identical, embryonic development proceeds unimpeded. Some Wolbachia sp. infects filarial nematodes. The wolbachia genes transferred into the insect beetle several million years ago, were found now to be disrupted, rendered transcriptionally inactive and turned into pseudogenes [582]. At least some of the wolbachia genes transferred into the mosquito *Aedes* sp. remained functional after an extended period of their horizontal transfer. In mosquitoes, the transferred wolbachia genes encoded receptors for malaria plasmodia in the insects' salivary glands. These wolbachia genes appear to have been originally acquired by the wolbachia genome from another insect host through lateral (horizontal) transfer [583]. The mosquito gene AAE-L004181 shows 50% aa identity with two wPip genes WP1348 and WP1346. Which one is the gene donor and to whom? Wolbachia-to-host transfer was proven [584]. The outer membrane proteins of wolbachia cells are inducers of innate immune reactions in insects, and are also apoptosis-inducers [585]. In drosophila sp., horizontally acquired transposons/retrotransposons abound, but not of wolbachia derivation. Insertins of LTR retrotransposons are the most frequent (90%), whereas non-LTR retroelements seldom (6%) succeed at their self-insertion [586, 587]. Almost the entire wolbachia genome is inserted in the bacteriome of the bedbug (*Cimex lectularius*). The wolbachia genome is vertically transmitted within the oocytes; bugs deprived of the wolbachia genome become infertile [588].

The wolbachia genomes inserted into the genome of insect hosts carry their prophages. Wolbachia phage WO-A is a pyocyaneus-like element. Wolbachia phage WO-B matures into particles, which spread horizontally between different strains of wolbachia within their insect hosts. The WO-B phage expresses genes that influence the biology of the insect host (virulence function gene; sex-specific expression genes). Wolbachia genomes are in the insects' sperm cysts and in the egg cells. The bacteriophage WO-B in the maternally inherited wolbachia genome is most active (replicative, temperate) in the larval stage of the insect hosts. Lytic phage activity may kill (lyse) the endoparasitic wolbachia [584, 589–592]. Whatever happened between wolbachia and the arthropod hosts millions of years ago, it is the scenery of a past mortal combat between host and parasite what is replayed today (as the Hubble telescope shows what happened in the universe milliards of years ago).

In another relationship, the arthropod Antarctic springtail (*Cryptopygus antarcticus*) acquired from bacteria its endo-β-1,3-glucanase gene [593]. The parasite of legume hosts, the pea aphid (*Acyrthosiphon pisum*) appears to have acquired a functional gene from a wolbachia; the gene product protein serves the aphid's endosymbiont, the *Buchnera aphidicola* [594]. The red-green colored carotenoids are encoded in pea aphids by genes of fungal derivation; after integration of these alien genes, the aphid genome duplicated them [595]. The 464 Mb genome of the pea aphid *A. pisum* shows extensive gene duplications, gene losses and new gene acquisitions. The expanded genes are set for chromatin modification, miRNA synthesis and sugar transport. Lost genes are those of the urea cycle and purin salvage and selenoprotein utilization, and some of the innate immune system, may be to be able to accommodate the bacterial endosymbiont *Buchnera aphidicola* (a gamma proteobacterium), which the aphid maternally transmits to its progeny. With this endosymbiont, the aphid shares aa synthetic purine metabolic pathways. The aphid possesses the aa synthetic and degrading genes and shares the products with the endosymbiont. In turn, the endosymbiont provides the purin metabolic pathway (purine nucleoside phosphorylase, adenosine desaminase: adenosine to inosine) for the salvage of purine nucleotides, except for guanosine, which is rendered by the aphid [596–598]. At least 12 genes in the aphid's genome are of bacterial origin (named in the article), but most of these genes are not of Buchnera, but are of wolbachia origin [594]. Buchnera and Hamiltonella are facultative symbionts to aphids; this type of endosymbiosis protects the aphids against pathogenic fungi, parasitoid wasps (*vide infra*) and heat strokes [599].

Female wasps use ichnoviruses to immunosuppress the caterpillars so, that in the caterpillars' body the wasp eggs may hatch and their larvae may develop. The caterpillars' immune reaction to the

injected foreign bodies (eggs) would consist of activated Toll-like receptors and phenolooxidase production. The ichnoviruses derive from ascoviruses (polyDNAviruses); descendants of iridoviruses. Ascoviruses injected into caterpillars would kill these hosts, but the ichnoviruses are attenuated and non-pathogenic. Ichnoviruses are restricted to replicate only in the female wasp's calyx cells. Ichnoviral genomes exist in the form of multiple circular DNA molecules. Ichnoviral particles assemble in the female wasps' genital tract. In the caterpillar host, the ichnoviruses do not replicate. The ichnoviral genes are not for encoding new viral particle structural proteins; the ichnoviral genome in the caterpillar encodes a number of proteins, which derive from wasp's genes inserted in the ichnoviral genome. In the female wasp's genome, most ascoviral genes were eliminated from the ichnoviral genome. The only one strain of ascovirus, which is able to replicate both in the wasp and in the caterpillar hosts is the DpAV4 (from *Diadromus pulchellus*). The DpAV4 is more of a symbiotic, than of a pathogenic virus. Symbiotic virus-host relationships promote reciprocal passive lateral transfers of genes between viral and host cell genomes. The viral genome undergoes recombination-primed replication in an environment, where an abundance of DNA sequences float freely. In the amoeba, the large dsDNA viruses (NCLDV, *vide supra*) chose this mode of genome replication. There, bacterial genomes are released from the microorganisms, which amoebae and their unicellular symbiotic algae feed on. The recombination-primed genomic replication promotes the integration of very short DNA segments (12 bp) with sequence homology to the amoebic or viral genomes. In these intracellular environments, passive lateral transfers of DNA segments to and from viral, host cell and bacterial chromosomes readily occur. In contrast, active lateral gene transfers show elements of dedication to conclude the selected process.

Wasps' genomes carry integrated sequences of ascoviral and ichnoviral genomes. In the female wasp's genome, the ascovirus loses its virulence genes and acquires somatic genes from its host. The non-replicating a-virulent ichnovirus encodes the wasp's proteins within its new host, the caterpillar. The wasp's proteins suppress the innate immune reactions of the caterpillar and render this host tolerant toward wasp proteins. The exact mechanisms of this symbiogenesis are not known. Endosymbiogenesis repeatedly occurred in the earliest stages of evolution, when proteobacteria became mitochondria and cyanobacteria became chloroplasts in the cells that engulfed them. The endosymbionts became domesticated servants (or slaves) of their host cells, as the cell nucleus expropriated the symbionts' genes one by one [600]. Female wasps domesticated the ascovirus: the servant's name is ichnovirus.

In conclusion for a most complex issue. Horizontal gene transfers are not limited to the sub-Darwinian threshold era, only the intensity of the events declines. Neither is the process confined to the transfer of one single gene. Insect genomes readily accommodate full rickettsial size genomes. The eukaryotic microalga, *E. huxleyi* (*vide supra*), harbors a large dsDNA virus, the EhV. The alga donated its entire sphingolipid/ceramide biosynthetic gene apparatus (seven genes) to its viral guest [158]. The biflagellated photoautotrophic protist, *Cyanophora paradoxa*, and *Euglena gracilis* (*vide supra*) acquired their transketolases from endosymbiotic green algae [522]. The cryptic viruses of beets and carrots are close to be identical with fungal partitiviruses. Naturally transmitted between related plants by seeds and pollens, the transmission of the entire genomic packages of these viruses, and whatever additional plant host cell genes the viral genomes might have picked up, to phylogenetically distant hosts occurs by horizontal transfers mediated by fungi [601]. Rice, maize, sorghum cells accept horizontally transferred genes by the 280,000 year old *gag-pol* retrotransposons called "Route66." Route66 was discovered in the genome of Japanese rice plants (*Oryza sativa*) in two copies residing on chromosomes 2 (nt 1 767 933 – 1772 818) and chromosome 6 (nt 26 706265 – 25 701 456) [602]. Pea aphids readily accept bacterial, genes (but not from their symbiont *Buchnera aphidicola,* it is from bacteria other than *Buchnera*) [603]. Three billion years after transgressing the Darwinian threshold: "are lateral gene transfers between prokaryotes and multicellular eukaryotes ongoing and significant?" [603]. Yes, they are, and very much so.

2.7 Epithelial-to-Mesenchymal Transition of Human Cancer Cells

2.7.1 Bacteria Inducing Inflammatory Cancers

2.7.1.1 Helicobacter Pylori: Cancers of the Soil and Seed

Bacteria acquire and transfer their virulence factors (toxins, adhesins, invasins, etc) through transposons, plasmids and bacteriophages (*vide supra*). Bacterial virulence factor genes are clustered in "pathogenicity islands" PI, Pais) (*vide supra*). Bacteria may delete, or amplify, or duplicate their pathogenicity island genes [604]. Gram-negative bacteria harbor distantly related PIs (*E. coli, Salmonella, Shigella, Vibrio cholerae Helicobacter pylori*). The toxin-coregulated type IV pilus (TcpA) may be encoded by cooperating phages CTXφ and VPIφ. The PI contains the phage genome that encodes the toxin-coregulated pilus (TCP). TCP functions as a colonization factor and as the receptor for phage CTXφ [63a,b] (*vide supra*). The toxin-coregulated type IV pilus (TCPA) expresses the coat protein of the VPIφ phage [63a,b, 605]. *Helicobacter pylori* (Hp) strains seldom succumb to phages; probably effectively eliminate them. The first isolation and photographic depiction of a lytic *H. pylori* phage (HP1) was certainly a sensational occasion. It is worthwhile to look up the original publications to view the electron microscopic depiction of the Hp phages [606, 607]. Genetically modified non-replicating phages lyse Hp, yet clinical phage therapy has not materialized [608]. In the mouse, the lytic filamentous phage M13 prevents the colonization of gastric mucosa by Hp [609]. Some stain of Hp release "rolling-circle replicating" highly promiscuous plasmids [610].

This author reviewed the human pathogenicity of *H. pylori* in the context of "the host confronting pathogens attacking lymphoid tissues" [213]. This bacterium accompanied mankind through its evolution, at least in the last stages of it: it was present 5,000 years ago in the stomachs of the ancient Egyptians. In its extraordinary variability, genomic segments of *H. pylori* strains readily recombine and exchange genes through horizontal transfer [611]. Major subpopulations of Hp are those of hpEurope, hpAfrica (subdivided to hpSAfrica and hpWAfrica), and hpEAsia. The subpopulation hpAmerind parasitizes native Americans. The genomes of the Amerindian (Venezuela) Hp and hpEurope were compared and revealed characteristics of the human Hp strains following the migration of the human race out of Africa, the population of Europe by *H. sapiens* (no information on *Homo neanderthalensis*), but the material from excavated graves would be available), and the movements of human populations through Asia and through the Bering passage-way to the Americas. Even human and murine Hp strains could be compared in an evolutionary scale [612]. The core genome of Hp substrains operates with 1,111 genes, but the gene number may increase to 1,531. The *cag* PI gene clusters may be acquired or deleted. Each Hp strain produces by recombinations highly individualized variants in each of their hosts. In cases of multiple subspecies *H. pylori* infections, interspecies horizontal gene transfers occur [613]. There are conserved and mobile regions in the Hp genome. Gene replacements and horizontal gene transfers directed the evolutionary changes within Hp strains [614]. Practically all infected persons harbor their own individual Hp strain [615].

The pathogenicity of *H. pylori* extends from the epithelial cell linings of the stomach to the reactive lymphocytes infiltrating it, as if it were carcinogenesis in the soil (the epithelium) and seed (the lymphocytes). The cytotoxin-associated gene product protein *cagA* → CagA is phosphorylated by a Src kinase. CagA inhibits E-cadherin and β-catenin complex formation resulting in the transfer of β-catenin from the cytoplasm into nucleus. The cells in which these events take place undergo the precancerous process of "intestinal metaplasia", with goblet cell mucin production in the gastric epithelial cells. When *H. pylori* acquires metazoan α2-macroglobulin genes through horizontal gene transfer, it uses the gene product proteins to suppress anti-bacterial immune reactions of the host [616]. Allelic diversity and extreme genetic variability of *H. pylori* creates individually modified substrains of the bacterium in their hosts [615].

In a "chronic inflammation-induced cancer" scenario, cell nuclear DNA suffers oxidative damage [617]. In these cells, K-ras mutations occur (Kirsten rat sarcoma oncogene). In response, there is first a polyclonal lymphoid cell proliferation. Monoclonally proliferating lymphoid cells emerge overexpressing the anti-apoptotic *bcl-2* gene cluster and undergoing the translocation t(14;18)(q32;q21) [618–620]. While Hp eliminates innate immune reactions by killing macrophages (*vide infra*), it disrupts adaptive immune reactions by generating $CD4^+CD25^+FoxP^+$ regulatory T cells for the elimination of immunoreactive immune T cells ($CD8^+$ T cells) [621].

The c-*myc* proto-oncogene is activated in the nucleus of Hp-infected gastric mucosal cells by ERK (extracellular signal-regulated kinase) and the c-Myc proto-oncogene (avian myelogenous leukemia oncoprotein) is phosphorylated in the cytoplasm. The chain reaction continues with the phosphorylation of c-Fos (Finkel mouse osteosarcoma oncoprotein). The c-Jun proto-oncoprotein (*vide supra*) joins in, resulting in the activation of a specific activator protein, AP-1. These proteins form cytoplasmic complexes, for example, the phosphorylated c-Fos/c-Jun complex binds the c-Myc promoter. The c-Fos/c-Jun complex translocates into he nucleus for further gene activations and gene silencing. The end result is polyamine gene activations with the production of ornithine decarboxylase (ODC), an apoptosis-inducer in macrophages [622]. The macrophages to be killed were lured on the site first by monocyte chemoattractant protein production (MCP-1) by the gastric mucosa [623]. When *H. pylori's* CagA protein translocates into lymphocytes, it assumes the role of an oncoprotein (*vide infra*) (Table 2.1).

The Hp *cag* PI by epigenetic mechanisms hypermethylates CpG (cytosine-post-guanine) islands in the genome of gastric mucosal cells resulting in translocation of NFκB from cytoplasm to nucleus for inflammatory reaction gene activation, AP-1 generation, activation of the PI3K (phosphatidyl inositol 3 kinase) pathway and proto-oncogenic, epithelial-to-mesenchymal transformation-inducing Wnt/β-catenin signaling [624, 625]. Pro-oncogenic and anti-oncogenic but not yet clearly identified microRNAs vie for superiorityin the gastric mucosal cells [626].

In the Spanish coastal town Ubrique the rate of Hp infection is high (54%) with Hp-antibody production shown in blood tests; these tests also showed high incidence (81%) of mutated p53 protein and ceruloplasmin levels in the blood of Hp-seropositive individuals. The mortality rate for gastric cancer exceeded twofold (20/100,000) that of communities with low Hp-related parameters [627]. Helicobacter DNA is present in pancreatic cancer tissue and helicobacter bacteria (but not other gastrointestinal bacteria) appear in hepatocellular carcinoma tissue samples [628]. Pyrosequencing and PCR reveal Hp Dna in liver tissue of patients with chronic cholestatic liver diseases and in gallstones [629a,b, 630a,b]. Since the observation of cytoplasmic vacuolization in gastric mucosal cells by Hp toxin to the documentation of oncogene mutations in these cells only 13 years went by [631–633]. In some patients with idiopathic thrombocyopenic purpura (ITP) and Hp antigens in their stool samples, antibodies to the Hp anticytotoxin-associated gene A product protein were found. After Hp eradication, the platelet levels returned to normal [634]. The association of Hp infection with certain types of ITP occurs world-wide [635–638].

The eradication of *H. pylori* is entirely possible with a combined antibiotics regimen (amoxicillin, clarythromycin or azithromycin, and metronidazole given with an omeprazole-like proton pump inhibitor) [639, 640]. Metronidazole- and tetracycline-resistant Hp stains are emerging in Iran [641].

2.7.1.2 Bacteroides Fragilis: Inflammatory Carcinogenesis with or Without It

This author recently reviewed work initiated at Johns Hopkins Hospital in Baltimore, for the Weekly Hungarian Medical Journal (Orvosi Hetilap) concerning the contribution of enterotoxigenic strains of *Bacteroides fragilis* to colon carcinogenesis [642a,b]. A comparison was suggested between the carcinogenic effect of *H. pylori* in the stomach and that of the enterotoxigenic *B. fragilis* in the colon.

Table 2.1 Helicobacter-induced gastric lymphoma and bacteroides enterotoxin-induced colonic adenocarcinoma

Gastric mucosa-associated lymphoid tissue lymphoma (MALT) in *Helicobacter*-infected stomachs	
Clinical course: Even antibiotic therapy refractory low grade MALTs seldom advance into high grade diffuse large B-cell lymphoma (DLBCL)	[730]
Complete genomic sequences of lymphomagenic *H. pylori* strains	[731, 732]
Chromosomal translocation t(11;18)(q21;q21) results in the formation of API12-MALT fusion oncoprotein. Oncoprotein releases cytoplasmic NFκB and activates Bcl10 nuclear gene	
MALT immunophenotype: $CD19^+CD20^+CD21^+CD79a^+CD5^-CD10^-CD23^-$	
Micro miRNA profile: Aberrant DNA methylation of p16/INK4a gene	
Methylated CpG islands:	[733, 734]
Translocations: t(1;14)(p22;q32) BCL10-IGH oncoprotein; t(14;18)(q32;q21) IGH-MALT1 oncoprotein. Oncoproteins activate NFκB, Toll-like receptors TLR2 & TLR6, chemokine receptor CCR2, clusters of differentiation CD69 & Bcl2. In translocation-negative lymphoma: IL-8 production, CD28, CD86 expression, ICOS (inducible T-cell costimulator) activation	[735]
Dysregulated NFκB pathway	[736]
Helicobacter virulence gene/gene-product protein CagA: CagA translocates (is horizontally transferred) into gastric epithelial cells and into B lymphocytes. Recipient cells phosphorylate (activate) CagA protein. CagA activates ERK and Bcl-2/Bcl-XL anti-apoptotic proteins. CagA is an oncoprotein	[737a,b]
Helicobacter-reactive host immune T cells: Are armed with FasL (ligand) and perforin cross-react in an autoimmune fashion with host cell ATPase autoantigens, kill mucosal cells causing atrophy of gastric mucosa. Immune T cells mobilized against MALT lymphoma B cells are defective in FasL and perforin expression and fail to eradicate B lymphoma cells. In a murine model of *Helicobacter*-induced gastric lymphoma, $CD4^+CD25^+FoxP3^+$ Treg cells were attracted into the tumor by chemokines CCL17/CCL22. The regulatory T cells eliminated immune T cells and promoted tumor growth	[738, 739]
Eradication of H. pylori: Antibiotics sensitive. Treatment results in lymphoma remission	[640, 740]
Commensal bacteria (*Bacteroides vulgatus, Fusobacterium varium*)	
Commensal bacteria adhere to, and enter the cytoplasms of colonic epithelial cells. In response, the cells produce IL-6, IL-8, TNF-α, macrophage/monocyte chemoattractant protein-1. The intranuclear NFκB p65 is phosphorylated (activated). These reactions occur in UC cells in vitro and in vivo, as illustrated	[741]
Bacteroides fragilis enterotoxin activates the REL protein (*vide supra*) heterodimer, NFκB. In response, chemokine (CCL2, chemoattractant for monocytes; CXCL1, growth-related oncogene-α (GRO-α); CXCL8/IL-8) gene overexpression induce neutrophil transmigration	[742]
Comments: Not addressed in this article is the strong possibility that bacteroides enterotoxin-stimulated colonic epithelial cells express FasL, with which they kill Fas receptor-positive host immune T cells. It is well documented that FasL-expressing tumor cells attract granulocytic infiltrations. Genetically engineered immune T cells from patients with metastatic colon cancer express bispecific cytotoxicity to $CEA^+/CD3^+$ colon cancer cells	[743–754]

In the cancer hospital M. D. Anderson in Houston, *B. fragilis* infections were severe and frequent [642a,b, 643] and in general continue to carry high morbidity and mortality [644].

The *B. fragilis* enterotoxin cleaves E-cadherin and activates the β-catenin/Wnt cascade. The enterotoxin induces neoangiogenesis and epithelial cell proliferation. The cell proliferation is driven by the STAT and MAPK cascades (signal transducer activation of transcription; mitogen-activated protein kinase). In mice the enterotoxin failed to induce Toll-like receptor or dendritic cell activations. Instead anti-apoptotic factors (NFκB and c-IAP2) are activated. Epithelial cells of the colon produce IL-8, an NFκB activator. The toxin-exposed colonic epithelial cells exude cyclooxygenase (Cox-2) and prostaglandin (PGE2). Reactive lymphoid cell infiltrates appear expressing IL-17 and the receptor for IL-23. Of the T cell factors, TCF-1 promotes, TCF-4 inhibits Wnt proto-oncogene activation [645–649]. Consequential to unopposed Wnt activity is the upregulation, amplification, or even mutation of the BLC9 (B cell lymphoma) and K-ras genes and overexpression of the EGFR in epithelial cells [650–654]. In contrast to *H. pylori, B. fragilis* is a phage-sensitive bacterium [655, 656] and responds to various antibiotic regimens [644].

In bacteria, and very prominently in *Bacteroides* spp., antibiotic-resistance is mediated by efflux pump P-glycoprotein-encoding genes, which are transferable by plasmids [657a,b, 658a,b]. The same mechanism is operational in chemotherapy-resistant cancer cells. This author could not find comparative studies for bacterial and eukaryotic (protozoal; cancer cells of vertebrates) efflux pump genomics and proteomics, but the mechanisms of action of these entities by ATP-binding cassettes, as well as their inhibition by selected compounds (phenothiazides, chlorpromazine, verapamil) are very similar in bacteria and in tumor cells [659–664]. The bacterial flora in the intestinal tract of gypsy moth larvae rapidly acquires and/or endogenously expresses efflux pump proteins for the mediation of multiple antibiotics resistance (but without any exposure to such antibiotics). Insect guts are environmentally rich in antibiotic resistance genes; there is a high potential for dissemination of such genes from host to bacterial symbionts, and in between members of the bacterial flora [665]. Disiloxanes (SILA-409, SILA-421) excelled as efflux pump inhibitors both in bacteria, where they also suppress plasmid-traffic of resistance genes (*vide infra*) and in multidrug resistant cancer cells [666–668]. The multidrug and toxic compound extrusion (MATE) family efflux transporter gene *bex*A was cloned from *Bacteroides thetaiotaomicron.* The BexA protein sequence is homologous to that of *Vibrio parahaemolyticus*. The *bex*A construct transferred cipro- and norfloxacin resistance to *E. coli* [669].

Most of the antibiotic-resistance genes of *Bacteroides* spp. have been recognized [670–674a,b]. The genes (*tet*Q, for tetracycline, *gyr*A for quinolone resistance, *erm*F for erythromycin resistance, *cfi*A for encoding a lactamase/carbopenemase for carbapenem, imipenem and metronidazole resistance, *cep*A for encoding cephaloporinase, *nim*B for nitroimidazole resistance) are transferred by conjugative transposons (CT, integrative and conjugative elements, ICE), plasmids, and phages, pHag1 and pHag2 [658]. The most prominent conjugative transposons for horizontal transfer of tetracycline and erythromycin resistance-encoding genes (*tet*Q, *erm*F) are the closely related CTnERL and CTnDOT integrases, especially the TcrEmrDOT (dot-blot hybridization, dot-plot) [675–681]. The promiscuous bacteroides conjugative transposons, CTnGERM, carrier of the erythromycin-resistance gene *erm*G, picked up the macrolide efflux pump gene *mef*A from *Streptococcus pyogenes* [682].

Vertically transmitted germ line mutations-induced carcinogenesis in the human colon is very well documented and the oncogenes are cloned and characterized. There are deleted tumor (colon cancer) suppressor genes (DCC from chromosome 18, p53 from chromosome 17p, MCC mutated colorectal caner gene from chromosome 5q), amplified, mutated or translocated oncogenes (c-*myc*, K-*ras*), unique mutated colon cancer-inducer gene (adenoma-polyposis coli, APC) and the Lynch syndrome I-inducer genes (named after yeast genes: PMS, postmeiotic segregation; MSH, Mut(mutated) S homolog; MLH, Mut L homolog, missense mutations). These are the hereditary colon cancer syndromes, the adenoma (polyposis) to carcinoma sequences, and the hereditary nonpolyposis colorectal cancers [683–687]. Colorectal cancers termed “sporadic” in stages II and III (without and

with regional lymph node metastases) present with different genetic signatures in the Affimetrix array [688].

Carcinogenic somatic mutations generated in the colonic inflammasomes are not inherited, and as such their inducers may be subject to horizontal acquisitions. The Lancet gave credit to Rudolf Virchow for proposing in 1863 that bacteria and the chronic inflammation they induce (leukoreticular infiltrates: "Phlogose und Thrombose im Gefäßsystem") may induce cancers [689]. The high pathogenicity and persistence of endo- and enterotoxigenic strains of *B fragilis* were recognized in the 1990s [690, 691]. The molecular pathogenesis of the bacteroides enterotoxin creates an environment highly conducive to malignant transformation of the mucosal cells of the colon (*vide supra*) [646–654]. The enterotoxin can re-arrange cellular cytoskeletons [692). It activates proto-oncogenes (c-*myc;* K-*ras* [693–695] and the human relative of drosophila mutated gene "legless," the BCL9 gene/gene-product protein (B cell lymphoma)). This gene-product protein stimulates the proto-oncogenic β-catenin/Wnt cascade, whose inhibitor is the dickkopf protein (DKK), which is often eliminated in the course of malignant transformation. This pathway of colon cancer oncogenesis may be shared between hereditary (APC tumor suppressor gene mutation) and inflammatory colon cancers [696]. It is not entirely clear what role DKK1-4 proteins may play in colon carcinogenesis. DKK-1 may be epigenetically inactivated and down-regulated, thus loosing its inhibitory effects on tumor cell growth [697, 698]. DKK2, 3, 4 may be upregulated; DKK-3, 4 are neo-angiogenic; DKK-4 inhibits host T cell enhancement and promotes tumor cell invasion [699–701]. In contrast, DKK-4 was found not to activate, but to inhibit β-catenin signaling, colon cancer cell cycle progression and growth [702]. Vitamin D3 (1α, 25-dihydroxyvitamin D) activates the gene of the Wnt/β-catenin antagonist DKK-1, thus inducing differentiation in human colon cancer cells; at the same time, the vitamin D compound inactivates the gene of the tumor promoter Wnt/β-catenin signaling pathway [703, 704].

Enterotoxic bacteroides further activates NFκB; induces IL-6, IL-8, IL-10 and TGF-β production; inhibits apoptosis, and induces neo-angiogenesis [646–654, 705]. In the inflamed colon, ectopic activation of cytidine deaminase (AID) by TNF-α *via* NFκB induction and/or by cytokines IL-4 and IL-13 results in p53 mutation [705], thus removing a major obstacle to colon carcinogenesis. Mutations of the p53 gene in colonic mucosal cells in ulcerative colitis (UC) may be contributory to carcinogenesis [706]. Colon cancer cells overproduce cyclooxygenase-2 (COX-2), a tumor cell growth promoter [707].

The causative factors of UC could not as yet be identified. High expression of heat shock protein (HSP47) in cancer cells in that condition is considered to be a unique feature [708]. The expression of RhoGDIα, the inhibitor of Rho-GTPases, disorganizes intestinal epithelial cells and is a known promoter of progression of breast and inflammatory colon cancers (rhomboid proteins; guanine triphosphatase; guanine nucleotide disassociation inhibitor; guanosine diphospho- (D-mannose)) [709, 710]. The gene "Wiskott-Aldrich (WA) syndrome protein and FKBP-like" (WAFL, tacrolimus-FK-binding protein) is overexpressed in the colon in inflamed colonic mucosa in UC; normally, it is a membrane traffic protein, whose role in carcinogenesis is unknown [711a,b]. Prominent role is ascribed to IL-6 and STAT3/SOC3 signaling pathway in UC-related carcinogenesis [712]. The activator of STAT2 signaling is IFN-αβ. STAT2 acted as a promoter of chemically-induced colorectal carcinogenesis; deletion of STAT2 was inhibitory to these processes. Thus, the proinflammatory mediator, STAT2 is now recognized as an uncovered co-carcinogenic secret agent [713].

Extracellular, cell-surface installed and intracellular innate immune faculties (Toll-like receptors, chemokines and cytokines; macrophages, dendritic cells, natural killer cells and Nod-like receptors) participate in colonic inflammatory and carcinogenic processes, either in a promotional, or in an inhibitory manner. Two extremely well referenced articles review the field with the conclusions that Nod-like receptors (nucleotide-binding oligomerization domains) protect against inflammatory carcinogenesis [714] and that innate and adaptive immune reaction cooperate in the intensity of the immune reactions, but divaricate in matters of pro- and anti-tumor reactions [715]. Homozygous

mutations in IL-10 receptor genes increased the propensity in patients to the development of colitis; thus, removal of a tolerogenic cytokine's actions promoted fulminant immune reactions to the intestinal bacterial commensals [716, 717]. These reports have avoided giving reference to carcinogenesis; there, not IL-10-mediated host tolerance, but intense immune reactions are needed, like in the IL-10R-mutated patients, or mice. An excellent tabulation lists innate immune responses, the IL-23/Th17-type pathway and other genes involved in inflammatory reactions of the human intestinal tract (chromosomal locations of the involved genes given). Two most impressive cartoons depict the interactions between the arms of innate and adaptive immune faculties. The article closes with therapeutic recommendations, as to the severe inflammatory reactions, but without. mentioning any favorable or adverse effects of the anti-inflammatory therapeuticals on the incidence of carcinogenesis [718]. In contrast, adaptive immune reactions mediated by $CD4^+$ T lymphocytes are mobilized in UC against dysplastic colonic mucosa, but not against normal mucosa [719].

The anti-inflammatory reactions that may promote or suppress the incidence of inflammatory carcinogenesis are the inhibitors of prostaglandins and cyclooxygenases (celecoxib) [707]. The pro-inflammatory transcription factor NFκB is inhibited by fluoro- and tribromsalans, sunitinib, lestaurtinib, ectinascidin, chromomycin and bortezomib [720]. The nuclear receptor PPAR (peroxisome proliferatory-activated receptor gamma) is an antagonist of NFκB upon its transfer from the cytoplasm to the nucleus in order to activate inflammatory reactions-encoding genes [721]. In mice with PPAR-positive colonic epithelial cells and lymphocytes, oral intake of conjugated linoleic acid (CLA) ameliorated inflammatory bowel disease and inhibited chemical carcinogenesis; CLA was ineffective in PPARγ-null mice. Successfully responding PPAR-positive mice had no macrophage infiltrations in the mesenteric lymph nodes; expressed low levels of TNF-α mRNA, and mobilized increased numbers of regulatory T cells [722]. In inflammatory bowel disease, IL-6 and its solubilized receptor (IL-6R) form complexes; these complexes react with CD130 of mucosal T cells (IL-6 trans-signaling). In the epithelial cells, IL-6 induces STAT3 signaling (*vide supra*). Myeloid cells in the inflamed colonic mucosa also secrete IL-6. Both anti-IL-6 and anti-IL-6R monoclonal antibodies and other IL-6 inhibitors are available for the suppression of IL-6-mediated pro- and anti-inflammatory reactions [723].

As to stem cells in the colonic mucosa, in response to the proper cytokines and growth factors, normal differentiation ensues. Inflammatory cytokines are genotoxic and mutagenic; the stem cells so treated emerge as cancer stem cells. The expression of CD133 is a clue for such a transformation [724–726]. Inflammatory rectal fistulous tracts could be cured by stem cell transplantation (bone marrow transplant from HLA-matched sibling to alemtuzumab- and fludarabine-preconditioned, gut-decolonized patient, whose graft-*versus* host disease was treated with prednisone). Full chimerism was established without GvHD [716]. From Russia comes a favorable report on improved clinical course of patients with UC treated with allogeneic bone marrow mesenchymal stem cells [727]. In Hungary, regeneration of the inflammatory damage in the colonic mucosa by stem cells migrating to the lesions was observed. As yet unidentified lymphocytic aggregates and the migrating stem cells appeared to have cooperated in the process [728]. If the thesis stands, that certain inflammatory cytokines may be genotoxic to stem cells embedded in the colonic mucosa [725], it is then essential to recognize those cytokines and cellular elements (Paneth cells) that sustain the integrity and the healthy homeostasis in the bowel mucosa [724, 729] (Table 2.1).

Inflammatory carcinogenesis was recognized long ago as a unique event in the induction of squamous cell carcinomas in Marjolin ulcers (Jean-Nicolas Marjolin, 1828). The concept now has been extended to prostate and breast carcinomas without an identified inflammation-inducer pathogen. Some colon adenocarcinomas may be generated by the inflammatory cascades induced by the *B. fragilis* enterotoxin. In gastric carcinoma- and lymphomagenesis, the *H. pylori* CagA gene product protein initiates the well defined cascade terminating in lymphoma- and/or adenocarcinoma induction [737a,b]. Cag A (cytotoxin-associated protein/antigen) may induce cell senescence or carcinogenesis in the gastric mucosa. In the carcinogenic pathway, c-Myc induces

microRNAs miR-17 and miR-20a for the suppression of p21 cyclin-dependent kinase leading to epithelial-to-mesenchymal transition [737a,b] (*vide infra*).

2.7.2 The Epithelial-to-Mesenchymal Transformation

Epithelial-to-mesenchymal transformation (EMT) of cancer cells is a complex act in the interest of increased virulence of the malignant pheno-genotype. The Ras oncoprotein activates TGF-β (transforming growth factor) production, which activates the *snail* genomic sequences followed by lymphoid enhancer factor (LEF) activation. The Bcl-3 protein translocates into the nucleus to react with N-cadherin DNA; in the cytoplasm, cyclin D is inactivated. When the WNT-induced signaling protein (WISP) is inhibited, E-cadherin expression is reduced in tumor cells, which gain invasiveness. The Snail and Twist proteins collaborate with *ras* and inactivate p53 and Rb (retinoblastoma) proteins, an anti-apoptotic event: tumor cells are now protected from apoptotic death. The SPARC protein (secreted protein acidic rich in cystein) promotes the nuclear translocation of β-catenin, which further increases the activation of LEF. This is the environment in which a subclass of tumor-promoter T-Lymphocytes is generated. This lymphocyte population induces (either in a causative, or in a coincidental manner) *ras* gene point-mutations in human breast cancer cells. These breast cancer cells metastasize to the regional (axillary) lymph nodes [755]. Sinkovics proposed the term of traitor/transforming T cells (T/T T cells) for this class of host T lymphocytes [756]. The generation of T/T T lymphoid cell clones have nor as yet shown in *B. fragilis* enterotoxin-exposed colon cells, but it is documented in the microenvironment of human breast cancer that a subpopulation of reactive T lymphocytes (T/T T cells) with upregulated ILEI genes (interleukin-like epidermal-to-mesenchymal transition inducer) produce TGF-β [755]. Colon cancer cells (*vide supra*) undergo EMT and express the molecular signatures of up-regulated vimentin, E-cadherin, Slug and Claudin protein families, forkhead transcription factors, and WNT signaling with down-regulated dickkopf (*vide supra*) [757]. This author proposed that ras-mutated colon cancer cells were induced by T/T T-like lymphocytes, which promote their metastases in the regional lymph nodes [642]. Tumor cells (pancreatic carcinoma cells) expressing FoxP3, thus imitating T_{reg} cells, neutralize, and protect themselves from the attack of, immune T cells [758]. While tumor cells undergo EMT, the tumor stroma (the extracellular matrix) either plays the role of the initiator, or changes in response. Non-coding RNAs (miR-200; miR-205) interact with vimentin in the stroma and with E-cadherin within the tumor cells. TWIST protein induces miR-10b, which drives breast cancer cells' invasiveness, whereas miR-335 antagonizes the process [759]. The mammalian cell microRNA, miR-146a, emerges as an inhibitor of innate immune reactions and a multiple tumor growth promoter; it is activated by NFκB [760] The tumor-promoting events of EMT are open to study, if the cascade of gene activations occurs within the tumor cell, or if a fusion of the epithelial tumor cell with a mesenchymal cell (as well documented in the case of melanoma and macrophages) resulted in the transfer of activated mesenchymal phenotype-inducer genes into the tumor cell from a mesenchymal cell of the stroma.

When human epithelial cancer cells express immunoglobulins (RAG1, 2; VDJ; IgA) [761], is it in the cells' own genome, where the genes that are usually kept silenced were activated in a germline fashion, or is it the result of a "natural hybridoma" (NH) formation that has had occurred? In the process of the original natural hybridoma (NH) formation, antibody-producing plasma cells fused with lymphoma cells [213, 762–766], a mesenchymal-to-mesenchymal cell fusion. Epithelial cells may fuse with lymphocytes or monocytes to gain pre-activated genes for a mesenchymal transition.

The original NH formation occurred in a murine lymphoma. The NH phenomenon was discovered by this author in the mid-1960s, understood clearly and reported in explicit terms that a mouse leukemia virus-carrier lymphoma cell fused with a plasma cell secreting antibody specific to the lymphoma cell as the lymphoma cell expressed retroviral envelope antigens. This cell line (Fig. 2.4a,b)

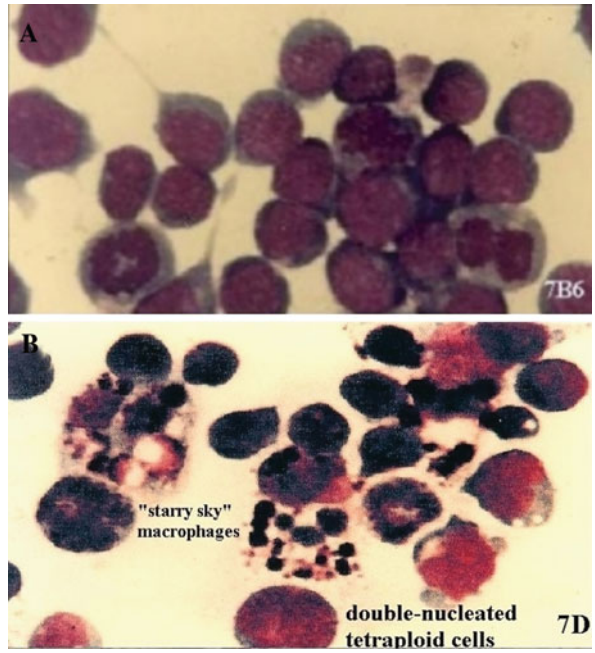

Fig. 2.4 (a,b) Spontaneous cell fusions in murine lymphoma observed in the mid-1960s at the Section of Clinical Tumor Virology & Immunology, M. D. Anderson Hospital, Houston, TX. The retrovirally transformed lymphoma cells showed budding retroviral particles (retroviral envelopes) in their cell membrane. The immune B (plasma) cells produced immunoglobulins specifically reacting with structural proteins of the virus particles budding from the lymphoma cells. The immunoglobulins neutralized infectious retrovirus in a spleen focus assay. The lymphoma cells and the immune plasma cells adhered to one another and fused. The fused products were tetra- or polyploid, grew in suspension cultures for over 10 years (**a**) and in the peritoneal cavity of mice, secreted the specific immunoglobulin, and were attacked by macrophages inducing the "starry sky" phenomenon (**b**). [767] The native spontaneous cell fusion event was duplicated in the peritoneal cavity of mice. Co-inoculated mixtures of lymphoma cells and immune plasma cells fused and produced bi-nucleated cells (**b**) [762–772]. In his first report on these fused cells, the author wrote in Lancet: "Tetraploid immunoresistant lymphoma cells in the mouse emerge by fusion of the diploid virus-producing lymphoma cell with a plasma cell producing virus-specific globulins. The resulting tetraploid cell will retain malignant growth potential and the genetically determined committedness of both parent cells – to produce leukemia virus, as coded for by the viral genome within the neoplastic cell, and to synthesize virus-specific globulins, as coded for by the genome of the plasma cell" [213, 765]. The USA National Cancer Institute replied in the mid-1970s to the author's grant applications: "approved without funding, due to low priority." The circumstances of this work were investigated, validated and credited for priority by Professor Milton Wainwright, University of Sheffield, Sheffield, England [819, 820]. Permission to re-publish is from Schenk Buchverlag, Passau and Budapest

was maintained in suspension cultures and as more ascites tumors for over 10 years, while it continued making the specific antibody [213, 762–772]. This author promptly proposed that the Reed-Sternberg cells of Hodgkin's disease may be B-cell natural hybridomas, and the Sézary cells of mycosis fungoides are T cell natural hybridomas [765, 769]. Molecular biology of RS cells so far provided no support for this theory, but there remains room for further reasoning in favor of natural hybridoma formation mediated by a fusogenic retrovirus (with or without EBV) in these pathological entities [213, 768–770].

When epithelial cells fuse with mesenchymal cells (EMT), the mechanism of fusion is not an explicit antigen-antibody reaction. The entire process of transdifferentiation of bone marrow stem cells may occur within the stem cell genome without fusion, by gene re-programming. In inflamed tissues, transdifferentiation of the stem cell may take the direction toward a malignant geno-phenotype [773]. In treated cancers, new stem cell type emerges; these are the "recurrent cancer stem cells"

(rCSCs). The rCSCs display strong resistance toward external chemo-radiotherapy by gaining strong anti-apoptotic faculties, and toward internal host immunity, thus practicing "oncogenic resistance." The phenotypic heterogeneity of these newly emerged rCSCs gives them the propensity to fuse with mesenchymal cells (monocytes) for their further advantage to invasiveness and metastasis formation [774].

2.8 Horizontal Transfer of Proto-Oncogenes

Peyton Rous at the Rockefeller Institute in New York City in 1908–1912 discovered tumor-induced neoangiogenesis, a monumental discovery, but not remembered, because of his another monumental discovery, that is, the Rous sarcoma virus. In 1908, the rapid reproducibility of lymphocytes upon stimulation caught Rous' attention [775]. In 1910 Rous wrote: "The fate of implanted tumor depends directly on whether it elicits from the host a vascularizing stroma. So, too, it is with implanted embryo" [776]. In 1910–1911, Rous transferred with cells and with filtrates a sarcomatous tumor in chickens (hens): the transmissible avian neoplasm, a sarcoma of the common fowl [777]. In 1912, Rous and Murphy observed lymphocytic activity in response to the transplanted sarcomatous tumors in the inoculated hens [778]. The Rous sarcoma virus and its sub-strains were the teachers of tumor retrovirologists world-wide for decades to come. It was the three editions of Ludwik Gross' textbook that provided the basic foundations of tumor virology for the early decades of that discipline [779]. Hidesaburo Hanafusa of the Rockefeller University, New York City, found the oncogenic genome of the Rous sarcoma virus (*v-src*) and the phosphorylated tyrosine kinases that it encodes in order to build the structural proteins of the virus particle; especially the *env*/Env gene and protein that was immunogenic. He realized that a cellular homologue of the viral oncogene existed (*c-src*) in the hosts of these tumors [780]. However, ahead of him at the University of California, San Francisco, the team of J. M. Bishop and H. E. Varmus, D. Stehelin and P. K. Vogt, established the DNA identity of the Rous sarcoma (and other avian sarcoma) proviruses in the cells of healthy hosts with that of the mature virions [781, 782]. The enzyme, the reverse transcriptase, which transcribes an RNA viral genome into a DNA provirus, was discovered by D. Baltimore and H. M. Temin and S. Mizutani [783]. Here is the most ostentatious (éclat!) example of gene switching: a host cell gene becomes incorporated into the DNA proviral genome of a retrovirus. It may remain independent within, or it may be fused with, a genomic sequence of the retrovirus, but when the retroviral genome is transcribed and translated, the usurped host gene and its gene product protein remain incorporated in the retroviral particle and expressed in the cell, respectively, that the virus transformed. If the usurped host gene encodes a growth factor, the retrovirus replicating in a malignantly transformed cell will overproduce that growth factor. Viral oncogenes and oncoproteins have been generated by gene transfer from a cellular genome into a viral genome.

While from fish to mammals (gibbon ape; woolly monkey) acute leukemias and sarcomas are caused by retroviruses, in the human host only one such retroviral pathogen could be isolated: the human T cell lymphotropic leukemia virus (HTLV) [784, 785].

In the 1970s, this author was engaged at the M. D. Anderson Hospital, Houston, TX. in efforts to isolate human sarcoma retroviruses. Cell free human sarcoma extracts and culture fluids could induce cell foci and antigenic conversions in human embryonic fibroblast cultures with occasional retroviral particles sighted, but up to this date no established human sarcoma virus could be isolated and identified. Human sarcoma cells certainly express "neoantigens" thus attracting cytotoxic lymphocytes (Fig. 2.2a,b). It is a matter of speculation how the hominoid genera and species (from *Australopithecus afarensis* to *Homo sapiens*) during their evolution succeeded in suppressing the leukemogenic and sarcomagenic retroviruses of their distant ancestors [213]. However, in Kaposi's sarcoma next to its causative herpesvirus (HHV-8) buds a reactivated endogenous retrovirus (Fig. 2.3a,b) [213].

Retrotransposons and the permanently inscribed genomic sequences (reversely transcribed DNA proviruses) of endogenous retroviral elements permeate the entire human genome, as relics of retroviral infections subdued in the distant and recent past. These are the viral genomes that may incorporate, propagate and horizontally spread genuine host cell genomic segments. The results of past and present endogenous retroviral activates extend from the evolution of the placenta and the telomeres, through the induction of autoimmunity and the reactivation and expression of certain endogenous retroviral elements in certain human cancers, the invocation of RNA interference and the tumor suppressor protein p53, to the ultimate primate evolution, including the development, present and future activities of the human brain [786–794].

2.9 Horizontal Gene Transfers for the Treatment and Cure of Cancer

The gene therapy of cancer was initiated by the replacement of the mutated or eliminated tumor suppressor genes (or whose gene product proteins were ubiquitinylated) by horizontal insertion of the wild-type genes. First, severe combined immunodeficiency (SCID) could be treated with bone marrow transplants. Haploidentical donor bone marrow transplants often induced graft-versus-host disease (GvHD) and failed to completely restore the function of B lineage lymphocytes. In clinical trials, children (infants) with adenosine deaminase deficiency (X-SCID) receive infused autologous bone marrow progenitor stem cells, which were transduced by a retroviral vector in vitro replacing the deficient gene. Restoration of the immune system occurs without GvHD [795]. In X-SCID the gamma c chain of the IL-2R is mutated (γ-c null) and is not signaling after stimulation with EBV. The cells with mutated IL-2R could be restored to function with a retroviral vector (G1γcSvNa) transducing the wild-type γ-c gene (c for common, because the IL-4R and IL-7R are also involved). In the transduced cells, the IL-2Rs signaled normally and phosphorylated the Jak1/Jak3 (janus kinase) tyrosine kinases [796]. However gene re-insertions by a murine leukemia retroviral vector carried a not foreseen major risk. Five of 20 patients developed acute T cell leukemia (first reported as "clonal T cell proliferation") after retrovirally vectored gene insertion. The vector retrovirus inserted its genome carrying the correct gene next to the T cell proto-oncogene LMO2, and thus activated it by the mechanisms of "insertional oncogenesis" [797–799]. The proto-oncogene LMO2 stands for "LIM only protein 2" and LIMs are mesenchymal (muscle, etc) proteins encoded by a family of genes (*lin/isl/mec*). The LMO2 gene is involved in T cell generation and in its activated state transforms from a proto-oncogene into an oncogene [800]. A single chain Fv antibody fragment was developed to specifically inhibit LMO2 [801], but its clinical usefulness is not yet known.

The Max Delbrück Institute of Molecular Medicine, Berlin, Germany, has taken the directives toward nonviral delivery approaches in human gene therapy. A plasmid expressing a transposase in *trans* position and a donor plasmid containing the DNA (gene) to be integrated are to be used in combination. The DNA to be integrated is flanked in cis position by the terminal repeat sequences of the transposase. The candidate transposons are the Tc1/mariner-type *Sleeping Beauty* (SB), the *Tol2* (both originally fish transposons) and the *piggybac* (PB). In order to increase the activity of SB, the transposase had to be reconstructed by exchanging its aa composition. Thus arriving at the hyperactive SB100X transposon/transposase, its increased activity in its integration process is over one hundred-fold elevated. The nonmariner-type Tol2 transposon can carry genes as large as 11 kb. While excess transposase could inhibit SB by "overproduction inhibition," Tol2 works better with more transposase at its disposal. The PB transposon is of insect origin (deriving from the lepidopteran noctuid, the cabbage moth, *Trichoplusia ni*). It was discovered because it inserted itself into the genome of the nuclear polyhedrosis virus, the baculovirus *Autographa californica* [802–804]. PB can transpose inserts of 14 kb (too large for any retroviral vector). While SB has no human relatives, PB has its related sequences dispersed in human chromosomes. It is a concern how these endogenous human PB

elements would react to the insertion of an exogenous PB transposons. The LTR sequences of SB100X are inert when it comes to activating host cell genes at the transposons integration site. This inactivity has been reinforced by adding an insulator sequence to the expression cassette of the transposons. In contrast, N-terminal DNA-binding domain fused with the transposon PB rendered the transposase more permissive as to its acceptance of DNA (gene sequences) for integration. Silent pluripotent stem cells also accept genes integrated by transposons for the re-programming of these cells. In induced pluripotent stem (iPS) cells, the c-*myc*× c-Myc activation carries the potential danger of oncogenesis. Some called c-*myc* "the oncogene from hell." Therefore activation of c-*myc* should be avoided (especially in the case of retroviral vectoring). The PB transposon could already safely deliver genes into mouse fibroblasts and thus reprogram them into iPS cells of endodermal, mesodermal and ectodermal lineages. The natural transposons/transposases become "designer transposases" opening up avenues toward regenerative medicine and cancer therapy by horizontally inserted genes [805].

After proving in mice that SB100X-inserted reporter gene-carrier hematopoietic stem cells function impeccably, human clinical trials have been initiated. In the first human clinical trial, SB100X will encode a chimeric T cell receptor for adoptive immune T cell therapy in patients with $CD19^+$ B lymphocyte lineage malignancies (malignant lymphomas). The new T cell receptor will consist of a specific CD19-reactive single chain v fragment linked to the CD28 endodomain which is fused with the cytoplasmic CD3-zeta (ζ) domain. It was already proven that T cells so reconstructed are cytotoxic to $CD19^+$ B lymphoma cells. The cytotoxic T cell clone will be expanded in vitro on irradiated $CD19^+$ lymphoma cells constantly stimulating it. Genetically engineered T cells will be infused during the period of recovery from myeloablative chemotherapy and autologous stem cell rescue [805–816].

2.10 Consequences

The once believed accidental, and exceptional horizontal (lateral) gene transfers and cell fusions have become accepted as one of the absolute rules of Nature. So much so, that when some vertically inherited genes have been mistakenly claimed to be horizontally acquired; the matter was quickly corrected [817]. Excessive exchange of genes outside of the vertical route by whatever other means (protocell fusions; protocell fusions fusogenic bacteriophage-mediated, naked DNA transfers; plasmid-, phage- and virus-mediated transfer) were the fundamental attributes and inherent characteristics of the living matter. The voracious acquisitions of alien genes in protocells, and in their immediate descendants prohibit a firm rooting of the Tree of Life below the Darwinian threshold.

To this author, the genome of the neoplastic cells resembles most the ancient wild-type premordial DNA: aggressive, an eager host (predator) of engulfed genes, and a most willing partner in gene fusions. It is immortal: it divides before its senescence (telomeric loss) and death could set in. Whereas the DNAs serving in multicellular hosts must have undergone through several "taming mutations" resulting in the acceptance of differentiation, service to the cell community, then senescence and death. However, within the stem cell compartments, silent remnants of the ancient wild-type DNAs prevail. In a blind rebellion, the stem cell DNA may divest itself from its superimposed role to differentiate and serve. Instead, it initiates a rebellious attempt at regaining its ancient immortality. The clinicians at the bed side diagnose the formidable disease: "cancer." However, the clinicians are not observing a "disease" as such. The inherent archaic DNA is rising to re-occupy its native territory, expand in the oceans and land as long as nutrients are provided and wastes are eliminated. If the environment freezes in the absolute temperature of the outer space, the DNA waits a milliard years until after it reaches another environment appropriate to its divisions, mutations, fusions and expansions. In another Cambrian sea, the tamed DNA-derivatives begin their service within multicellular organisms, while harboring in their sequences some silent remnants of their wild-type ancestry. "Cancer" is not a

disease as such; it is the ancient wild-type DNA's inscribed and inherent faculty for its immortality in any shape or form under any circumstances.

The explosive evolution culminating in the armada of creatures in the Cambrian sea, slowed down horizontal gene transfers under the newly installed rules and regulations of vertical inheritance, in which the retention of point-mutated, amplified, recombined and duplicated genes represent the driving force. From this point on, there is a clearly recognizable Tree of Life. Even those highly disciplined cells that form the Tree of Life would not refrain from accepting a useful alien gene whenever a rare opportunity still arises. In other instances, an uninvited retrotransposon or a virus will intrude and implant a new gene into a rigidly organized genome. The host, its defenses overcome, tries to reduce these sequences to pseudogenes, or actually takes use of them as new useful genes; other inserted sequences remain inert for millenia. F. Bushman provided a tabulated list of human genes potentially derived from transposable elements (including telomerase, human endogenous retroviruses, placental syncytins and *rag*s 1 and 2) [435].

Provoke the genomes of plants, domesticated animals and human beings living on Mars: the discipline of the rigidly regulated genomes will melt. In response to the challenge, the genomes will re-open the gates for the acceptance of mutations, gene duplications and new genes. That would be the course of natural evolution. However, gene therapists will intervene to produce a heavy set human race on a planet with low gravitational force, with allowances to anaerobic (Warburg) glycolysis, and with the lipid metabolism of the arctic (polar) bear, the seal (or the penguin). Processes of the lateral (horizontal) gene transfers from archaea and prokaryota to eukaryota and from eukaryota to multicellular eukaryota, substantially restricted, but remaining active, will continue as long as cells evolve on Earth (or on Mars).

2.11 Summary

Voracious lateral (horizontal) exchanges of alien genes between the ancestor proto-spheroplasts of archaea, prokaryota and the first unicellular eukaryota, occurring below the Darwinian threshold, render the rooting of the Tree of Life close to be impossible. A fusogenic phage might have mediated the primordial fusion between crenarchaeal and prokaryotic protoplastic cells to form the first ancestral eukaryotic cell(s). Descendants of fusogenic mycoplasma phages and extant proto-spheroplasts of crenarchaeota and prokaryota may be able to repeat this seminal experiment of Nature in the laboratory. If not by free-swimming DNA retrotransposons encoding the genes for the enzymes that carried out insertions and excisions, horizontal gene transfers were mediated by viruses. Plasmids and bacteriophages served as ancient vehicles of laterally transferred genes. The ancestors of the large dsDNA cytoplasmic viruses (the mimivirus and its relatives) contributed to eukaryogenesis in the "Virus World." These very large viruses remain in existence through some 3 billion years as parasites and/or symbionts to the descendants of the first unicellular eukaryotes. Above the Darwinian threshold, as speciation and the rules of vertical inheritance established themselves, horizontal gene transfers gained another route: the cell fusions, whether hostile or symbiotic, promoting the exchange of alien genes. Some of the most important genes encoding the adaptive immune system in the first chondrichthyes sharks in the Cambrian sea were acquired through horizontal insertions The reticuloendothelial retrovirus inserted its genome into Marek's herpesvirus, or into the fowl pox virus. There is a "criminal collusion" between herpes- and retroviruses dating back to tens of millions of years of co-evolution in the same hosts (simians, primates, *Australopithecines,* hominoids and *Homo*) in Africa.

How did the original cholera vibrio acquire its cholera toxin genes? What is the oceanic or estuarial environment from which the cholera vibrio and its phages emerged? The donor of the cholera toxin genes (blooming zooplanktons; a dinoflagellate; a crustacean) swims free and unrecognized, while the cholera vibrio spreads around the globe. Of ancient marine mycobacteria, the genes that

encode the mycolactone toxin that causes the Buruli ulcer might have been acquired from an ancient amphibian (a frog) by the ancestor of *M. marinum* and passed by plasmids from it to the later becoming human-pathogen *M. ulcerans*. The genes encoding antibiotics and antibiotic-resistance, and those of "pathogenicity islands" appear to have emerged after speciation evolved, above the Darwinian threshold. Nevertheless, these gene clusters remain the subjects of horizontal transfers between prokaryota.

Eukaryotic cells, including those of the human brain, accept horizontally inserted genes (viral genes, such as those of the Bornavirus). A review with selected references is provided on horizontal gene transfers from archaea and prokaryota to eukaryota and from eukaryota to eukaryota. The genes (operons) of photosynthesis were transferred from cyanobacteria to algae through lateral routes (from engulfed cytoplasmic chloroplasts to the host cell nucleus) and from there to terrestrial plants by vertical inheritance, or into the apicoplasts of plasmodia and other apicoplexan unicellular eukaryota from red algae by horizontal transfers. The female wasps demonstrate superb ingenuity in converting pathogenic ascoviruses into, non-pathogenic in-the-wasps, ichnoviruses, which are immunosuppressive in the caterpillars. There, the hatching of the wasps' fertilized eggs after their insertion culminates without immune rejection. Some insect cell nuclei carry from generation to generation the entire genomes of rickettsia-like bacteria (Wollbachia) and exchange genes with them. The insertion of cellular proto-oncogenes into retroviral genomes (proviral DNAs) is the most ancient form of natural oncogenesis. Inflammation-induced carcinogenesis is analyzed against the backgrounds of chronic bacterial infections with *Helicobacter pylori* and *Bacteroides fragilis*, and that of epithelial-to-mesenchymal transformation of pre-cancerous stem cells and of cancer cells. In cancerous tissues "natural hybridoma" formations may occur by fusion between lymphoma cells and antibody-secreting plasma cells (first observed, understood and reported in 1970 by this author), or between epithelial cancer cells and mesenchymal (lymphocytes, monocytes) cells. Of these, fusion of human melanoma cells with host monocytes/macrophages the most ostentatious example; here again, a reactivated endogenous retrovirus in the melanoma cell may be the fusogen. Gene therapy with horizontally transferred tumor suppressor genes utilizing transposons as vehicles may induce remissions in, or even cure of, human cancers.

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Chapter 3
Class III Viral Membrane Fusion Proteins

Marija Backovic and Theodore S. Jardetzky

Abstract Members of class III of viral fusion proteins share common structural features and molecular architecture, although they belong to evolutionary distant viruses and carry no sequence homology. Based of the experimentally determined three-dimensional structures of their ectodomains, glycoprotein B (gB) of herpesviruses, G protein of rhabdoviruses and glycoprotein 64 (gp64) of baculoviruses have been identified as class III fusion proteins. The structures are proposed to represent post-fusion conformations, and they reveal trimeric, elongated, rod-like molecules, with each protomer being composed of five domains. Sequences which interact with target membranes and form the fusion peptides are located in two loops found at one end of the molecule. Class III fusion proteins are embedded in viral envelope with the principal function of catalyzing fusion of viral and cellular membranes, an event that is essential for infection to occur. In addition, they have been implicated in processes such as attachment to target cells and viral maturation. G protein is the only class III fusion protein for which structures of both pre- and post-fusion states have been determined, shedding light on the mechanism involved in the conformational change and membrane fusion. Whether similar structural organization of class III fusion proteins translates into a common mechanism involved in carrying out membrane fusion remains to be investigated.

3.1 Introduction

3.1.1 Definition of Class III Fusion Proteins

Viral membrane fusion proteins have been grouped into three classes (class I, II and III) based on their key structural features. The proteins described in this chapter, the members of class III fusion proteins, have been identified recently. Despite the lack of sequence conservation, they share a common three dimensional organization distinct from the one found in class I and II type fusion proteins. The unique structural features of class III fusion proteins include: (1) presence of five domains composed of both α- and β- secondary structure elements, unlike the predominantly helical class I, or class II proteins, made mostly of β-sheets; (2) a common secondary structure topology in which each domain is an insertion into another domain (described in detail in Section 3.2.2); (3) an elongated, centrally located α-helix, that forms a trimeric coiled-coil.

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3.1.2 Viruses Encoding Class III Fusion Proteins

Based on the experimentally determined structures, viral membrane proteins identified as class III fusion proteins are: glycoprotein B (gB) of herpesviruses (Herpes Simplex virus type 1 (HSV-1) and Epstein-Barr virus (EBV)), protein G of Vesicular Stomatitis virus (VSV) and glycoprotein 64 (gp64) of Baculoviruses.

Herpesviridae are a large family of double-stranded DNA viruses, counting more than 200 members that infect humans and a wide range of invertebrates and vertebrates [1]. They are notable human pathogens. HSV-1 infections cause benign epithelial labial lesions although they can result in life-threatening conditions such as encephalitis. EBV is an oncogenic herpesvirus associated with development of malignancies such as Burkitt's and Hodgkin's lymphoma, and nasopharyngeal carcinoma [2]. Herpesviruses carry a large linear genome of 100–200 Kb, which is packed in an icosahedral capsid. The nucleocapsid is immersed in a protein rich matrix called tegument, which is enveloped by a lipid bilayer decorated with gB spikes and a dozen of other surface glycoproteins. Herpesvirus virions appear as quasispherical particles in electron micrographs, with a diameter of 120–260 nm, depending on the thickness of the tegument.

VSV belongs to the *Rhabdoviridae* family of the negative-strand RNA viruses, which infect plants, insects, and a variety of animals, from which the virus can be transmitted to humans (rabies being the best known human pathogen) [3]. The virions of Rhabdoviruses form bullet-shaped 180 nm × 75 nm particles. The single RNA molecule forming the genome is ∼11 Kb long, and is coiled in a tight complex with a nucleocapsid protein, giving rise to a helical ribonucleoprotein structure that is surrounded by the cell-derived membrane. Unlike herpesviruses, which contain a range of proteins embedded in the membrane, the sole type of spike present in the envelope of rhabdoviruses is made of the protein G.

Members of the family *Baculoviridae* are double-stranded DNA viruses, whose circular genome ranges in size from 90 to 160 Kb. Baculoviruses infect insects, and have been found in more than 500 insect species. Their genome is packed with a nucleoprotein core into a capsid, which is surrounded by a membrane giving rise to rod-shaped virions, 250–300 nm long and 30–60 nm wide. The major surface glycoprotein associated with the envelope is gp64.

Viruses encoding class III type fusion proteins are evolutionarily distant, and they differ markedly in shape, size, virion structure and genome organization. Their fusion proteins show no sequence conservation, however they share a common structural architecture as described below. Whether the structural homology translates into a shared mechanism that these proteins utilize to cause membrane fusion is the topic of ongoing investigations.

3.1.3 The Multiple Roles of Class III Fusion Proteins

Herpesvirus gB, rhabdovirus G and Baculovirus gp64 share a common role of catalyzing fusion of viral and cellular membranes. The fusion event allows viral entry into the cells, and is essential for infection to occur. Rhabdoviruses and budded virions of Baculoviruses enter cells by endocytosis, where the low pH environment of the endosome triggers fusion of the viral envelope and endosomal membrane, resulting in the release of viral genome into the cytosol [4, 5]. Herpesviruses enter cells primarily by fusion of the viral and plasma membrane, although endocytosis, both pH-dependent and pH-independent, has been reported as a mode of entry when certain herpesviruses are presented with specific cell types (reviewed in detail in [6]). It is worth noting that while gp64 and G are necessary and sufficient to carry our membrane merger, herpesviruses, in addition to gB, require presence of a non-covalent hetero-dimeric complex made of glycoproteins H and L (gH/gL). It has been proposed that gH/gL mediates hemifusion of the outer lipids leaflets, while gB is required for resolution of the hemifusion intermediate [7].

Class III type fusion proteins are involved in processes other than fusion, suggesting that these proteins evolved to execute multiple functions. There are numerous reports implicating class III fusion proteins in intracellular processes such as viral maturation and egress of virus from the infected cells. For example, HSV-1 gB promotes fusion between the virion and outer nuclear envelope during the virion trafficking from the cell nucleus to cytosol [8]. The carboxy-terminal domain (CTD) of the human cytomegalovirus (HCMV) gB is critical for viral maturation [9], while the CTD of EBV and HHV-8 gB are required for lytic replication and production of infectious particles [10, 11]. gp64 is essential for efficient virion budding as well [12].

Some class III fusion proteins also mediate virus binding to cells. gB of herpesviruses allows the virus to attach to the heparan sulfate moieties expressed on target cells [13], and HSV-1 gB in addition associates with paired immunoglobulin-like type 2 receptor (PILR) alpha [14]. The latter interaction is required for infection, even though HSV-1 has another receptor binding protein gD, which binds to herpes virus entry mediator (HVEM) and Nectin-1 cellular receptors. HCMV gB interacts with the epidermal growth factor receptor, which serves as one of its entry receptors [15, 16], while HHV-8 gB binds to integrin $\alpha_3\beta_1$ [17]. A receptor for entry of Baculoviruses has not been identified, but gp64 has been reported to bind to a cellular receptor [18], with the putative receptor binding domain being located in the N-terminal region of the gp64 ectodomain [19]. Phosphatidylserine was believed to serve as a receptor for VSV G for a long time, but this has been recently disputed [20, 21]. There is also no clear consensus which cellular factors act as receptors for the rabies virus, a human rhabdovirus. Gangliosides, phospholipids, nicotinic acetylcholine receptors are among the ones proposed (reviewed in [22]).

3.2 Structural Features of Class III Viral Fusion Proteins

3.2.1 Global Structural Organization of Class III Fusion Proteins

Before introduction of class III type proteins, all viral fusion proteins had been categorized as class I or II fusion proteins [23–25]. Class I proteins, the best characterized being influenza virus hemagglutinin, contain functionally critical α-helices, and in the post-fusion conformation contain a prominent trimeric α-helical coiled coil [26]. They are present as trimers in both pre- and post-fusion states. The fusion protein E of tick borne encephalitis virus was the first viral protein, whose structure was shown to be radically different [27] from any described class I fusion protein, prompting the definition of a new class of fusion proteins (class II). Class II fusion proteins are mostly made of β-sheets, and unlike class I proteins, which remain trimeric during the conformational change from pre- to a post-fusion state, class II proteins are present as pre-fusion dimers that convert to post-fusion trimers.

Class III fusion proteins are transmembrane proteins, composed of a large ectodomain, followed by a single transmembrane region and a smaller cytosolic domain (C-terminal domain or CTD). The X-ray structures of the ectodomains of HSV-1 gB [28], EBV gB [29], VSV G [30, 31] and Baculovirus gp64 [32] became available in the past four years. VSV G is the only class III protein for which the structures of both pre-fusion and post-fusion (low-pH) states have been solved. Based on the structural homology of gB and gp64 with the latter form of G, their conformations were proposed to represent post-fusion states as well. Since there is currently limited functional data (see Section 3.4.2) and no experimental structural data describing the pre-fusion forms of gB and gp64, the following section will focus on the description of the putative post-fusion forms of the class III fusion proteins.

Crystallized ectodomains of all class III type proteins form post-fusion trimers (VSV G is a trimer in pre-fusion state as well). There is a centrally located > 40 residue long helix, that forms a coil (reminiscent of the coiled coils found in class I fusion proteins), and around which the three protomers wrap in a left-handed twist, forming an elongated rod-like molecule (Fig. 3.1). In addition to

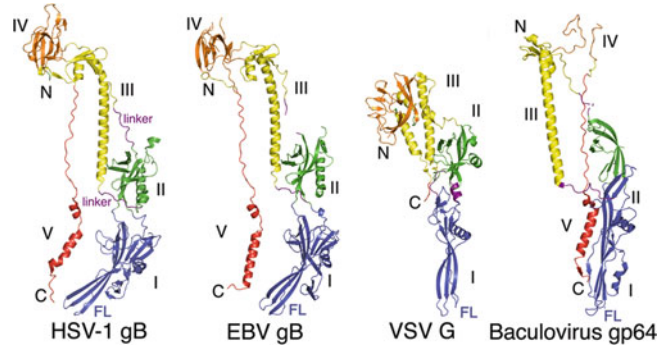

Fig. 3.1 Structures of class III fusion proteins. Three-dimensional, X-ray structures of the post-fusion, monomeric ectodomains of HSV-1 gB, EBV gB, VSV G and Baculovirus gp64 are shown. Domains are colored as in Fig. 3.1, and are labeled with roman numbers I to V. N and C are used to designate the amino and carboxy termini of the crystallized ectodomains. Linkers are colored in *purple* in all four panels. FL is used to designate the "fusion loops". The cartoon representations were generated in Pymol [71], using the following Protein Databank identifiers: 2GUM for HSV-1 gB, 3FVC for EBV gB, 2CMZ for VSV G and 3DUZ for Baculovirus gp64

the helical secondary structure elements, class III proteins contain β-sheets, the mixture of α and β structures giving rise to the distinct molecular architecture.

Ectodomains of class III fusion proteins share a similar secondary structure topology and three dimensional arrangement (Fig. 3.1). Yet, there are significant differences in the size of the ectodomains and their individual domains. G and gp64 ectodomains are more compact (∼450 residues), compared to the gB ectodomain which contains more than 700 residues. Correspondingly, the G and gp64 crystallized spikes are shorter (12.5 and 15 nm, respectively), while gB trimers spikes are ∼16 nm long. Conservation of the core structures suggests a common evolutionary origin, while the differences in the size and complexity of some domains indicate that class III proteins might have evolved to carry out functions specific to the viruses they belong to.

3.2.2 Domain Organization of Class III Fusion Proteins

Ectodomains of class III fusion proteins are composed of five domains. Domain I or the "fusion domain" contains a bipartite fusion peptide, composed of two loops, which carry the residues that insert into target membranes. In the post-fusion conformation, the fusion loops are exposed and located at one end of the rod-like trimer, in close proximity of the ectodomain C-terminus (domain V) (Fig. 3.1), which proceeds the transmembrane region (the latter not being part of the crystallized constructs). The whole domain I is inserted in between two β-strands of domain II (Fig. 3.2), which has a plekstrin homology (PH) fold. PH domains serve as binding surfaces for phospholipids and for protein ligands [33, 34], indicating that domain II of class III fusion proteins may interact with other proteins involved in the fusion process (for example, with the gH/gL complex or receptor binding proteins in herpesviruses). Domain II is embedded within domain III, which contains the prominent central α-helix contributing to most of the trimerization contacts. Domain III is inserted into domain IV (Fig. 3.2), located on the opposite end of the molecule from domain I and fusion loops (Fig. 3.1). Domain IV is formed of β-sheets, and exhibits the highest variability in size and structural arrangement (in gp64 it is largely disordered). Domain V is an extended segment of a polypeptide chain, that runs along the long side of the trimeric molecule, and in gB and gp64 inserts into the crevice formed by two other protomers, thereby contributing to an extensive trimerization surface. The VSV

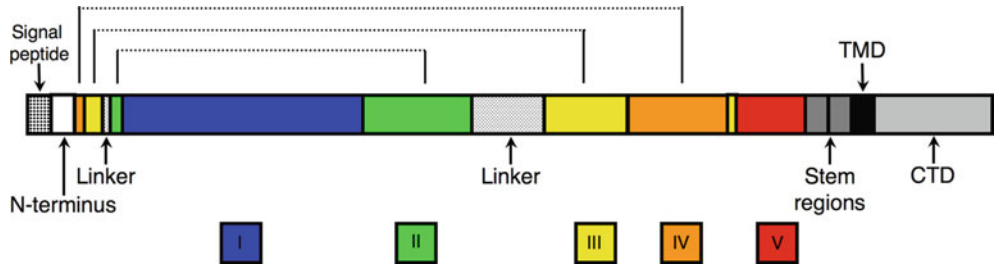

Fig. 3.2 Schematic representation of the domain organization of class III fusion proteins. The sequence of EBV gB is shown as an example. Other class III fusion proteins follow a similar global domain arrangement, although with different domain sizes and boundaries. *Brackets* shown on *top* are used to illustrate the insertion of domain I (*blue*) into domain II (*green*), which is on its own embedded in domain III (*yellow*), that is inserted in domain IV (*orange*). Domain V, which leads to the membrane-proximal stem regions, is shown in *red*. TMD and CTD designate transmembrane domain and C-terminal domain, respectively. The N-terminal region of gB, which is flexible and was not resolved in the HSV-1 and EBV structures, is shown as a *white* box and labeled as "N-terminus". Linkers are flexible regions that connect domains, and are believed to be important for conformational changes

G ectodomain lacks most of domain V, as it has been removed by proteolytic cleavage, employed to cleave the protein from the virion surface. More detailed description of the gB, gp64 and G domain structures, sizes and boundaries is reported in [35].

3.3 Functionally Important Regions of Class III Fusion Proteins

3.3.1 Fusion Loops

Fusion peptides are defined as stretches of polypeptide chain that interact with target membranes, allowing the fusion protein to be anchored in both membranes, the viral envelope via the transmembrane domain and the target membrane through the fusion peptide. Further structural changes, which occur in the fusion protein intermediate that bridges the two membranes, are proposed to bring the membranes in close apposition, resulting in their merger. Fusion peptides are secluded in the pre-fusion conformation, and become exposed to interact with the target membrane upon receiving an activating fusion trigger. Class I proteins have fusion peptides that are typically located at the N-terminus of a subunit, while the fusion peptide of class II proteins is internal and located in a loop between two β-strands. Fusion peptides of class I and II proteins are highly conserved sequences, rich in small, apolar residues (A, G, L, I), which have a high propensity to insert into lipid bilayers (reviewed in [36]).

The fusion peptide of VSV G has been located by mutagenesis [37, 38], and the structure of G ectodomain pre-fusion state [31] revealed two internal loops, located at the end of the trimer, and in a "pointy" conformation, similar to what has been observed in the fusion peptides of class II fusion proteins. The bipartite fusion peptide of G is composed of residues W^{72}, Y^{73}, Y^{116} and A^{117}. Mutagenesis studies demonstrated that the aromatic residues W^{72}, Y^{73}, Y^{116} are critical for the ability of G to mediate fusion, while A^{117} is less important [39]. Direct association of the region carrying the fusion peptide of G with lipid bilayers was observed by photolabeling studies [40].

Loops in gB, which are structurally analogous to the fusion loops of G, were proposed to form gB putative fusion peptide [28]. Mutagenesis studies confirmed that substitutions of some of these residues (shown as underlined) in HSV-1 gB (V**W**FGHR**Y**$^{173–179}$ and RVEAF**HR**Y$^{258–265}$) [41] and EBV gB (GWYA$^{111–114}$, GWLIWTY$^{192–198}$) [42] abolished the ability of gB to mediate fusion, supporting the idea that these loops play an important role in fusion. Finally, mutations of the hydrophobic

as well as charged residues (shown in bold) in the HSV-1 gB fusion loops diminished the binding of the resulting recombinant proteins to cells and naked liposomes, demonstrating that the proposed regions of gB interact with membranes directly and constitute the gB fusion peptide [43].

The fusion peptide of EBV gB contains more hydrophobic residues than HSV-1 gB, consistent with the tendency of the EBV gB recombinant ectodomains to form rosette structures [44], typically found in post-fusion preparations of class I and II fusion proteins. Rosette formation in the latter cases is driven by hydrophobic interactions of the exposed fusion loops, and indeed EBV gB ectodomains form simple trimers, which could be crystallized, only when its residues $WY^{112–113}$ and $WLIW^{193–196}$ are substituted by the analogous residues, HR and RVEA, found in HSV-1 gB.

Residues in gp64, forming loops analogous to the fusion peptide of G and gB, are $GGSLDPNT^{79–86}$ and $NNNHFA^{149–154}$ [32]. Substitutions of the hydrophobic, but also polar residues within the fusion loops (shown as underlined) result in the loss of ability of the gp64 variants to induce syncytium formation [32].

Unlike fusion peptides of class I and II fusion proteins, the bipartite fusion peptides of class III proteins are not conserved sequences. They however locate to the structurally homologous loops, which are found at the end of the spike molecule, and contain aromatic, polar residues (such as W and Y) and histidine residues, which are often found at membrane interfaces [45]. This indicates that while class III protein fusion loops may not be inserting deeply into the lipid bilayer (as peptides of class I and II proteins abundant in residues such as A, I, L, and G), they may associate with membranes in a more superficial manner, sufficient enough to destabilize the integrity of lipid bilayers and promote fusion.

3.3.2 Membrane-Proximal (Stem) Regions and C-Terminal Domain (CTD)

Membrane-proximal or stem regions of gB and G are around 40 residue long segments that precede the transmembrane domain. They are rich in hydrophobic residues, indicating that they may interact with membranes and play a role in fusion. Deletions made in the stem region of VSV G cause profound decrease in cell:cell fusion and reduce virus infectivity [46]. Grafting of the G stem regions onto heterologous fusion proteins enhances their fusion activity, and stem regions together with the CTD and the transmembrane domain (i.e. lacking the ectodomain) are sufficient to mediate fusion penetration, supporting the idea that the G stem regions have inherent fusogenic potential [47]. The last 12 residues of the G stem were also shown to be necessary for efficient virus assembly, possibly because they promote virus release by destabilizing membranes at the sites of budding [48].

The involvement of gB stems in fusion has not been systematically investigated. It is however curious that the gB proteins that have a lower content of hydrophobic and aromatic residues in their fusion loops (indicating weaker propensity to interact with membranes), have higher abundance of such residues in their membrane-proximal regions [44]. It is possible that fusion loops and stem regions complement each other in destabilizing the membranes, but this hypothesis remains to be tested.

The CTD of gB modulates the fusion activity of the ectodomain, resulting in some cases in hyperfusion or null fusion phenotypes, and demonstrating the existence of cross-talk between the gB ectodomain and its CTD through the membrane. For example, a truncation in the CTD of EBV gB results in a protein which can cause fusion in the absence of the gH/gL complex [49], and deletions in the CTD of HSV-2 gB can yield protein variants with dramatically enhanced cell fusion activity [50]. Experiments using the recombinant CTD of HSV-1 gB demonstrated that CTD associates with membranes, preferentially binding to the negatively charged lipid heads [51]. The CTD truncations that caused a hyperfusion phenotype bound poorly to artificial liposomes, suggesting that a stable CTD interaction with lipid bilayer may serve as a negative regulator of fusion.

The stem region and CTD of gp64 (around 20 and 7 residues, respectively) are shorter than those of gB and G. Deletion of the CTD only moderately decreases production of infectious virus, and has a more dramatic effect on budding efficiency, suggesting involvement of the CTD in viral maturation [12].

3.4 Molecular Basis of the Class III Type Proteins Fusion Mechanism

3.4.1 Activation Triggers for Class III Viral Fusogens

Herpesviruses enter cells through fusion of the viral and plasma membranes, in a process that is triggered by binding of a viral surface protein to a specific host-cell encoded receptor. This interaction is essential for entry as it provides an activation signal for the fusion machinery composed of gB and the gH/gL complex. Herpes simplex viruses utilize gD to bind to Nectin-1 or HVEM receptor (reviewed in [13]), while gp42 of EBV interacts with MHC class II expressed on B lymphocytes, the target cells for EBV [52]. Both gD and gp42 are membrane anchored proteins, however their soluble, recombinant ectodomains activate fusion in virus-free cell:cell fusion assays as well [53, 54]. gD and gp42 have been proposed to undergo conformational changes upon binding to cellular receptors [55, 56], suggesting that the structural alterations may provide the activation signal to the gB-gH/gL fusion machinery. How the fusion trigger is transferred to the fusion machinery, and to which of its components is not understood. pH has also been suggested to serve as a fusion trigger in cases where herpesviruses enter cells by endocytosis [57, 58].

The G fusion protein of rhabdoviruses and gp64 of Baculoviruses are activated by exposure to the acidic pH of the endosome, which occurs after the virus is endocytosed by target cells. G and gp64 have a unique capability of undergoing a reversible conformational change [59–61], and both proteins can be reactivated to induce fusion after acidification [60, 62]. This is in contrast to all other known fusion proteins, where the post-fusion state is the more thermodynamically stable one, regardless of pH, and whose conformational rearrangement is irreversible. It has been proposed that the reversible conformational change may serve to maintain the fusion proteins in a fusion-competent state after they pass through the acidic Golgi compartment, during protein trafficking to the cell surface.

3.4.2 Structural Rearrangements of Class III Fusion Proteins

VSV G is the only class III fusion protein whose structure has been solved for both the pre-fusion and post-fusion states [30, 31]. During its conformational change, which is triggered by exposure to the acidic pH of the endosome, individual domains of G relocate in a manner that results in conversion of a more compact pre-fusion (8.5 nm) to an extended post-fusion trimer (12 nm). Unlike class I and II fusion proteins, in which individual domains undergo significant refolding, the domains of G mostly retain their structure. The domain repositioning is rather a consequence of structural alterations occurring in flexible linker regions that connect domains. As a result, domain I, which carries the fusion loops, relocates 16 nm from one to the opposite side of the molecule (reviewed in detail in [22, 35]). The conformational change is proposed to be a consequence of the pH-induced protonation and deprotonation of key histidine residues, leading to a loss of a network of interactions that hold domain I and membrane-proximal regions together, thus initiation domain I repositioning.

Baculovirus gp64 contains a number of conserved histidine residues, and a similar mechanism driving the conformational change has been proposed [32]. Interestingly, in addition to the conserved intra-molecular disulfide bridges, gp64 contains an inter-chain disulfide bridge (Cys^{26}-Cys^{372}) which is located at the top of the central helix, connecting domains III of two protomers. It could be expected

that the inter-molecular disulfide imposes restrictions on domain movement and reorganization, thus being important for the fusion mechanism. Elimination of the inter-chain disulfide however was shown not to affect fusion and virion budding. However, the gp64 variant lacking the disulfide bond cannot rescue a gp64null bacmid [63], suggesting a still unknown role of the inter-chain disulfide in viral infectivity.

As mentioned in Section 3.4.1, the post-fusion, low-pH form of G and gp64 can be reactivated to adopt a fusion-component state by exposing the protein to elevated pH. Whether gB undergoes a similar reversible conformational change is not clear, and data suggesting the ability of gB to adopt different conformational states has become available just recently. Structural changes in murid herpesvirus gB were detected upon exposure to low pH [64], as well as in HSV-1 gB [65]. In the latter case, the observed conformational changes in the antigenic structure were reversible, and could be detected in purified, recombinant gB material as well. pH-dependent entry of herpesviruses via endocytosis has been reported for certain herpesviruses in combination with specific target cell types, for example in the case of HSV-1 entry into human epithelial cells [58]. Whether gB undergoes a conformational change during receptor-mediated entry, which is the predominant entry pathway of herpesviruses, still needs to be determined.

Unlike Baculoviruses and rhabdoviruses which have a single fusogen protein, herpesviruses have evolved a more complex machinery that in addition to gB contains the gH/gL complex. The structure of the gH/gL ectodomain from HSV-1, EBV and pseudorabies have become available recently [66–68], and revealed a protein with a fold that does not resemble any known fusion protein. Functional data show that while the gH/gL complex mediates hemifusion, gB is required for fusion to proceed to formation of a fusion pore thus allowing capsid entry [7]. gH/gL and gB form transient complexes, but only when gD (as membrane-anchored or soluble, recombinant ectodomain) is added, indicating that the fusion complex assembles only when gH/gL, gB or both components of the fusion machinery receive an activating signal from the receptor-binding protein gD [69]. Finally, by using a panel of monoclonal antibodies that bind to distinct domains of gB, it was suggested that the fusion process begins with the insertion of gB fusion loops into target membrane, followed by a gB–gH/gL interaction, and eventually fusion [70]. What the molecular mechanism of these events and what kind of structural changes, if any, occur in gB and gH/gL to drive fusion, are some of the questions that need to be answered.

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Chapter 4
Human Trophoblast in Trisomy 21: A Model for Cell–Cell Fusion Dynamic Investigation

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Abstract Trophoblastic cell fusion is one essential step of the human trophoblast differentiation leading to formation of the syncytiotrophoblast, site of the numerous placental functions. This process is multifactorial and finely regulated. Using the physiological model of primary culture of trophoblastic cells isolated from human placenta, we have identified different membrane proteins directly involved in trophoblastic cell fusion: connexin 43, ZO-1 and recently syncytins. These fusogenic membrane retroviral envelop glycoproteins: *syncytin-1* (encoded by the HERV-W gene) and *syncytin-2* (encoded by the FRD gene) and their receptors are major factors involved in human placental development. Disturbances of syncytiotrophoblast formation are observed in trisomy 21-affected placentas. Overexpression of the copper/zinc superoxide dismutase (SOD-1), encoded by chromosome 21 as well as an abnormal hCG signaling are implicated in the defect of syncytiotrophoblast formation. This abnormal trophoblast fusion and differentiation in trisomy 21-affected placenta is reversible in vitro by different ways.

4.1 Human Placenta and Trophoblast Differentiation

In mammals, embryonic development requires a placenta that forms by implantation of the blastocyst in the maternal organism. The placenta is an autonomous and transitory organ which allows nutritional and gas exchanges between the fetus and the maternal organism. In addition, the endocrine and immunological functions of the placenta are essential in pregnancy and for fetal growth. The human placenta is characterized by extensive invasion of trophoblasts into the maternal uterus allowing direct contact of trophoblasts with the maternal blood (hemomonochorial placentation) [1], and by the extent and specificity of its hormonal production [2]. In this tissue, steroid and protein hormones such as human chorionic gonadotropin (hCG), human placental lactogen (hPL), placental growth hormone (PGH), are produced in large amounts, unparalleled in other mammals [3].

In the human placenta, the trophoblast differentiates along two major pathways both critical for normal placental function [2] (Fig. 4.1). In the extravillous trophoblast invasive pathway, the cytotrophoblastic cells of the anchoring villi in contact with the uterus wall proliferate, detach from the basement membrane and aggregate into multilayered columns of non-polarized cells that invade the uterus wall (Fig. 4.1). These cells, which compose the extravillous cytotrophoblast (EVCT), invade the endometrium, the first third of the myometrium and the associated spiral arterioles. During the first trimester of pregnancy these cells form plugs that occlude the tips of the spiral arteries. This

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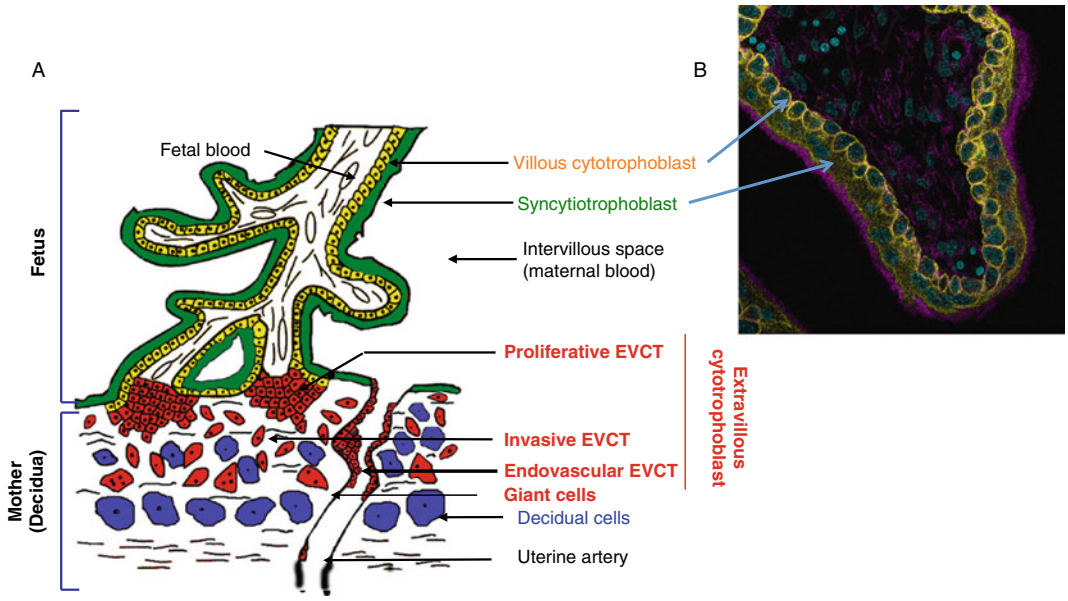

Fig. 4.1 Human chorionic villi. (**a**) Schema of human chorionic villi. In humans, the chorionic floating villi are in contact with the maternal blood in the intervillous space. In these villi, cytotrophoblastic cells differentiate by fusion to generate the syncytiotrophoblast. In the anchoring villi, the cytotrophoblastic cells proliferate and invade the decidua. The extravillous cytotrophoblastic cells (EVCT) invade the uterine stroma and differentiate into giant cells and invade also the lumen of uterine arteries. (**b**) First trimester chorionic villi with a continous cytotrophoblastic cell layer and syncytiotrophoblast with its microvillous membrane

trophoblastic endovascular invasion is of major importance for feto-placental physiology: intra-arterial plugs of endovascular trophoblasts prevent, until the 12th week of gestation, access of maternal blood to the intervillous space and therefore protect the conceptus from excessively high oxygen levels during this very critical stage of development. At the beginning of the second trimester these plugs delete progressively allowing maternal blood to circulate into the intervillous space. In the villous trophoblast pathway, the trophoblast covers the mesenchymal core of the chorionic villi (fibroblasts, fetal vessels, macrophages also called Hofbauer cells). In this pathway, the cytotrophoblastic cells of the floating villi proliferate, differentiate and fuse to form a syncytiotrophoblast (ST) that covers the entire surface of the villi (Fig. 4.1). The syncytiotrophoblast layer plays a major role throughout pregnancy, since it is the site of numerous placental functions, including ion and nutrient exchange and the synthesis of steroid and peptide hormones required for fetal growth and development. This multinucleated syncytiotrophoblast is regenerated along pregnancy by a continuous turnover process including proliferation of underlaying mononuclear cytotrophoblasts (CT), fusion of these cytotrophoblasts into syncytiotrophoblast and progression toward apoptosis.

4.2 Membranes Proteins Involved in Human Trophoblast Fusion and Differentiation

Villous trophoblast differentiation can be reproduced in vitro. Purified cytotrophoblastic cells isolated from human placentas adhere to plastic dishes, aggregate and then fuse, forming a multinucleated syncytiotrophoblast with pregnancy-specific hormonal production (i.e. the human chorionic gonadotropin hormone: hCG) [4, 5] (Fig. 4.2).

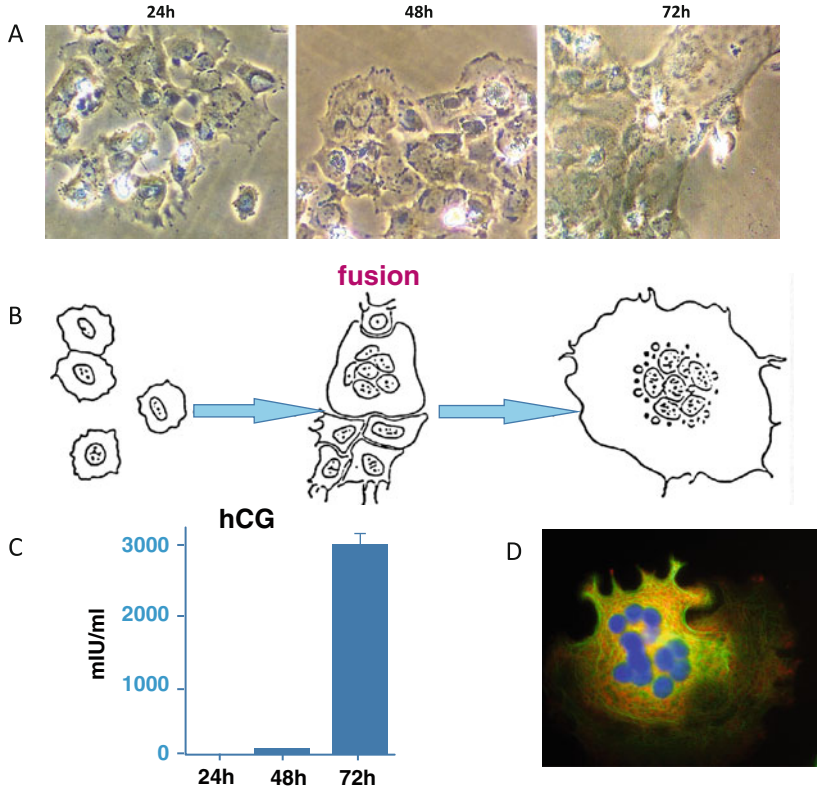

Fig. 4.2 In vitro human trophoblastic cells differentiation. (**a**) Morphological differentiation of isolated cytotrophoblast cultured on plastic dishes. After 1 day pseudopodia of CT are making contact with neighboring CT. After 2 days, CT are mainly aggregated. After 3 days, large syncytiotrophoblast (ST) are observed with central nuclear mounts. (**b**) Illustration of the in vitro human trophoblast differentiation. (**c**) Levels of hCG secreted into the culture medium at the indicated times. (**d**) Syncytiotrophoblast formed in vitro. Nuclei are stained *blue* with DAPI. Human placental lactogenPL known to be expressed mainly by the syncytiotrophoblast is detected by *red* fluorescence

This physiological model coupled with antisense or siRNA strategy has allowed to identify membrane proteins required for human trophoblast fusion: cadherin 11 [6], CD98 [7], ADAMs12 [8], connexin 43 [9], ZO-1 [10]. Among these factors some are highly or specifically expressed in the human placenta such as retroviral envelop proteins recently shown to be directly involved in trophoblast differentiation [11].

In humans, two *env* genes carried by endogenous proviruses belonging to the HERV-W and HERV-FRD families, and designated *syncytin-1* (*syncytin*/HERV-W) and *syncytin-2* (*HERV-FRD*), respectively, have been identified for which convincing evidence of their involvement in placental physiology could be provided [12–14]. The HERV-W locus is located at chromosome band 7q21-q22 and the HERV-FRD at chromosome band 6p24.1. *Syncytin* genes have been highly conserved in evolution, from the time when the respective HERV-W and HERV-FRD retroviruses carrying them entered the primate lineage 20 and 40 Myr ago, respectively, and they display remarkably few polymorphisms in the extant human population, both facts providing strong evidence for purifying selection [12, 15, 16]. Both the human syncytin-1 and -2 proteins were found to be highly fusogenic in *ex vivo* cell–cell fusion assays, but the cells prone to fusion in each case being different suggested different receptor usage [12]. Transcriptional regulation of the *syncytin-1* gene has been extensively investigated. The placenta-specific transcription factor Glial-Cell Missing 1 (GCM1; also known as GCMa)

has been shown to regulate *syncytin-1* expression. Moreover a selective and temporal unmethylation of ERV-W in placenta during the first trimester may allow *syncytin-1*-mediated cell fusion [17]. Transcriptional regulation of the *syncytin-2* gene has been recently investigated [18], GCM1 is also a critical factor for trophoblastic cell fusion, through transcriptional regulation of GCM2. In addition GCM1 may also play a role in the epigenetic regulation of *syncytin-2* gene expression.

Both the receptors for *syncytin-1 and -2* have been found. That of *syncytin-1* was identified as a sodium-dependent neutral amino acid transporter variously designated as ASCT2/ATB°/SLC1A5 [13]. The receptor for *syncytin-2* was identified as encoding a multi-pass transmembrane protein, named Major Facilitator Superfamily Domain Containing 2 (MFSD2) that belongs to a large family of putative carbohydrate transporters conserved in evolution. This gene was mapped to chromosome 1p34.2 Other Env proteins, ERV3 (HERV-R) were found in placenta [19, 20] but their role, if any, in the physiology of the organ remains to be established.

4.2.1 Syncytin-1

By in situ hybridization on term placental sections, HERV-W family mRNAs were initially detected only in the ST of the villous trophoblast [14]. However, depending on the specificities of antibodies and/or the various staining methods used there is little consensus regarding *syncytin-1* localization in the villous trophoblast: syncytiotrophoblast, cytotrophoblast. Interestingly, in vitro isolated primary CTs from early and term placentas express the *syncytin-1* transcripts [11]. More recently according to Muir [21], in all first and second trimester villous tissues examined, *syncytin-1* is not confined to the ST, but is also detected strongly in the underlying CTs. Using two different monoclonal antibodies, we have confirmed this double localization [22]. *Syncytin-1* was also immuno-localized in all the cell types of the extravillous phenotype lineage [23]. *Syncytin-1* induces the formation of syncytia upon interaction with the type D mammalian retrovirus receptor, ASCT2 (see above). Using a polyclonal antibody, the type D mammalian virus receptor was localized in the various trophoblasts with the villous and extravillous phenotypes [23]. The colocalization of *syncytin-1* and its receptor in some trophoblastic cells that do not fuse (proliferative, intermediate and endovascular extravillous trophoblastic cells) suggests that *syncytin-1* and its receptor appear to be required but are not sufficient for trophoblastic cell fusion.

4.2.2 Syncytin-2

Using a highly specific monoclonal antibody *syncytin-2* is detected only in the cytoplasm of some cytotrophoblastic cells of the villous trophoblast [24]. Interestingly, this localization highlights the modification of cytotrophoblastic cells shape from cuboidal in early placenta to flat with cytoplasmic processes in term placenta [24]. Furthermore, in vitro detection of *syncytin-2* transcripts is restricted to villous CTs and decreases significantly with time in culture (Fig. 4.4). In vitro immunostaining is also observed in some aggregated CTs, with a stronger staining at the intercellular boundaries [25]. Consistent with these results, *syncytin-2* transcripts were recently detected in situ only in CTs [26, 27]. In situ hybridization of human placental tissue using an MFSD2-specific probe provided evidence for *syncytin-2* receptor expression at the level of the ST. Taking into account the difference in expression of *syncytin-2* and its receptor MFSD2 between trophoblastic cell types, a model can be proposed in which an oriented process of cell–cell fusion takes place with “in-fusion” of the mononucleated CT expressing *syncytin-2* into the ST expressing MFSD2 [26]. *Syncytin-2* is highly fusogenic when overexpressed by transfection in various cultured cells and recently its role in villous trophoblastic cells fusion was demonstrated using siRNA strategy [28].

4.2.3 *Syncytin-1 and Syncytin-2 Expression During In Vitro Trophoblast Differentiation*

Using our well-established in vitro model of villous and extravillous CT isolation and culture, we investigated the levels of *syncytin-1* and *syncytin-2* transcripts in these cells. *Syncytin-1* is expressed at a higher level in villous as compared to extravillous CTs [23], whereas *syncytin-2* is only expressed in villous CTs. Interestingly, during in vitro fusion and differentiation of villous CTs into ST, a striking difference in expression was observed between the two syncytin genes. While *syncytin-1* expression increases with cell aggregation and fusion, *syncytin-2* transcripts decrease upon fusion of CTs into ST (Fig. 4.4).

4.3 Abnormal Trophoblast Fusion and Differentiation in Trisomy 21

Trisomy of chromosome 21 (T21), which causes the phenotype known as Down syndrome, is the major known genetic cause of mental retardation and is found in around 1:800 live births.

Little is known about placental development in this aneuploid condition despite the fact that the trophoblast carries the genetic abnormality. Different histological observations have pointed out that trisomy 21 may be associated with villous hypovascularity [29], intrastromal cytotrophoblastic cells, and abnormalities of the trophoblastic layer [30]. Indeed an increased percentage of two-layer trophoblast is observed in T21 placenta suggesting a delay in villous maturation and trophoblast differentiation [31].

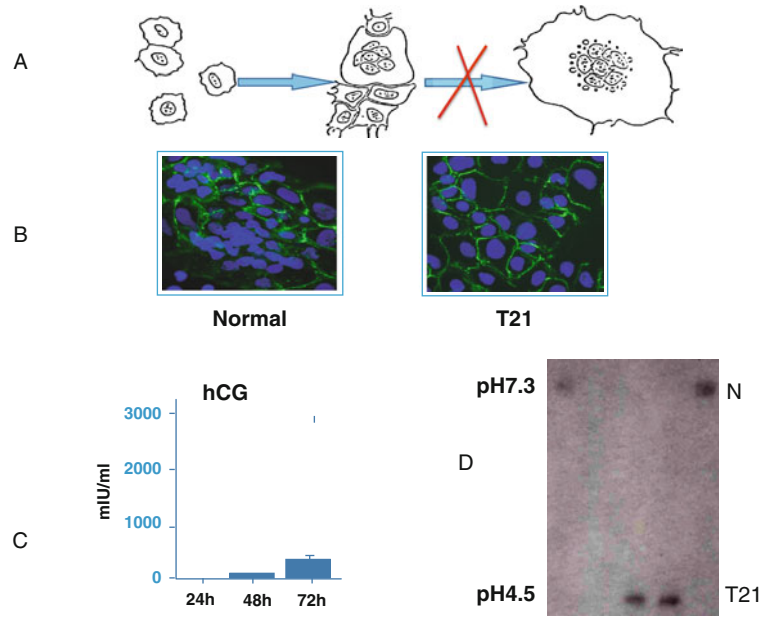

Fig. 4.3 In vitro differentiation of human trophoblastic cells isolated from Trisomy 21-affected placentas. (**a**) Illustration of the absence of cell–cell fusion observed in cultured human trophoblast isolated from trisomy 21-affected placentas. (**b**) Differentiation of CT into ST, at 24 and 72 h of culture, with normal and T21 cells. The cells were visualized by immunostaining with an anti-desmoplakin monoclonal antibody. Nuclei were counterstained by DAPI. At 72 h, normal CT had fused, as immunofluorescence staining of the cell boundaries disappeared, owing to the formation of a large syncytium containing many nuclei. T21 cytotrophoblasts were still aggregated and had not fused. (**c**) Levels of hCG secreted into the culture medium at the indicated times. (**d**) Detection by immunoelectrofocusing and immunoblotting using an anti hCG polyclonal antibody [35] of a highly acidic form of hCG in the T21 cell culture medium

Cultured cytotrophoblasts, isolated from T21-affected placentas, aggregate but fuse poorly or belatedly [32, 33] (Fig. 4.3). Indeed in our unique experience of primary culture of human cytotrophoblastic cells isolated from second trimester control ($n = 44$) and trisomy 21-affected placentas ($n = 71$), we observe that this abnormal trophoblast fusion and differentiation occurs in more than 90% of the primary culture of T21 cells. This is in agreement with previous histological observations pointing to an increased percentage of two layered trophoblast in trisomy 21-affected placentas [30, 31] (Fig. 4.5). In addition, this in vitro defect or delay in syncytiotrophoblast formation is characterized by a dramatic decrease in the synthesis of syncytiotrophoblastic pregnancy-associated hormones [34] and by the secretion of an hyperglycosylated hCG with low bioactivity [35] (Fig. 4.3). Overexpression of the copper/zinc superoxide dismutase (SOD-1), encoded by chromosome 21 [36, 37] is implicated in this abnormal trophoblast fusion and differentiation. Indeed SOD-1 mRNA expression ($p < 0.05$), protein levels ($p < 0.01$), and activity ($p < 0.05$) are significantly higher in trophoblast cells isolated from trisomy 21-affected placentas than in trophoblast cells from gestational age matched normal placentas. In addition experimental over-expression of SOD-1 in normal cytotrophoblasts impairs ST formation [36]. This defect in ST formation is associated with a significant decrease in mRNA transcript levels and secretion of hCG and of other pregnancy specific hormones. In addition Pidoux et al. [38] demonstrated that human trophoblast differentiation is impaired by abnormal hCG signaling. In trisomy 21 there is a marked decrease in the number of mature hCG receptor (LH/CG-R) molecules expressed at the surface of the cytotrophoblasts associated with the secretion of an abnormally glycosylated hCG with low bioactivity.

4.4 Cell–Cell Fusion: A Dynamic Process

Several steps are needed before two trophoblastic cells can fuse. Firstly, the cells must leave the proliferative stage and express genes and proteins involved in the fusion process. Secondly, the cells must recognize and interact to their fusion partner. Thirdly, the cells must communicate together, allowing signals exchange. Lastly, the cells can fuse. Actually, we hypothesize that the trophoblast cell–cell fusion mechanism involves several partner proteins within a fusogenic complex, in a multi-factorial and dynamic process. This fusogenic complex remains to be characterized and besides the previously mentioned membrane proteins all the constituent proteins of this complex need to be identified.

In addition this dynamic process may be tightly regulated and coordinated. As shown in Fig. 4.4, it appears that *syncytin-2* and ZO-1 are highly expressed in isolated CTs and that their expression rapidly decrease during ST formation. On the other hand, *syncytin-1* and Cx43 mRNA increase with cell aggregation and fusion and then slightly decrease. Proteins expression follows the same pattern (data not shown). In addition, these results illustrate the striking difference in *syncytin-1 and -2* localization: *syncytin-2* only in some CT and *syncytin-1* in all CT and ST.

As shown in Figs. 4.3 and 4.4, CT isolated from T21 affected placenta, aggregate normally but do not fuse or fuse poorly. In these cells, transcript levels of Cx 43, ZO-1, *syncytin-1 and -2* do not vary with time in culture (Fig. 4.4). Proteins expression follows the same pattern (data not shown).

4.5 In Vitro Reversibility of Abnormal Trophoblast Fusion and Differentiation in T21

Interestingly, we have recently demonstrated that the addition of a recombinant hCG (rhCG, 10^{-8}M) to the culture medium of T21-affected CT induced syncytiotrophoblast formation, the final stage of villous trophoblast differentiation [38]. Similar results were obtained in the presence of 8 bromo-cAMP (P. Gerbaud, personal data).

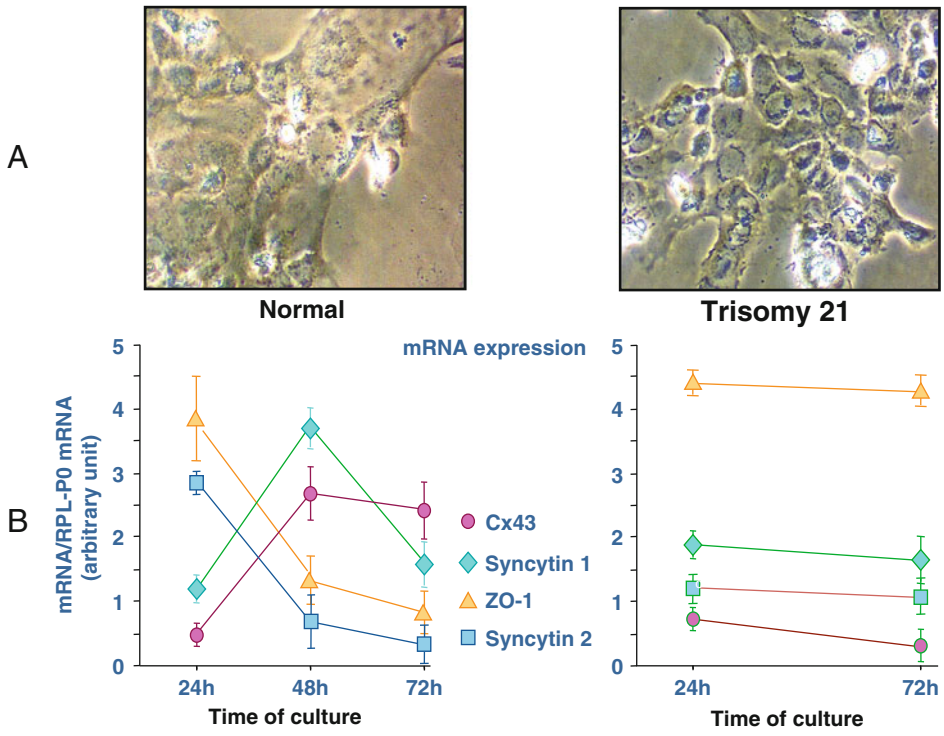

Fig. 4.4 Evolution of membranes protein expression during in vitro differentiation of trophoblastic cells isolated from normal and trisomy 21-affected placenta. (**a**) Morphological differentiation during in vitro culture of normal (*left panel*) and T21 trophoblastic cells (*right panel*). Cytotrophoblastic cells were purified from three distinct age matched (second trimester) normal and T21-affected placentas and separately cultured. The cells were visualized under phase contrast light microscopy. At 72 h, normal cytotrophoblastic cells had fused resulting in the formation of a large syncytium containing numerous nuclei. In contrast, T21 cytotrophoblasts were still aggregated and had not fused. (**b**) Real-time RT-PCR analysis of *syncytin-1*, *syncytin-2*, ZO-1 and Cx43 mRNA during in vitro differentiation of normal (*left panel*) and T21 trophoblastic cells (*right panel*). Total mRNA were extracted after 24 and 72 h of culture. Data are expressed as the level of each mRNA normalized to that of RPL-P0 mRNA (with permission of Malassine et al. [25])

This suggests that in trisomy 21 the PKA (protein kinase A) signaling might be affected including an abnormal binding of cAMP to the regulatory subunits of the enzyme in relation with the abnormal oxidative state of the cell [39], a decrease of phosphorylation of target proteins and/or an abnormal subcellular localization due to a deficient binding to anchoring proteins [40].

In situ the syncytiotrophoblast is bathing in the maternal blood at the beginning of the second trimester after the progressive disappearance of trophoblast plugs. Therefore the continuous turn over of villous trophoblast might be regulated by different factors: the quality of the pool of progenitors cells present within the cytotrophoblast layer [41], circulating factors from maternal blood, such as IGF2 recently showed to stimulate cytotrophoblast proliferation [32], but also from the cross talk between the mesenchymal cells of the chorionic villi and the trophoblast. Indeed we recently showed that conditioned media obtained from mesenchymal cells of normal placenta induced a reversibility of abnormal T21 cytotrophoblastic cells fusion by a non cAMP-dependent protein kinase pathway. Using a commercial protein array we analyzed cytokines secreted in conditioned media by both control and T21-affected mesenchymal cells. Among them activin-A is highly secreted by these mesenchymal cells but at significantly ($p < 0.01$) lower level in case of T21. Activin-A stimulated T21-trophoblast fusion.

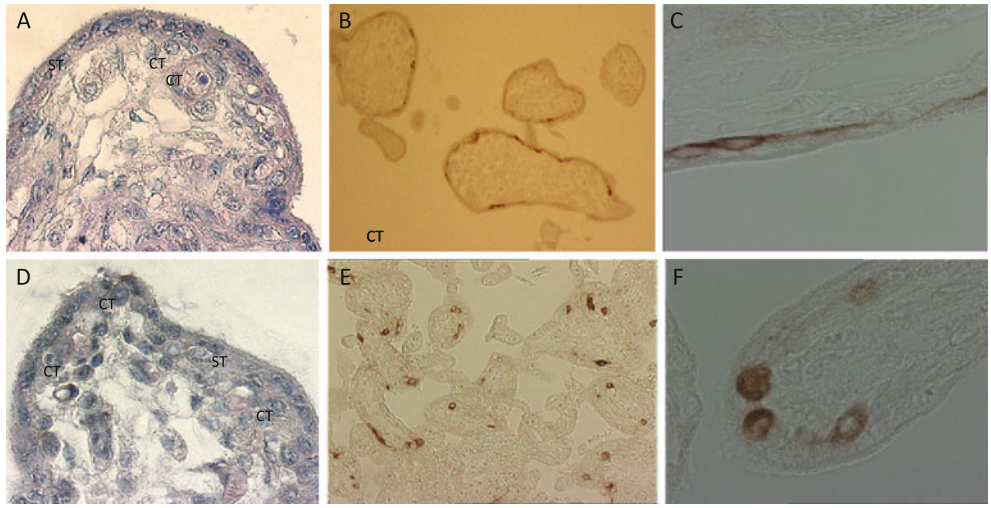

Fig. 4.5 Microscopic morphology of second trimester chorionic villi of normal placentas. (**a**) (19 weeks of amenorrhea) and trisomy 21-affected placentas (**d**) (18 weeks of amenorrhea). In normal placenta, a large amount of cytotrophoblastic cells (CT) have fused into a thin multinucleated syncytiotrophoblast (ST). In trisomy 21-affected placenta, many cuboidal cytotrophoblastic cells (CT) are still present beneath the syncytiotrophoblast (ST) increasing the thickness of the trophoblastic layer. Immunohistochemical analysis of *syncytin-2* (HERV-FRD Env) in age-matched second trimester (19 weeks) normal (**b**, **c**) and T21-affected placentas (**e**, **f**). Immunostainig with anti-*syncytin-2* antibody shows positive reactivity in elongated cytotrophoblastic cells in normal placentas and cuboidal cells in trisomy 21-affected placentas

These results show that abnormal T21 trophoblast fusion and differentiation are reversible in vitro. An abnormal paracrine cross talk between the mesenchymal core and the trophoblast could be involved in the delay of trophoblast fusion and differentiation but also in placental development observed in T21 placentas. This is well illustrated by the immunolocalization of syncytin 2 in control and T21-affected placentas (Fig. 4.5). Indeed *syncytin-2* immunostaining highlights the modification of CT shape from cuboidal in first trimester placentas to flat with cytoplasmic processes in term placenta [24]. As illustrated in Fig. 4.5 in second trimester control placentas, cytotrophoblasts are elongated, while in T21-affected placentas, these cells are still cuboïdal.

In conclusion the study of placental differentiation in trisomy 21 and the use of a well established in vitro model of trophoblast fusion, allow us to demonstrate that the abnormal cell behavior and differentiation in T21 can be reversed at least in vitro. We highlight the abnormal glycosylation of hCG in trisomy 21 impairing its biological activity and therefore inducing a delay in trophoblast differentiation. We also demonstrate that soluble signals are produced from the mesenchymal core of the villi and are involved in the trophoblast turnover. The pertinence of this trophoblast model also suggests that in trisomy 21 abnormal glycosylation process might be a key event involved in the abnormal phenotype.

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Chapter 5
Cell Fusion and Hyperactive Osteoclastogenesis in Multiple Myeloma

Franco Silvestris, Sabino Ciavarella, Sabino Strippoli, and Franco Dammacco

Abstract Multiple myeloma (MM) is a hematologic malignancy whose progression may account for uncontrolled osteoclastogenesis promoted by the malignant plasma cells within the marrow microenvironment. Osteoclasts are multinucleated cells derived from the fusion of myeloid progenitors such as monocytes/macrophages, in response to specific differentiation factors released within the marrow niche, that are significantly deregulated in MM. In this malignancy DC-STAMP, a major fusogen protein enrolled by pre-osteoclasts, is highly expressed by peripheral macrophages, whereas dendritic cells and myeloma plasma cells show high fusogenic susceptibility and under specific conditions transdifferentiate to osteoclasts. In particular, the malignant plasma cells, besides altered ploidy, expression of cancer stem cell phenotype and high metastasizing capability, are able to express phenotypic markers of osteclasts, namely the proteolytic enzymes for the bone matrix, and to activate the β3 transcriptional pathway leading to ERK1/2 phosphorylation and initiation of the bone resorbing activity. Thus, based on the imbalanced osteoclast formation and activity that involve cells constitutively uncommitted to osteoclast differentiation, both homotypic and heterotypic cell fusions in myeloma marrow microenvironment represent a major pathogenetic event that drives the development and progression of the skeleton devastation typical of the myeloma bone disease.

5.1 Introduction

Cell fusion involved in both cancerogenesis and tumor progression was originally described in the early 1900s by Otto Aichel, who postulated that spontaneous amalgamation of cell membranes between either homotypic or heterotypic cells forming a new cellular element is a biological event mediated by fusogenic factors such as viruses, that may result in chromosomal abnormalities leading to cancer development [1, 2]. At variance from the homotypic cell fusion that is considered a recurrent event in physiology, the heterotypic fusion is a rare phenomenon whose relevance in cancerogenesis has been recently revitalized as one of the driving forces in tumor development. Moreover, somatic hybridization of heterotypic cells to produce hybridomas secreting monoclonal antibodies [3] supports the in vitro reproducibility of this phenomenon and emphasizes its role in vivo, in particular in pathologic conditions including cancer [4, 5].

Spontaneous cell fusion has been reported in a number of in vitro models of human cancers [6], while its in vivo occurrence in animal models is estimated to be as low as 1% [7]. Its efficiency in cancer development seems correlated to the malignancy degree of the tumor cells [8] and their relative

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fusogenicity appears to be dependent on several factors [9, 10]. A primary mechanism is related to the structurally enveloped viruses that interact with cell membranes and stimulate the tendency of infected tumor cells, as those of both liver and cervical cancers, to prearrange their reciprocal membrane integration leading to the cell fusion [11]. In this context, fusogenic proteins functionally expressed by tumor cells after their viral infection exert a critical function. For instance, syncytin, an Env protein of HERV-W human retroviruses acting as mediator of the cell fusion in trophoblasts, has been demonstrated to prime both breast cancer cells and endothelial cells to homotypically fuse between themselves [12]. Another fusogenic membrane protein is CD44, a transmembrane receptor physiologically expressed by osteoclasts during their differentiation and formation of syncytia, but commonly detectable on the cancer cell surface and usually considered as a marker of poor prognosis and cancer stemness phenotype [13].

Chronic inflammation of cells has been shown to promote cell aggregation and then heterotypic fusion between hematopoietic precursors and cardiomyocytes, hepatocytes, skeletal myocytes and Purkinje cells during their tissue repair and remodeling programs [14]. Since the tumor microenvironment resembles an inflamed site that chemoattracts inflammatory cells, the fusion between tumor and hematopoietic marrow cells is strictly dependent on mechanisms that finely regulate the inflammatory response against the tumor and involves a number of soluble factors and mediators of the inflammation [15].

Cell fusion in tumor development and progression is also induced as an effect of tumor cell phagocytosis by macrophages [16]. Finally, as a mechanism driving the clonal expansion of tumor cells, formation of syncytia usually accelerates the proliferative rate in fused cells that ultimately undergo uncontrolled cell replication typical of the majority of hematologic and solid tumors. However, although the cancerogenesis implies the upregulation of the cell cycle, accidental cell fusions by somatic hybridization have been reported to occasionally induce its arrest in in vitro experimental tumor models [17]. Indeed, fused cells expressing the oncogenes E1A and H-RAS, overrule the cell cycle arrest and become tumorigenic cells after chromosomal rearrangement [18].

By using lineage-specific markers it is also possible to recognize tumor cells deriving from heterotypic fusions in culture, whereas the fusion events occurring in vivo in human tumors are usually undetectable for the lack of genetic tracing markers. In renal cancers, developed in patients transplanted with allogeneic bone marrow, tumor cells showed kariotypes of the bone marrow donors that were probably inherited by somatic fusion of cancer cells with the marrow cells [19]. Similarly, osteoclasts in bone biopsies of patients with multiple myeloma were shown to include nuclear cytogenetic alterations with typical chromosomal translocation of the malignant cells [20].

Other aspects supporting the cell fusion as a recurrent event in cancer development include premature chromosomal condensation as a marker for heterophasic cell fusion in a number of tumors [21], the formation of polykaryons in culture [22], as well as the hyperploidy observed in cells from ovarian, prostate, colon and breast tumors that typically correlate with their highly malignant behavior and progression [23].

Thus, although in the absence of clear-cut evidence of the pathogenetic role of cell fusion in cancer development, data accumulating in the last few years support the pivotal role of this event in cancerogenesis. Here, we will summarize the main studies related to this phenomenon in MM, including our own work.

5.2 Cell Fusion in Multiple Myeloma

Despite the introduction of new therapies, both cancerogenesis and molecular pathogenesis of multiple myeloma (MM) are still undefined. However, a number of genotypic and phenotypic aspects emphasize the potential role of the cell fusion in MM development.

5.2.1 Ploidy Abnormalities

A primary evidence that MM may represent the product of homotypic and heterotypic cell fusions is provided by the nuclear content of malignant plasma cells. Genetic aberrations including structural and/or numerical chromosome abnormalities have been consistently described in MM cells [17, 18] and their recurrence in the disease evolution from the stage of monoclonal gammopathy of undetermined significance (MGUS) to overt MM, has provided new models for studying the disease pathogenesis and progression [19].

Recurrent MM cell abnormalities, detectable in approximately 90% of all MM patients at different clinical stages, include chromosomal translocations and euploidy alterations, such as deletions, monosomies, hyperdiploidy, and trisomies. Recurrent chromosomal translocations include t(4;14), t(11;14), t(14;16), and t(14;20), as well as translocations of the immunoglobulin heavy and light chain loci [20]. These gene alterations have been primarily implicated in the pathogenesis of MM since FISH-based cytogenetic analysis has demonstrated these aberrations in nearly 50% of MGUS patients, approximately 70% of those with intramedullary MM, 80% of those with primary plasma cell leukemia, and in more than 90% of human MM cell lines [21–23]. On the other hand, aneuploid MM is frequently associated with the trisomies of chromosomes 3, 5, 7, 9, 11, 15, 19 and 21, whereas the most common monosomies variably affect chromosomes 13, 14, 16 and 22. Overall, chromosome analysis in MM clusterizes patients into four typical patterns including hypodiploid, pseudodiploid, hyperdiploid, and near tetraploid states, but none of these chromosomal abnormalities is predictive of the disease progression. However, both numerical and structural aberrations occur in the early phases of MM development and patients bearing specific chromosomal abnormalities undergo different clinical outcomes, thus reflecting the genetic instability of myeloma in both clinic and prognostic heterogeneity of the disease [24, 25]. The mechanisms underlying these karyotype abnormalities are at present unknown, albeit telomerase dysfunctions, spindle checkpoint abnormalities, centrosome aberrations as well as cell fusion are thought to variably contribute to the genetic instability that primes the plasma cell of MGUS to switch from a precancerous phenotype to a highly malignant one.

The potential contribution of accidental cell fusion in MM development has been recently attributed to the “mitotic catastrophe” (MC), namely a fusion-related mechanism of cell death underlying tumor aneuploidy [26]. MC, in fact, results from premature or inappropriate mitosis of cells with deregulated checkpoints of the cell cycle. After an incomplete DNA replication and abnormal chromosome segregation, cells undergoing the MC acquire a tetraploid genotypic pattern and can generate aneuploid progenies as a consequence of subsequent asymmetric divisions. Thus, a minority of these abnormal cells could survive and contribute to oncogenesis. Typically, the cell fusion between mitotic and interphase cells usually results in the formation of giant non-viable cells with numerous chromatin-dispersed micronuclei, that is the morphologic pattern of the MC. These giant cellular elements have been described in different tumor models [27, 28]. With regard to MM, by analyzing the cell cycle in RPMI8226 MM cell line, we have found similar multinucleated giant cells in myeloma, thus supporting the hypothesis that fusion events resulting from the MC may also occur in this hematologic malignancy. Figure 5.1 depicts myeloma giant cells undergoing abnormal division with the typical morphologic pattern of spindle alteration and chromosome missegregation that precede the MC.

5.2.2 Centrosome Amplification

Both alignment and segregation of chromosomes during cell mitosis are events directly dependent on the correct function of centrosomes. These are nuclear structures committed to the microtubule organization during the mitotic spindle formation and their duplication cycle is tightly controlled by

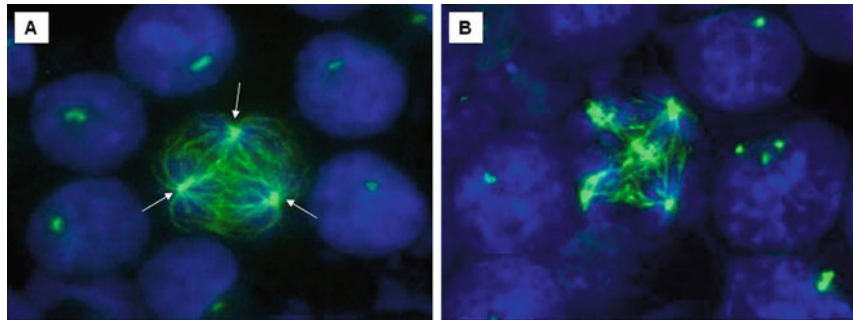

Fig. 5.1 Altered ploidy and mitotic catastrophe in myeloma cells. (**a**) Malignant plasma cells of the RPMI-8226 cell line show abnormal multipolar mitosis with multiple mitotic spindle poles including supernumerary centrosomes (*arrows*). The cell displays three tubulin-organizing centers with parallel abnormal chromosome segregation suggestive of cell death via mitotic catastrophe or asymmetric division, resulting in generation of aneuploid daughter cells (50× magnification). (**b**) Giant metaphase in RPMI-8226 cells with thickened chromatine aggregates along a four-pole spindle organized by abnormal centrosomes, both as number and volume (50× magnification)

the cell cycle phases. Centrosome amplification is an abnormality characterized by increased number and size of centrosomes with consequent impairment of their structure and function. The occurrence of extranumerary centrosomes results in the formation of multipolar and asymmetrical mitotic spindles, causing chromosome segregation errors and aneuploid cell progenies. However, although these cells undergo apoptosis, several of them may survive and further generate genetically instable cell populations. Therefore, the centrosome amplification has been emphasized as a critical event that promotes aneuploidy in a number of solid and hematologic malignancies, including MM [29–32]. In this context, two studies focusing on recurrent MM centrosome abnormalities showed that both number and volume of centrosomes were significantly higher in MM than in MGUS cells, whereas the percentage of malignant cells including centrosome abnormalities increased progressively from MGUS to overt MM [32].

The occurrence of centrosome amplification in myeloma cells has been correlated to poor prognosis since patients with supernumerary centrosomes have a shorter survival [33, 34]. Nevertheless, neither a clear correlation between centrosome amplification and a defined gene expression profile in MM, nor the specific mechanisms underlying the formation of supernumerary centrosomes in MM and other cancers have so far been elucidated. However, a role in centrosome-mediated cell aneuploidization may be exerted by cell fusion. Within the tumor environment, fusion events often give rise to genetically instable cell hybrids with high tumorigenic potential. In fact, fusion of elements with pre-existing oncogenes or tumor-suppressor-gene mutations produces tetraploid hybrids bearing alterations in the cell cycle checkpoints and/or centrosome aberrations. Further abnormal divisions of these cell populations may select aneuploid cell subsets that can become fully malignant. Thus, the cell fusion-mediated tetraploidy may precede aneuploidy of certain MM cell subsets with ultimate priming of their malignant potential.

5.2.3 Myeloma Stem Cells

Stem cell-like cancer cells with continuous self-renewing and differentiation capacity are under intensive investigation for their involvement in initiation, growth, recurrence and drug resistance in many tumor types, including MM [35]. As demonstrated in a few solid tumors including breast cancer, these cell hybrids derive from the cell fusion between normal and cancer cells and are able to maintain unaltered several malignant properties such as metastatic capacity, drug resistance and stemness.

Within the MM microenvironment, high amounts of inflammatory cytokines, growth factors and functional molecules induce a persistent inflamed state that is favorable for cells to adhere between themselves and fuse. Thus, the chronic inflammation of the MM marrow milieu and the accumulation of malignant cells in the presence of other cell types including resident stem cells, monocytes, macrophages, lymphocytes, fibroblasts, endothelial cells, as well as osteoblasts and osteoclasts, may represent a crucial event in promoting cell fusion. In this context, a non-aneuploid tumor stem cell may easily fuse with a normal marrow resident cell and generate an aneuploid hybrid while, alternatively, an aneuploid myeloma cell may fuse with a normal marrow stem cell leading to an aneuploid hybrid cell, namely a myeloma cancer stem cell. However, despite the lack of data supporting this theory, myeloma stem cells probably derive from rare fusogenic events occurring within the bone marrow of MM patients. Their occurrence has been variably detected for the expression of a typical stemness phenotype as Oct-4/$CD19^+$/$CD44^+$ cells lacking the CD138 molecules [36].

In myeloma stem cells, as well as in other tumor models, pharmacologic treatments usually enhance fusogenic susceptibility for at least two reasons: (a) the consistent drug-related cell destruction and (b) the accelerated recruitment of immune and inflammatory cells that enhance the degree of local inflammation [37]. For this reason, the hybridized cancer stem cells derived from cell fusions within the bone marrow become resistant to chemotherapeutic agents and give rise to a progeny unresponsive to other drugs. This hypothesis that is applicable to the resistance to melphalan by myeloma stem cells, is supported by the evidence that fusion-derived cells from 5-fluorouracil- and methotrexate-resistant mammary tumor cell lines also become refractory to other cytotoxic drugs including melphalan [38]. Alternatively, hybrid cells still maintaining their sensitivity to cytotoxic drugs may become unresponsive to a number of apoptogenic stimuli as an effect of a wide variety of fusion-related gene mutations [39]. Overall, the high rates of genotype abnormalities underlying drug resistance in cancer [40] suggest that the contribution of cell fusion to this process should not be underestimated.

5.2.4 Macrophage Fusion and Metastatic Potential of Malignant Plasma Cells

Cancer cell fusion has also been reported as an efficient way for tumor cells to acquire a metastatic phenotype, as primarily demonstrated by fusing tumor cells with macrophages both in vivo and in vitro [6]. Macrophages, either resident or recruited by tissues in response to inflammatory stimuli, possess an intrinsic fusogenic ability for the constitutive expression of DC-STAMP (dendritic cell-specific transmembrane protein), a membrane protein that physiologically promotes their homotypic membrane fusion for generating osteoclasts [41]. This function is particularly effective in MM whose progression includes the accelerated osteoclastogenesis as the major event driving the osteolytic disease of the skeleton.

On the other hand, macrophages also exert a significant role in enhancing the metastatic potential of several tumors as a direct effect of their fusion with cancer cells. Cell hybrids able to maintain cancer cell proliferative features while expressing the high migratory capability typical of marrow-derived cells such as macrophages have been described in vivo in specific cancers including renal [42] and colon carcinoma [43], and melanoma [44], and are easily obtainable in vitro after fusion with bone marrow cells [45]. Furthermore, transplanted bone marrow cells fused with tumor resident cells have been shown to promote formation of distant metastases in a mouse tumor model [6], while the experimental fusion between lung-disseminated mammary cancer cells and lung-resident macrophages generate long-living cells with highly metastatic potential to the lung [46]. Such a metastatic organotropism of fused cells is apparently dependent on the original tropism of tumor cells undergoing their cell fusion with macrophages, since hybrids from bone- or lung-tropic tumor cell lines are capable of metastasizing to these organs in animal models of tumor progression [47]. However, myeloma hybrids obtained in vitro by fusing malignant plasma cells with macrophages showed higher tropism to lungs than those derived from fusion with normal B cells [46].

5.2.5 Upregulated Osteoclastogenesis

Tumor progression in MM typically includes the formation of multiple bone erosions and osteolytic lesions leading to pathological fractures that represent the hallmark of the disease and are directly dependent on hyperactive osteoclastogenesis promoted by the tumor cells. Osteoclasts are bone resorptive cells deriving from precursors that belong to the monocyte/macrophage family. There is a general agreement that the principal physiological osteoclast progenitors are the bone marrow macrophages that, under specific stimuli by a number of cytokines and soluble factors, undergo homotypic cell fusions generating large polykaryons that terminally differentiate to bone resorbing cells by producing matrix proteolytic enzymes [48]. Several fusogenic proteins are enrolled by osteoclast precursors in the generation of polykaryons. As mentioned, DC-STAMP is currently considered a "master fusogen" for osteoclast formation [49]. This 65 kDa membrane protein is a seven-transmembrane domain receptor acting as reciprocal homo-activator of cell fusion between pre-osteoclasts. Its single expression by one of the fusing cell partners may be enough to effect the fusion [50]. It was originally identified on human monocyte-derived dendritic cells and is thought to exert the major role in osteoclast cell fusion since DC-STAMP knockout mice are defective in multinucleated osteoclasts and, moreover, develop osteopetrosis [51].

Furthermore, MFR/SIRPα (macrophage fusion receptor/signal-regulatory protein alpha) and CD200 molecules are membrane glycoprotein members of the immunoglobulin superfamily, that bind CD47 and the CD200 receptor respectively, to activate homotypic fusions between marrow macrophages or with other cells of the myeloid lineage [52, 53].

An excess of osteoclast formation in MM is thus correlated with the enhanced cell fusion between marrow macrophages in relation to the high membrane expression of fusogenic proteins activated by soluble factors within the tumor milieu. In this context, we have found that DC-STAMP is overexpressed by macrophages of patients with severe osteolytic MM (unpublished observation), whereas no data are presently available on MFR/SIRPα and CD200.

5.3 The Origin and Role of the Osteoclasts in MM

The formation of osteolytic lesions resulting in pathological fractures, spinal cord compression and intractable pain, namely the myeloma bone disease (MBD), is a hallmark of MM and is primarily regulated by the excessive osteoclast differentiation and hyperactivity.

In normal conditions, recruitment and differentiation of osteoclast progenitors are controlled by the interplay of regulatory molecules expressed by the marrow stromal component. RANK-L (receptor activator of nuclear factor kB-ligand) and M-CSF (macrophage-colony stimulating factor) are major osteoclastogenic factors that respectively drive the maturation and survival of pre-osteoclasts. Their marrow levels are finely balanced to correct the excess of osteoclast activity by decoy ligands as OPG (osteoprotegerin) for RANK-L, in relation to the cellular physiologic requirement of ionized calcium that is removed from skeleton storage. However, increased marrow concentration of RANK-L and M-CSF are variably observed in several inflammatory disorders as well as in bone chronic degenerative diseases as osteoporosis.

5.3.1 Enhanced Marrow Levels of Osteoclastogenic Factors

In MM the production of locally acting osteoclastogenic and resorptive factors by both stromal cells and osteoblasts is enhanced by a number of molecular interactions between the malignant plasma cell clone and the accessory cells within the marrow microenvironment. Cross-talk by functional

molecules results in the formation of a neoplastic unit that includes a number of chronically activated marrow cells as stromal cells, macrophages, dendritic cells, T and B lymphocytes and, of course, malignant cells. This functionally self-maintaining neoplastic unit induces a perturbation of the marrow cytokine homeostasis leading to a persistent increase of powerful inflammatory cytokines, namely IL (interleukin)-1β, IL-3, IL-6 (which acts as survival factor for myeloma cells), IL-11, TNF (tumor necrosis factor)-α and β, RANK-L, M-CSF, PTHrP (parathyroid hormone-related protein), HGF (hepatocyte growth factor), bFGF (basic fibroblast growth factor), metalloproteases and MIP (macrophage inflammatory protein)-1α [54].

Such deregulated marrow levels of these cytokines accelerate the recruitment and differentiation of resident macrophages, enhancing their proliferation, cell fusion, inhibition of apoptosis and functional hyperactivity while OPG is functionally inhibited within the myeloma marrow milieu. The reason for this is not clear, though functional deregulation of stromal cells following their assimilation within the neoplastic unit may lead to a major transcriptional defect. However, soluble OPG is also neutralized by myeloma cells within the marrow through their overexpression of syndecan-1 (CD138) molecules. These include a transmembrane proteoglycan enriched with heparin sulphate groups that binds OPG through its heparin domain, resulting in its internalization and subsequent degradation within the lysosomal compartment of the malignant plasma cells [55]. Therefore, the transcriptional and post-translational decrease of OPG strongly reduces the potential of neutralizing RANK-L, and this results in the persistence of predominant osteoclastogenic differentiation and hyperactivation.

RANK-L is expressed and released by malignant plasma cells, which in turn increase its marrow levels and reinforce osteoclast differentiation [56, 57]. Although this aspect has been disputed since several investigators failed to detect RANK-L at both protein and RNA levels either on primary malignant plasma cells or cell lines [58], studies of the last few years including data from our own group [59] support the production of this potent osteoclastogenic factor by myeloma cells. The major production of RANK-L in the myeloma marrow microenvironment belongs to cells of the osteoblast lineage, whereas the amount of RANK-L produced in vitro by myeloma cells is apparently low to independently drive the hyperactivity of the osteoclast differentiation and function.

Comparison of patients with active MBD and multiple osteolytic lesions to those with minimal skeleton involvement and inactive disease reveals an inverse correlation between marrow and/or serum levels of OPG with the severity of the bone devastation [60, 61]. Such a reverse RANK-L/OPG ratio suggests that hyperactive marrow macrophages exert a major pathogenetic mechanism of MBD, though its primary event includes the excess of osteoclastogenic factors predominantly released by stromal cells and osteoblasts within the myeloma neoplastic unit.

5.3.2 Osteoclast-Like Myeloma Polykaryons

While the upregulated osteoclastogenesis in MBD is reflected by increased marrow and serum levels of bone resorption markers [62], histo-morphometric studies suggest that the osteoclast accumulation in proximity to, or within the osteolytic lesions is not high enough to explain the dramatic skeleton devastation of MM, whereas conglomerates of plasma cells with highly malignant morphologic features including multinuclearity, usually occur in these sites as well as in marrow myeloma cell nests. This observation supports the hypothesis that malignant plasma cells can directly take part in bone destruction and, in fact, their osteoclast-like behaviour has been demonstrated in vitro [63], while other data [64] suggest the ability of these cells to form in vitro polykaryons with functional properties similar to osteoclasts.

Myeloma cell polykaryons are observed in MM in less than 3% of patients and have been variably associated with different isotypes of myeloma, including the non-secretory [64], Bence-Jones [65],

IgAk [66] and other phenotypes [67]. However, although implying a cell fusion event, there is no clear explanation for the observed multinuclearity, whereas their association with a poor prognosis and severe progression of MBD has been postulated. Other observations have linked multinuclearity with a potential phenotype heterogeneity particularly including the monocytoid lineage as defined by both morphologic and phenotypic criteria [68]. Extension of this research to several myeloma cell lines has disclosed a broad spectrum of myelomonocytic, NK and T cell markers, including the CD5 molecule, a T-cell antigen expressed by a subset of B cells involved in autoimmune and immunoproliferative diseases [69].

Heterogeneity of MM has been assessed using molecular approaches, documenting in U-266 cells (an IgEλ myeloma line) the expression of myelomonocytic markers as well as others belonging to the NK cell, neuronal or dendritic cell lineage, all associated with the deregulated response to IL-6 and the lack of Pax-5, a master gene of the B cell lineage [70]. Myelomonocytic nuclei in the presence of multilineage markers confirmed the tendency of these cells to undergo multinuclearity morphology transformation that implies a mechanism of either homotypic or heterotypic fusion with cells of the myelomocytoid lineage.

In addition to multinuclearity, osteoclast-like myeloma cell polykaryons also produce TRAcP (tartrate-resistant acid phosphatase) in extended cultures [71]. This enzyme is specifically produced by mature osteoclasts for the degradation of the extracellular bone matrix, and is uniformly distributed in myeloma cell polykaryons as diffuse or granular staining of intracellular components. Figure 5.2 shows some morphological and functional aspects of myeloma cell polykaryons including multinuclearity, expression of TRAcP and bone-resorbion on calcium phosphate substrate. Further similarities of myeloma polykaryons with osteoclasts involve other functional aspects of those cells. A functional form of the calcitonin receptor expressed by primary myeloma cells and myeloma cell lines as U-266, belongs to the molecular variant expressed by osteoclasts [72], whereas myeloma adherent polykaryons rearrange the cytoskeleton to form a ring of fibrillar actin identical to the sealing zone assembled by osteoclasts to define the bone area to be resorbed [16]. Thus, it is postulated that multinuclear giant cells may exert bone erosive activity.

Involvement of myeloma polykaryons in the generation of bone erosion is an actively debated topic. Animal studies point to their direct participation, but this has not been clearly demonstrated in humans. We have shown that U-266 and MCC-2 myelomas [73] fuse and exert osteoclast-like activity in vitro when extensively cultured in the presence of several osteoclastogenic cytokines [71]. After 3–4 weeks of culture, adherent cells from both lines display typical osteoclast-like aspect as $TRAcP^+$ polykaryons with extended pseudopodes and condensed chromatin in their nuclei. When incubated on calcium phosphate or dentine substrate as models of both inorganic and organic bone matrix, these cells extensively produce pits and erosive lacunae. To explain this osteoclast-like function, we have explored the activation pathways of osteoclasts in myeloma polykaryons and found that these cells enrol the αvβ3 integrin pathway to initiate and propagate intracellular signals, leading to the production of proteolytic enzymes as happens in osteoclasts. In particular, when the RGD (arginine-glycine-aspartic acid) binding domain expressed by the β3 subunit of the integrin is linked to either osteopontin or vitronectin, both expressed by the bone matrix, the intracellular pathway leading to ERK1/2 phosphorylation is activated to drive the cFos transcription factor that induces the expression of TRAcP, cathepsin K, carbonic anydrase, vacuolar ATPase and metalloproteases for bone proteolytic degradation. Stimulation of $β3^+$ myeloma polykaryons with osteopontin results in ERK1/2 phosphorylation and activation of the osteoerosive properties of these cells, whereas this is not inducible in β3-silenced MM [74]. Since αvβ3 integrin contributes to the cell fusion of osteoclasts, its property to activate osteoclast-like functions in myeloma polykaryons implies its indirect involvement in the fusion of myeloma cells, thus promoting the formation of giant multinuclear myeloma cells, indistinguishable from normal OCs, that probably participate in osteolysis also in vivo.

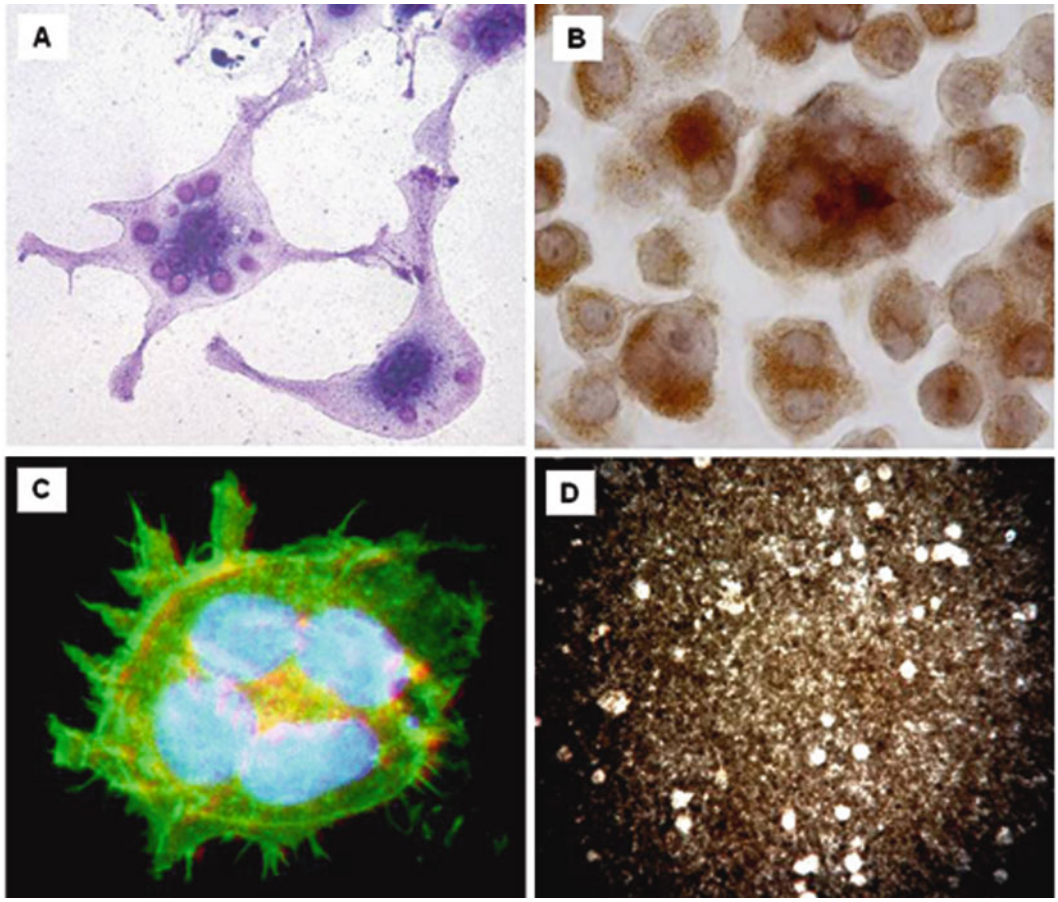

Fig. 5.2 In vitro generation of myeloma polykarions with bone-resorbing capacity. (**a**) Multinucleated giant myeloma cells were generated in vitro from RPMI-8226 cell line after 3–4 weeks of culture and stained by May-Grunwald-Giemsa. Their origin is attributable to the increased activity of fusogenic proteins expressed by these cells and to their high degree of aneuploidy as major event favoring their homotypic cell fusion (50× magnification). (**b**) Osteoclast-like morphologic aspect of adherent myeloma polikaryons expressing TRAcP, a major proteolytic enzyme of osteoclasts engaged in the bone matrix degradation. The cell displays six nuclei containing dense chromatin and multiple nucleoli. In addition, there are cytoplasmatic vacuolized areas and fine cytoskeletal prolongations to resemble the osteoclast morphology, whereas fine granulations of TRAcP are dispersed within the cytoplasm (50× magnification). (**c**) Triple fluorescence staining of a myeloma cell polykaryon detecting the membrane cytoskeleton F-actin (*green*), DNA in nuclei (*blue*) and cytoplasmic λ-chains (*red*). Detection of multinuclearity in keeping with condensation of membrane F-actin and presence of intracytoplasmic λ-chains, suggests that this cell was originally a light chain-secreting B cell undergone osteoclast-like transformation in relation to multiple in vitro events, including the cell fusion (100× magnification). (**d**) A representative image of the experimental bone resorption obtained by culturing RPMI-8226 polykaryons on calcium phosphate substrate resembling the inorganic bone matrix. Multiple large erosive lacunae were detected by Von Kossa staining after 3 weeks of culture (20× magnification)

5.3.3 Osteoclast-Myeloma Hybrids

In addition to homotypically fused myeloma polykaryons, the fusion between myeloma cells and myelomonocytic cells may generate heterotypic hybrids. Both cytogenetic and immunohistochemistry analyses have convincingly demonstrated chromosomal derangements typical of malignant plasma cells in the nuclei of multinucleated cells with osteoclast morphology. As mentioned, a variable percentage of osteoclast-like cells from MM patients show, in vitro, additional nuclei with t(4;14)

and t(11;14) of malignant plasma cell derivation that are transcriptionally active and fully integrated with the normal nuclei. These osteoclast-myeloma hybrids occur in approximately 30% of the ex-vivo osteoclast population and are detectable in vivo, together with mononucleated myeloma cells in proximity to the osteolytic lesions [15]. Further ex vivo observations demonstrate that clone-derived osteoclast-myeloma hybrids show a significantly higher richness in nuclei per cell with respect to normal osteoclasts, thus implying high bone-resorbing potential [75].

Moreover, these hybrids have been generated in co-cultures of osteoclasts with myeloma cells. Thus, as a result of their tendency to fuse, myeloma cells have been suspected to "corrupt" normal osteoclasts, transfer their malignant DNA and enhance their function in vivo. In keeping with this interpretation, a peculiar localization of tumor cells within marrow niches has been shown in bone marrow biopsies from MM patients with severe MBD. In the majority of bone samples, MM cells appear to penetrate into the marrow cavity and make direct contact with resident osteoclasts. In subsequent morphometric analysis, the population of malignant plasma cells infiltrating the niche showed the highest percentage of osteoclasts containing nuclei with myeloma translocations, thus proving that the occurrence of heterotypic fusion events between myelomas and OCs in vivo significantly correlate with the extent of physical osteoclast-myeloma interactions within the marrow milieu [76]. This may definitely support the role of malignant plasma cells in activating cell fusion with heterotypic cells and their contribution to the deregulated osteoclastogenesis in MM.

5.4 New Evidence of Cell Fusion in MM

New experimental evidence focuses on the deregulated cell fusion events enhancing osteoclastogenic derangement in MM.

5.4.1 Increased Expression of Fusogenic Proteins

Apart from the already mentioned mechanisms, both recruitment and fusion of marrow monocytoid pre-osteoclasts in myeloma microenvironment are also sustained by the persistent inflammatory state of the bone marrow that enhances the cellular expression of fusogenic molecules including DC-STAMP, as well as the production of RANK-L and MIP-1α by either malignant plasma cells or stromal cells. Work from our laboratory supports the involvement of a forceful myeloid cell fusion in the myeloma microenvironment, since peripheral macrophages from MM patients with active osteolytic bone disease express significant levels of DC-STAMP. As shown in Fig. 5.3, the real time PCR revealed in these cells, from all patients with overt MBD, a DC-STAMP mRNA transcription level at least ten-fold higher then the basal value. It appeared definitely upregulated as compared to patients with smoldering MM or with MGUS. Furthermore, in co-cultures of DC-STAMP$^+$ peripheral blood-derived monocytes/macrophages with autologous primary myeloma cells from similar patients with MBD, the malignant cells expressing membrane RANK-L molecules, rapidly accelerated both fusogen tendency and maturation to functional osteoclasts in those cells even in the absence of stromal cells [56].

5.4.2 Osteoclast Transdifferentiation of Dendritic Cells

Additional evidence from several investigators and from ourselves also supports the hypothesis that other cells of the same osteoclast ontogeny, namely the dendritic cells, albeit uncommitted to the osteoclastogenic fate, may undergo both multinucleation and acquirement of bone-resorbing function

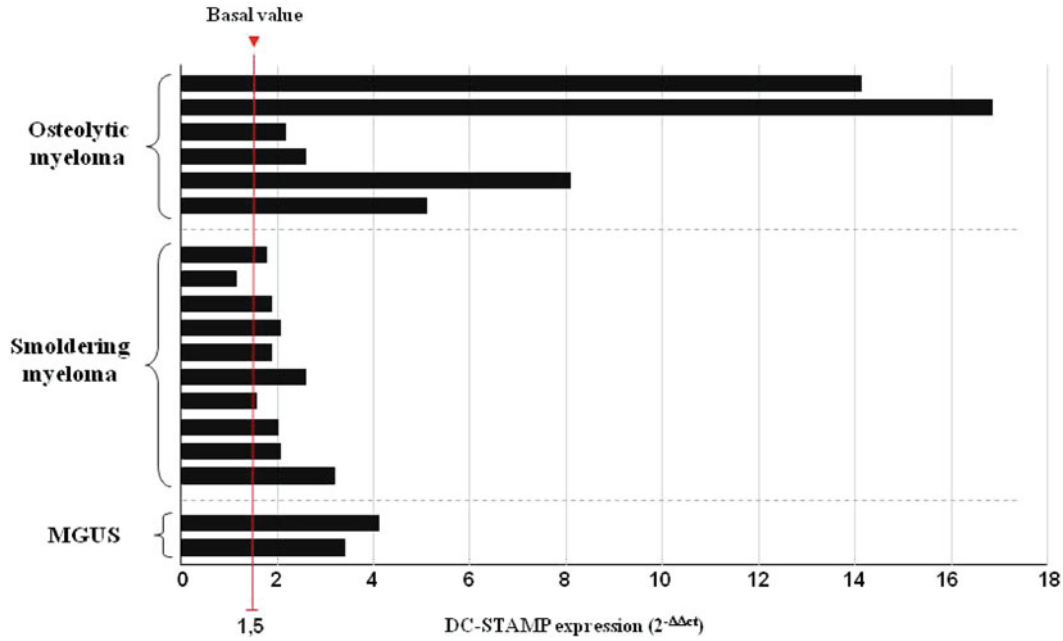

Fig. 5.3 DC-STAMP mRNA increased expression in macrophages from myeloma patients. Quantification of DC-STAMP transcription by real-time RT-PCR in macrophages from patients with MGUS, smouldering and osteolytic MM revealed a significant up-regulation in almost all samples. However, MM patients with severe bone disease reach the highest increase, up to 15-fold with respect to the basal value and definitely higher than those with MGUS and smoldering MM. Relative "fold-change" representation of the transcript expression was carried out by normalizing relative mRNA transcription to values of β-actin as control for each nuclear extract

in a powerful conditioning tumor microenvironment as provided by the MM marrow. It has been demonstrated that the in vitro RANK-L stimulation of immature $CD11c^+$ dendritic cells is enough to prime the osteoclast transdifferentiation of these cells and that this may also occur in vivo is shown by the observation that in chronic inflammatory disorders such as rheumatoid arthritis, extensive subcondral bone erosions are dependent on the excess of dendritic cells recruited within the joint compartment by the persistent flogosis of synovium [77, 78]. Dendritic cells have also been investigated in MM in relation to their potential contribution to the pathogenesis of MBD. They have been found near malignant plasma cells in the tumor microenvironment of both murine plasmocytoma and human MM [79, 80]. It is interesting to emphasize the clonogenic effect induced on the malignant cell growth by the cell-to-cell contact that triggers several interactive molecular pathways alternatively expressed by dendritic cells or myelomas [81]. Based on the high expression of stromal derived factor (SDF)-1 by myeloma cells, $CXCR4^+$ dendritic cells are chemoattracted into the marrow microenvironment and the subsequent cell-to-cell interaction with the tumor cells through the TACI/April axis promotes the expansion of the malignant clone. In this cell cross-talk, however, it has been identified a reciprocal activation of a further osteoclastogenic pathway that engages the thrombospondin-I receptor on dendritic cells by CD47 molecules overexpressed by malignant plasma cells [82]. Triggering of this molecular pathway results in efficient osteoclast differentiation of immature dendritic cells.

In line with these findings, we have demonstrated that dendritic cells from patients with MM are highly susceptible to undergoing homotypic cell fusion and osteoclast differentiation once co-cultured with RANK-L-expressing myeloma cells [83]. This implies that there are at least two different osteoclastogenic pathways on dendritic cells that are initiated by thrombospondin-I receptor and RANK respectively. Thus, enrichment of malignant plasma cells expressing CD47 and/or RANK-L within the

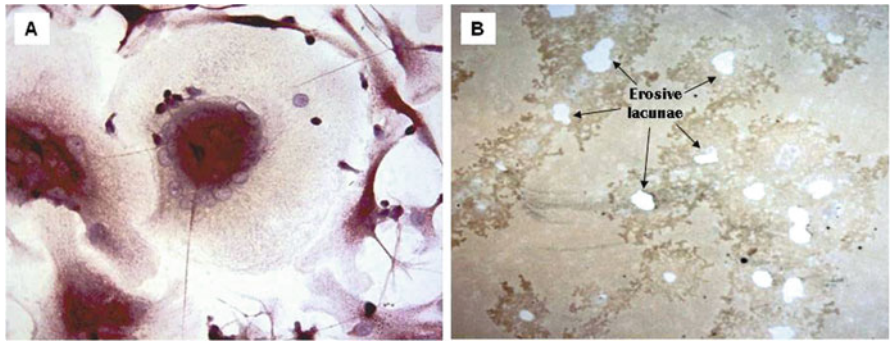

Fig. 5.4 Dendritic cell-derived osteoclast-like cells exert bone-resorbing activity. (**a**) Intense TRAcP expression in multinucleated giant cells obtained by co-culturing immature dendritic cells with RANK-L$^+$ malignant plasma cells. After 2 weeks of incubation, dendritic cells undergo homotypic fusion, acquire multinuclear morphology, polarize and produce a diffuse granulation of intracellular TRAcP detectable by specific staining (50× magnification). (**b**) These cells are also capable to exert bone resorptive activity in vitro, as revealed by the pits of erosion on experimental dentin substrates observable by light microscopy (30× magnification)

myeloma marrow milieu primes the formation of dendritic cell polykaryons, supporting the hypothesis that the hyperactive osteoclastogenesis in MM is also sustained by cells of the same myeloid derivation as osteoclasts, but constitutively uncommitted to differentiate into bone resorbing cells. Figure 5.4 illustrates the peculiar patterns of dendritic cells derived from peripheral macrophages transdifferentiated to osteoclasts after co-incubation with RANK-L-expressing myeloma cells. As can be seen, both morphological and functional patterns of transdifferentiated cells, including multinuclearity, expression of TRAcP, and bone resorptive capacity, appear typical of osteoclasts.

5.4.3 Functional Osteoclast-Like Activity of Malignant Plasma Cells

Several studies have focused on the real nature of cells that contribute to the bone erosion in MM. Based on the relative paucity of osteoclasts detectable within the MM osteolytic lesions and in the light of emerging data on cell fusion in this hematologic tumor, malignant plasma cells themselves seem to exert osteoclast-like function and actually become bone resorbing cells. It has been suggested that clonogenic B cell may express phenotypic markers shared by myeloid cell lineage and that, under appropriate stimulation, they may dedifferentiate to generate monocytes that in turn homotypically fuse and give rise to mature multinucleated osteoclasts [84]. Alternatively, based on the close proximity of myeloma cells to osteoclasts in bone lytic lesions, their functional cell-to-cell contact may drive their fusion even in vivo, as confirmed by cytogenetic assessment of chromosomal aberrations typical of malignant plasma cells in myeloma-osteoclast hybrids detected in vivo in MM patients. We have provided evidence that the fusogenic tendency and the susceptibility to generate osteoclast-like polykaryons by malignant plasma cells are also regulated by the activation status of the β3 chain that drives their osteoclast-like transdifferentiation. In fact, microarray analysis exploring the gene expression profile of αvβ3$^+$ MM cells revealed that the majority of genes enrolled by the β3 pathway in osteoclasts were significantly upregulated in those cells. It is conceivable that the increased expression of β3 related genes by myeloma cells concurs to drive their adhesion, fusion, multinucleation and expression of the osteoclast-like bone resorbing program [74]. Therefore, besides the continuous production of a variety of soluble factors accelerating the maturation of normal osteoclast precursors, different cellular elements may act as potential "fusogenic partners" within the MM marrow microenvironment and strongly reinforce the osteoclastogenic process. These cellular partners of

Table 5.1 Potential cellular fusion partner in MM

Cellular fusion partners	Molecular mediators		References
Macrophage/macrophage	RANK M-CSF CD200/CD200R	DC-STAMP SIRPα/CD47 CD44	[50, 53]
Dendritic cell/dendritic cell	RANKL/RANK M-CSF DC-STAMP TSP-1		[77, 81, 82]
Plasma cell/osteoclast	DC-STAMP MIP-1α SDF-1		[15, 16]
Myeloma stem cell/macrophage	MIP-1α SDF-1 TNF-α	IL-6 IL-8 MCP-1	[37]
Plasma cell/plasma cell	$\alpha_v\beta_3$-Integrin		[74]

the cell fusion, along with the molecular components enrolled in this deregulated event in MM, are summarized in Table 5.1.

5.5 Conclusion

The hypothesis that enhanced cell fusion may regulate the pathogenesis of osteoclast hyperactivity in MM and contribute to the myeloma related bone loss is supported by multiple evidence showing the increased expression of fusogenic proteins, along with the accelerated osteoclastogenesis involving peripheral monocytes, marrow pre-osteoclasts, myeloid precursors and malignant plasma cells. The contribution of tumor plasma cells to bone destruction may account for the multiple interactions occurring with other cells of the marrow milieu that promote adhesion, cell fusion, multinuclearity and bone resorbing effect in vitro. Such a high fusogenic condition of MM bone marrow may facilitate the abnormal differentiation of resident cells, including dendritic cells and plasma cells themselves, resulting in a chronic amplification of the osteoclast function.

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Chapter 6
Cell Fusion Hypothesis of the Cancer Stem Cell

Xin Lu and Yibin Kang

Abstract A major advance in recent cancer research is the identification of tumor cells with stem cell-like properties. Cancer stem cells (CSCs) often represent a rare population in the tumor mass and possess the exclusive ability to initiate the growth of a heterogeneous tumor. The origin of CSCs remains elusive and is likely to be cancer type specific. One possible but under-appreciated potential mechanism for the generation of CSCs is through fusion between stem cells and differentiated cells. The cell fusion hypothesis of CSCs adds an important functional underpinning to the potential multifaceted roles of cell fusion in the initiation and progression of cancer.

6.1 Introduction

Cell fusion is a normal physiological process that occurs in diverse organisms and plays essential roles in fertilization and development of various organ systems. When cell fusion goes awry, however, it may lead to aneuploidy and cancer. While the idea of cell fusion as a key driver of oncogenesis dates back to early twentieth century [1], its popularity only lasted for a short period of time before the mutation theory of cancer took the central stage. Recent findings in stem cell biology regarding tissue regeneration and somatic cell transdifferentiation have revitalized the cell fusion hypothesis of cancer [2–4]. Cell fusion produces hybrids that are equipped with doubled amount of chromosomes and centrosomes, a haphazard condition that can lead to abnormal chromosomal segregation and aneuploidy. Compared with the classic model of oncogenesis through linear accumulation of mutant alleles, cell fusion efficiently creates nonlinear assortments of genetic rearrangments and associated phenotypic alterations. Integrating the cell fusion hypothesis into the conceptual framework of cancer biology may help connect many hallmark features of cancer, such as the fusogenecity of tumor cells, aneuploidy, cellular and genetic heterogeneity, multi-drug resistance and metastasis [2–4].

One major recent breakthrough in cancer research is the discovery of cancer stem cells (CSCs) in leukemia and solid cancers. Although CSCs may not be a universal phenomenon for all cancer types [5], their existence helps explain the clinical observation of cellular heterogeneity of tumor tissue, drug resistance and cancer recurrence [6, 7] and may provide a novel avenue for the development of cancer therapeutics. The origin of CSCs has been a topic of speculations and intense research efforts. Although still lacking in experimental evidence, cell fusion has been proposed as a

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potential mechanism for the generation of CSCs [4, 8–10]. In this chapter, we will review the current understanding of cell fusion in cancer, the biological properties of CSCs, and finally, the cell fusion hypothesis for the generation and function of CSCs.

6.2 Cell Fusion: A Hidden Force in Cancer Progression

Cell fusion occurs in a wide range of physiological and pathological conditions [2, 4, 8, 11, 12]. Cell fusion can be classified as homotypic or heterotypic fusion. Homotypic fusion occurs between two or more cells that are committed to the same fate and is an essential process for the formation of placenta syncytiotrophoblasts, osteoclasts, myotubes, and macrophage-derived giant cells [8, 11]. Heterotypic fusion occurs between two different types of cells, and exists in many experimental settings, although the evidence showing heterotypic fusion in a strictly unperturbed physiological condition is rare [11]. Heterotypic fusion can result in either a heterokaryon (hybrid with distinct nuclei) or a synkaryon (hybrid with a common nucleus after nuclear fusion). The first well known report on heterokaryons was by Harris and colleagues, showing that induced fusion between human Hela cells and mouse Ehrlich ascites tumor cells generated hybrids with phenotypes distinct from either parental cells [13]. Induction of heterokaryon formation was later used as a technique to study cell fate plasticity and gene expression. Recently, heterotypic fusion was shown to occur spontaneously in vivo in mice between bone marrow derived cells and differentiated cells from organs such as liver, brain and heart [14–17]. These studies provide a cell-level mechanism for the previous observed phenomenon termed transdifferentiation. Compared with heterokaryons, synkaryons are more likely to undergo continuous divisions. Meanwhile, the formation of synkaryons is usually accompanied by the chromosome loss [8]. The classic example of synkaryon is the hybridoma cell derived from fusion of murine myeloma cells with B cells [18]. Spontaneous synkaryons were identified in vivo as cells derived from the fusion between hepatocytes and bone marrow derived cells in a process of experimental liver regeneration [14, 19]. For tissue repair and regeneration, it was proposed that, compared with differentiation of tissue-specific stem cells, fusion between bone marrow cells (or other cell types) with parenchymal differentiated cells possesses the advantage of correct positioning [11]. An example of fusion-mediated tissue repair is the fusion of transplanted dermal fibroblasts or bone marrow cells with muscle cells, which could generate heterokaryons and restore the muscular function in mutant mice with muscular dystrophy [20–23]. Tissue regeneration involving cell fusion may not simply be an idiosyncratic event in mouse models, because heterokaryons resulted from cell fusion between transplanted bone marrow cells with skeletal muscle cells had been detected in a patient with Duchenne muscular dystrophy [24].

Despite the possible contribution to tissue repair and regeneration, spontaneous cell fusion does not come without risks. In fact, nearly 100 years ago, cell fusion between leukocytes and resident somatic cells was already speculated as a potential initiation event of cancer due to its potential of rearranging chromosomes [1]. The cell fusion hypothesis of cancer was further developed through the following decades to include the concept that fusion between tumor cells and leukocytes may lead to metastasis [25, 26]. For a period, cell fusion as a mechanism of tumor progression was an active topic of research, and a number of reports showed that the metastasis phenotype could indeed be acquired through spontaneous or induced cell fusion. In a classic experiment, in vivo spontaneous fusion of human lymphoma or glioma cells with hamster host cells was found to give rise to more malignant cells [27]. However, with the rapid advance in the discovery of viral and cellular oncogenes in the 1980s, the oncogenic mutation theory of cancer took central stage and interests in the cell fusion hypothesis took the back seat until very recently, when the cell fusion theory of cancer was revisited [2, 3, 28].

There is a strong technical barrier for direct detection and back-tracing of parental cell lineages of fused cells in human cancer (or any human tissue), especially for heterotypic fusion events. Therefore,

definitive evidence of cell fusion in human cancer is rare. The most striking evidence was probably the clinical cases that patients who developed renal-cell carcinoma years after allogeneic bone marrow transplantation from donors of the opposite gender had tumor cells containing chromosomes from both the recipient and the donor, possibly through fusion of tumor cells with transplanted bone marrow cells [29]. Other lines of evidence also implicated cell fusion as a possible route in tumor progression. First, aneuploidy, which may result from cell fusion, was observed in virtually all of the over 5,000 solid human cancer samples analyzed [30], and cells from metastases are often more aneuploid than cells from primary tumors [31]. The correlation between increased DNA content and enhanced malignant behavior has been observed in ovarian cancer [32, 33], prostate cancer [34], colon carcinoma [34] and breast cancer [35, 36]. While cell fusion does not generate aneuploidy initially, it leads to tetraploidy that was observed to frequently precede aneuploidy in cancer both in clinical samples [37] and experimental models [38]. Second, binuclear and multinuclear cells are frequently observed in cancerous tissue [39], and cell fusion is one of the mechanisms that may generate such cells [38, 40]. Third, extra copies of centrosomes (i.e., supernumerary centrosomes) have been described as a common phenomenon in various types of cancer and linked to aneuploidy and chromosomal instability through multipolar mitosis [41]. Apparently, cell fusion is one of the likely reasons for the generation of supernumerary centrosomes. Fourth, some studies identified expression of non-epithelial markers in carcinoma-type cells, suggesting a possible adoption of other lineage markers through heterotypic cell fusion. For example, a study in small cell lung carcinoma found that the lung cancer cells shared several surface antigens normally present in macrophages [42]. Finally, the presence of premature chromosome condensation, a typical result of heterophasic cell fusion, in human tumor cells provides a possible footprint of the past cell fusion events in these cells [43–49].

Compared with the clinical implication, results from experimental model systems offer more direct evidence and functional understanding on cell fusion [2, 4, 10, 28, 50, 51]. Tumor cells are usually much more fusogenic than their normal counterparts. The frequency of in vivo cell fusion can be up to 1% in experimental tumor models [2, 52]. Moreover, fusion efficiency can be proportional to the malignant level of tumor cells [31]. In mouse models, it is concluded that both tumor–tumor [31, 32] and tumor-lymphocyte hybrids [28, 53, 54] can be isolated in vivo, and they displayed enhanced metastatic ability [32, 54], multi-drug resistance [32, 55], resistance to drug-induced apoptosis [55], and modified cellular function [28]. Macrophages are most frequently implicated as the host fusion partner possibly due to their inherent fusogenicity [56] or phagocytotic ability [50]. Our recent study showed that spontaneous fusion between two tumor cells with distinct organ tropism of metastasis generated hybrids with the ability to metastasize to both organs [52], highlighting the potential contribution of cell fusion to converge genetic and phenotypic properties from both fusion partners. This feature of cell fusion also inspired the speculation that cell fusion might be involved in the generation of CSCs [8, 10], as discussed further below.

6.3 CSCs: A Conceptual Advance in Cancer Biology

The essence of the definition of CSCs is the ability to regenerate the heterogeneous tumor from which they were isolated [6, 7]. While the CSC model generally infers a hierarchical organization of epigenetically distinct tumorigenic and non-tumorigenic tumor cells [5], it is also recognized that multipotency of lineage differentiation is not a necessary property of CSCs [7]. In addition, CSCs are not necessarily rare [57, 58]. By using highly immunocompromised NOD/SCID *Il2rg*$^{-/-}$ mice, Morrison and colleagues found that about 25% of unselected patient-derived melanoma cells could form xenograft tumors, demonstrating high frequency of tumor-initiating cells in melanoma [58]. Whether cancers with high frequency of tumor-initiating cells should be considered along the framework of the CSC model is still under debate [5].

The existence of CSCs was first reported in acute myeloid leukemia based on the surface marker expression pattern $CD34^+CD38^-$ [59, 60]. Significant advance in CSC research in recent years has brought out several exciting discoveries, including the prospective isolation of CSCs in various solid tumors such as cancers of breast [61], brain [62], colon [63–65], head and neck [66], pancreas [67, 68], skin [69], liver [70], lung [71], prostate [72] and ovary [73]. Existence of CSCs in genetically engineering mouse models was also reported in leukemia [74–76], mammary carcinoma [77–79], and squamous cell carcinoma [80], confirming that CSCs exist in spontaneous tumors and can be detected through syngeneic transplantations.

The concept of the CSC represents a major advance in the understanding of cancer biology and therapeutics. CSCs that survive conventional cancer treatments may cause relapse after the initial remission. Several reasons may explain the resistance of CSCs to chemotherapy, including their slow proliferation rate, high expression level of ATP-binding cassette (ABC) drug pumps, the intrinsic high expression of anti-apoptosis proteins and the more efficient DNA repair mechanism [7]. The slow proliferation or quiescent nature of CSCs in certain cancer types may also account for their resistance to molecular therapies that target proliferating cells. For example, imatinib, a drug targeting the oncogenic translocation BCR-ABL in chronic myelogenous leukemia (CML), may spare CSCs, because BCR-ABL is not required for the survival of quiescent CML stem cells [81, 82]. It should be noted that CSCs are not necessarily proliferating more slowly than the rest of the tumor mass [61, 67]; neither are they necessarily more resistant to therapies compared with the differentiated tumor cells [5, 83]. The variable nature of CSCs in different cancer types emphasizes the importance of comprehensive characterization of these cells and careful selection of targeting strategies for each cancer type.

Multiple signaling pathways that are important for self-renewal of normal somatic stem cells have been suggested to be deregulated in CSCs and promote uncontrolled self-renewal capacity [7, 84]. Wnt pathway plays an essential role in the initiation of CSCs in leukemia [85–87]. In breast cancer, Wnt/β-catenin pathway, which may be activated through *PTEN* loss, promotes enrichment of normal and malignant human breast stem cells [88]. Hedgehog (Hh) pathway was shown to be essential for maintenance of CSCs for CML [89, 90]. Emerging evidence also suggests the importance of Hh pathway in maintaining stem cell-like properties in solid tumor CSCs, such as breast cancer [91], glioblastoma [92, 93], and colon cancer [94]. Notch pathway activity plays an essential role in stem cell function and differentiation. Blocking Notch pathway through genetic or pharmacological approaches was shown to deplete CSC phenotype in preclinical models of colon cancer [95], breast cancer [96] and glioblastoma [97]. Other signaling pathways shown to contribute to CSC initiation and maintenance include TGF-β [98], hypoxia-inducible factors (HIFs) [99], bone morphogenetic protein (BMP) [100] and Bmi-1 [91, 101, 102].

A corollary from the CSC hypothesis is that metastasis originates from migrating or disseminated CSCs [103, 104]. Emerging evidence supports this idea. In pancreatic cancer, a subset of CSCs, characterized by $CD133^+CXCR4^+$ marker expression pattern, were found to determine the metastasis phenotype [68]. Surface marker analysis on disseminated tumor cells in bone marrow from breast cancer patients showed an enrichment of the CSC population ($CD44^+CD24^{-/low}$) from <10% in primary tumors to about 72% in disseminated cells [105]. Therefore, better understanding of CSCs may facilitate treatment of both the primary tumors and metastases.

Another important question about CSCs is in regards to their cellular origin. While CSCs share similarities with somatic stem cells in their self-renewal and differentiation properties, they may originate from not only stem cells, but also progenitor cells or differentiated cells. In either case, the cell of origin is a single cell that is transformed. For example, murine leukemia stem cells could be derived from committed granulocyte macrophage progenitors through oncogenic activity of the fusion protein MLL-AF9 [75]. Limited reactivation of hematopoietic stem cell self-renewal genes is sufficient to drive the unlimited proliferating potential [75]. Medulloblastoma in mice can be initiated by deletion of *Patched* and subsequent activation of the Sonic Hedgehog (Shh) pathway, in either neural stem cells

or granule neuron precursors, although tumors with neural stem cells as the origin displayed notable accelerated growth [106]. For glioma, murine neural stem cells and astrocytes were found equally permissive to transformation by combined loss of *Ink4a/Arf* and EGFR activation [107]. More recently, genetic deletion of *BRCA1* in mouse mammary epithelial luminal progenitors was shown to produce tumors that phenocopy human *BRCA1* breast cancer and resemble the majority of sporadic basal-like breast tumors. In contrast, genetic targeting of BRCA1 in the basal cells which are know to include a population of mammary stem cells, generated tumors that express the molecular profiles but do not present histological features of basal breast cancers [108]. Taken together, these studies suggested that cancers may be originated from cells of different differentiation status and may display distinct biological properties, prognoses and treatment responses.

6.4 The Cell Fusion Hypothesis of CSCs

Recently, cell fusion has been speculated to be involved in the generation of CSCs, as proposed first by Bjerkvig et al. and later extended by Dittmar and colleagues [8, 10]. Here, we incorporate their hypothesis and extend the discussion about how cell fusion might promote the genesis of CSCs. The observation that bone marrow derived cells can fuse with local somatic cells in vivo in the event of tissue damage and inflammation raised the interesting possibility that cell fusion might provide a means for the generation of CSCs and neoplasm [8]. In terms of fusion partners, several possible scenarios can occur: (1) both fusion partners are local with one of them being a tissue stem cell; (2) a bone marrow derived stem cell fuses with a local differentiated cell; (3) a bone marrow derived non-stem cell fuses with a local stem cell; (4) a disseminated cells from a different tissue origin fuses with a local stem cell (Fig. 6.1a–d). Although not a necessary condition, all these scenarios involve the participation of a normal stem cell and a differentiated cell based on the expectation that the stem cell may pass on the self-renewal capacity to the fused progeny, whereas the genetic and epigenetic composition of the differentiated cell allows the maintenance of the tissue lineage and appropriate positioning. On the other hand, if the oncogenic mutations happen to confer the stem cell-like property to one of the fusion partners, fusion between two differentiated cells (with at least one of them containing additional oncogenic mutations to promote full malignancy) can also generate a CSC (Fig. 6.1e). In all cases, the cell fusion model of tumor initiation requires that at least some of the oncogenic mutations are dominant in nature, otherwise cell fusion would lead to the suppression of the oncogenic potential.

An advantage of the cell fusion model is that it can readily explain aneuploidy, a feature frequently observed even at the early stage of tumor development, because tetraploidy and supernumerary centrosomes – the natural products of cell fusion – are conditions predisposing cells to aneuploidy through chromosome rearrangements [37, 38]. An aneuploid genome might further promote malignancy and tumor heterogeneity by clonal selection of random chromosomal gains and losses in favor of cells with the defining properties of cancer, including unlimited growth, resistance to apoptosis and senescence, angiogenesis and metastasis [109].

The cell fusion model may also help explain the puzzle that metastasis appears to be a hardwired property for some of the primary tumors [110, 111]. Whereas metastatic ability used to be considered as a capacity acquired late during tumor progression [112, 113], recent transcription profiling studies on breast cancer identified primary tumors with "good-" and "poor-prognosis" signatures that are predictive of the low or high risks of developing distant metastasis [114, 115]. In addition, dissemination of tumor cells was found to occur sometimes very early during primary tumor growth [116–118]. These findings prompted the postulation that the tendency to metastasize is largely determined by the nature of mutations acquired relatively early during the multistep tumorigenesis [110]. The cell fusion model of tumorigenesis suggests an alternative, even though not mutually exclusive, explanation: the

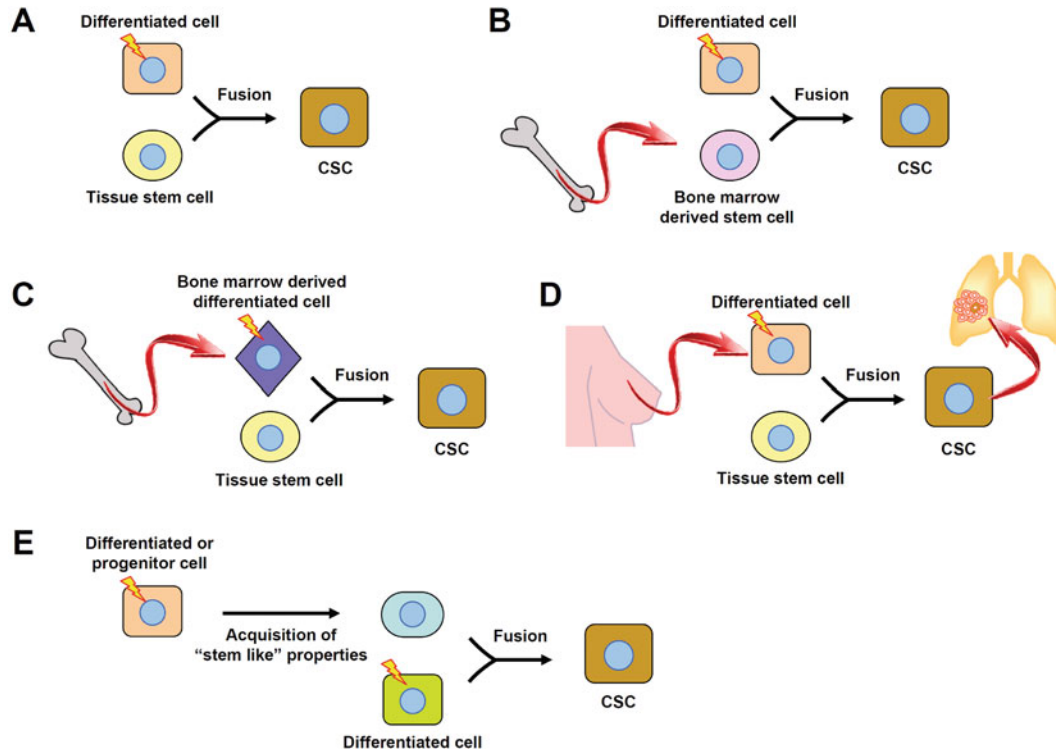

Fig. 6.1 Multiple possible scenarios to generate CSC through cell fusion. (**a**) Both fusion partners are local resident cells. A differentiated cell carrying oncogenic mutations may fuse with a local tissue stem cell to produce a CSC. (**b**) A bone marrow derived stem cell may fuse with a local differentiated cell with oncogenic mutations to produce a CSC. (**c**) A bone marrow derived non-stem cell with oncogenic mutations may fuse with a local stem cell to form a CSC. (**d**) Pre-neoplastic cells may be disseminated cells from a different distant organ (e.g. breast) and fuses with a local stem cell (e.g. in the lung). Further accumulations of oncogenic mutations may lead to the formation of CSC, and may produce metastatic disease without a primary tumor. (**e**) Some oncogenic mutations in a differentiated or progenitor cell may confer stem cell-like properties to one of the fusion partners, which upon fusion with another differentiated cell with appropriate (complementary) oncogenic mutations can also generate a CSC with full oncogenic potentials

specific lineage of fusion partners in the process of CSC generation may predetermine the capacity of metastasis. If one of the fusion partners belongs to a cell type with intrinsic migratory ability (e.g., macrophages), the fused cells would be more likely to be hardwired with cell motility programs that allow early dissemination and distant metastasis. On the other hand, if both fusion partners are of local epithelial origin, the fused cells might require a longer evolution process to acquire the necessary functional mediators to complete all steps of metastasis. Supporting this hypothesis, fusion between tumor cells and macrophages indeed has been discovered in a significant number of in vitro and in vivo studies and has been associated with increased metastatic ability [3].

The cell fusion hypothesis may also have important implications in our understanding of the molecular and cellular basis of metastasis organotropism. While metastasis is a general term to describe cancer spread to distant organs, different cancers do not metastasize to organs with equal likelihood. Instead, each type of solid cancer has a unique spectrum of preferred target organs [119]. For example, while the majority of colon cancer metastases occur in the liver, prostate cancer almost exclusively spreads to the bone [120]. Even for the same cancer type, different patients display variable metastasis patterns and kinetics. To explain these clinical observations, Steven Paget proposed the "seed and soil" hypothesis a century ago. The hypothesis posits that tumor cells only grow in the distant organs with compatible microenvironment that are conducive to productive tumor-stroma interactions crucial for

the formation of metastasis [113, 119, 121]. Tumors results from cell fusion may display metastatic organ tropisms related to the cellular properties of fusion partners. For example, fusion of myeloma cells with B lymphocytes resulted in hybrids metastasizing to the spleen and liver, while fusion with macrophages led to metastasis to the lung [122]. Our recent finding that organotropism is a phenotype transmittable through cell fusion [52] also supports this hypothesis. Experiments in mice already suggest bone marrow derived cells can fuse with hepatocytes, cardiac myocytes, Purkinje cells and oligodendrocytes [14–17], although the oncogenic consequence of such events has not be explored. Future studies are needed to determine whether cell fusion is a prominent mechanism for oncogenesis and whether difference in fusion partners produce cancers with distinct metastasis organotropisms.

The cell fusion hypothesis of CSCs remain largely speculative, because experimental evidence directly proving (or disproving) them, especially in human conditions, is lacking. One argument against the cell fusion hypothesis is that there is no evidence that the CSCs found in human or animal models are tetraploid in nature. However, tetraploid hybrids may undergo multipolar division and quickly become aneuploid. By the time the CSCs are prospectively isolated from tumor samples, the time window for detecting tetraploidy may have already passed. Furthermore, reduction division offers another possible route for exiting the tetraploid state. This possibility is validated in a liver regeneration mouse model in which fusion-derived polyploid hepatocytes underwent ploidy reductions to generate diploid daughter cells [123]. The transition from diploidy to tetraploidy and back to diploidy was also observed in human fibroblasts [124]. Taken together, the lack of detectable tetraploid CSCs by itself cannot nullify the cell fusion hypothesis. In fact, recent findings actually provide additional supports for this hypothesis.

First, chronic inflammation was shown to dramatically increase the frequency of cell fusion between bone marrow derived cells and various somatic cells such as cardiomyocytes, hepatocytes and Purkinje cells [125, 126]. Meanwhile, chronic inflammation may foster a cancer-prone condition for the inflicted organ, since alleviating chronic inflammation (e.g., by long-term uses of non-steroidal anti-inflammatory drugs) reduces risk of colon cancer in patients and breast cancer in rodents [127, 128]. The pro-malignancy nature of chronic inflammation may be in part attributed to the increased frequency of cell fusion that may eventually lead to tumor initiation.

Second, centrosome orientation and correct segregation of mother and daughter centrosomes in mitosis provide an interesting mechanism for the asymmetric division of stem cells [129, 130]. Supernumerary centrosomes as a direct consequence of cell fusion may confuse the mechanisms to discern mother and daughter centrosomes and disrupt asymmetric stem cell division. Combined with another possible consequence – multipolar division and aneuploidy, supernumerary centrosomes may lead to somatic stem cell dysfunction and transformation. One prediction of the cell fusion hypothesis is the appearance of supernumerary centrosomes at early stage of cancer progression, which indeed has been validated for in situ ductal carcinomas of breast [131]. In addition, supernumerary centrosomes were observed in inflammatory tissue [132] and during tissue repair [133], both of which may involve cell fusion events.

Finally, a number of proteins have been implicated as functional mediators in physiological cell fusion events. While their role in cancer cell fusion is unknown, it is interesting to note that some of these molecules have been associated with CSC phenotypes and cancer progression. CD44, a cell surface receptor known to be involved in cell fusion of macrophages during osteoclastogenesis [134], is frequently overexpressed in cancer cells and linked to poor prognosis [3]. Interestingly, CD44 is a surface marker that enriches for CSCs for several cancer types such as breast cancer [61], prostate cancer [72], pancreatic cancer [67], head and neck cancer [66], and colorectal cancer [65]. This connection supports the proposition that CD44 might be involved in CSC generation through fusion of epithelial cells with bone marrow derived cells, specifically cells of the monocyte/macrophage lineage. Cytokine IL-4 is required for fusion of myoblasts [135]. IL-4 is also produced by $CD133^+$ colon CSCs to mediate growth and chemoresistance [136], again supporting the idea that cell fusion might be involved in CSC formation or function. Other fusogenic proteins, such as SDF-1[137] and MCP-1

[138, 139] have well characterized functions in cancer progression and metastasis [140, 141] and may also find their roles in cell fusion-mediated CSC generation and functionality.

6.5 Conclusions

We provided a brief summary of the current understanding on how cell fusion might be linked to cancer, especially with the focus on the generation of CSCs. To test the cell fusion hypothesis of CSCs, experiments should be designed to harness the elegant fusion-tracing technique involving Cre-mediated activation of a LacZ reporter [15] to elucidate the functional significance of cell fusion in tumor initiation. Whether cell fusion is required for tumorigenesis should also be tested using inducible cell ablation techniques through which fused cells can be conditionally eliminated in well developed tumors or metastases in experimental animal models. Furthermore, the gold standards for testing CSC functions need to be applied to test whether fusion-induced cancer cells possess CSC properties. Ultimately, cell fusion hypothesis has to be tested in clinical settings, which still remains as a formidable challenge.

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Chapter 7
Expression of Macrophage Antigens by Tumor Cells

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Abstract Macrophages are a heterogeneous cell population of the myeloid linage derived from monocytes. These cells show two different polarization states, M1 and M2 macrophages in response to different micro environmental signals. Tumor associated macrophages (TAM) represent the M2 type and promote tumor progression. These cells express antigens that more or less are specific for macrophages like: CD14, CD68, MAC387, CD163, and DAP12. In a series of recent studies it is shown that cancer cells may express these antigens and CD163, MAC387 and DAP12 may be expressed by e.g. breast cancer cells. Thus, 48% of the breast cancers expressed CD163 that is a scavenger receptor normally expressed by macrophages alone. The corresponding figure for rectal cancer is 31%. The expression of CD163 is correlated to early distant recurrence in breast cancer and local recurrence in rectal cancer and reduced survival time in both conditions. Expression of macrophage antigens in breast- and colorectal-cancers may have a prognostic relevance in clinical praxis. One explanation to these findings is that resemblance with macrophages may indicate a more invasive phenotype due to genetic exchange between the primary tumor cells and associated macrophages. This is further supported by the finding that expression of DAP12, a macrophage fusion receptor, in breast cancer is associated with an advanced tumor grade and higher rates of skeletal and liver metastases and overall shorter distant recurrence free survival. Another explanation to the changed phenotype is a genetic exchange between the cells by exosome-mediated transfer.

7.1 Introduction

Macrophages are a heterogeneous population of cells derived from monocytes and originate from mesoderm. During embryogenesis, macrophages appear first in the yolk sac, then in the liver, and finally in the bone marrow. Blood monocytes arise in the bone marrow from precursor monoblasts and enter tissues where they mature into a heterogeneous population of cells called macrophages. These cells and there descendents are common in the liver (Kuppffer cells), intestine, lungs (alveolar macrophages), bone (osteoclasts) and brain (microglia). Monocytes may also mature to dendritic cells.

Macrophages may develop into two different polarization states in response to the micro environment. M1 macrophages are proinflammatory and characterized by the release of inflammatory cytokinins and antimicrobial activity. M2 macrophages are releasing anti-inflammatory molecules like IL-4, IL-13 and IL-10 and support angiogenesis and tissue repair. Tumors associated macrophages (TAM) represent the M2 type and promote tumor progression [1–5].

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Cell–cell fusion is an important function of macrophages and results in the formation of osteoclasts and multinucleated giant cells (MGC) [6]. In vitro hybridization of cancer cells and macrophages is reported in several studies [7–11]. Busund and coworkers showed that co-culture of peritoneal macrophage and Meth A sarcom cells resulted in hybrids with intermediate morphological and functional features between the two original cells. The hybrids grew more slowly in vitro than the sarcoma cells [12]. In vivo, however, the hybrids produced tumors that grow faster and had better developed vasculature than the parental Meth A sarcoma tumors. Pawelek and coworkers reported similar results after hybridization of human monocytes and melanoma cells. The hybrids had increased metastatic potential and again expressed morphologic and genetic characteristics from both maternal cells [13–17].

Heterotypic cell fusion occurs between bone marrow derived-cells (BMDCs) and somatic cells, such as hepatocytes, cardiomyocytes, endothelial cells and skeletal muscle cells [18–20]. Terada and coworkers described that spontaneously fused BMDCs could adopt the phenotype of the recipient cells [21]. During normal in vivo conditions cell fusion is a rare process. Inflammation and irradiation, however, have been shown to induce frequent heterotypic cell fusion between myeloid/lymphoid cells and non-hematopoietic cells. Johansson and coworkers [22] reported that chronic inflammation, induced from severe dermatitis or autoimmune encephalitis, resulted in fusion of BMDCs with Purkinje neurons and formation of binucleated heterokaryons in a significantly higher frequency [22].

To prove the existence of cell fusion and its clinical significance by studying surgically removed tumors from patients is difficult. Cancer cells mainly have the same genetic material as other somatic cells. Cancer cells also have high plasticity, have different genetic expression and may transdifferentiate to other tissue types. Although available data do not prove that tumor – host cell (macrophage) hybrids are generated during malignant transformation, the combined results from several studies support that cell fusion may occur in tumors [23]. The morphologic and phenotypic similarities between hybrids and maternal cells have been an important approach in the research field of cell fusion.

On the basis of cell fusion theory, previous experimental studies and the assumption that the macrophage-cancer cells fuse to create hybrids that will express the characteristics of both parent cells, we investigated the expression of macrophage markers in tumor cells in breast and colorectal cancer clinical specimens. Macrophage antigen expression in cancer cells was studied in relation to clinicopathological data [24, 25].

There are several macrophage markers which have been used both clinically and for research purposes to identify macrophages. Few of these markers are entirely specific for macrophages which complicate the interpretation of the histopathological analysis. These markers have been used to identify macrophages and macrophage subsets in different conditions and pathological process like chronic inflammation, infections and malignancy. Studies which examined the prevalence and clinical significance of macrophage antigen expression in cancer cells in solid tumors are limited. Some of the classical markers used to identify macrophages in tumor tissue are discussed below.

7.2 Macrophage Antigen CD14

CD14 is a 55-kDa glycophosphatidylinositol-linked protein that exists either as a membrane (mCD14) or in soluble form (sCD14). Soluble CD14 appears after shedding of mCD14 or is directly secreted from intracellular vesicles by hepatocytes and monocytes. mCD14 is attached to the cell surface by a glycosyl-phosphatidylinositol (GPI) anchor and is mainly expressed by macrophages. It is expressed to less extend also by neutrophil granulocytes, dendritic cells, B cells, fibroblasts as well as endothelial and epithelial cells [26–31]. CD14 acts as a co-receptor to toll-like receptor 4 (TLR4) for detection of bacterial lipopolysaccharide (LPS). It can bind LPS only in the presence of lipopolysaccharide-binding protein (LBP). LPS is the main ligand for CD14 but other bacterial products like lipoetichoic

acid, uronic acid polymer and spirochete lipoprotein can also activate CD14 pathway. sCD14 compete with mCD14 for LPS binding and thus reduce the effects and responsiveness to LPS. sCD14 occurs in human milk, where it is believed to regulate microbial growth in the infant gut. In the case of cells lacking mCD14, such as epithelial and endothelial cells, soluble CD14 (sCD14) endowed the cells with responsiveness to LPS [32, 33].

7.3 Macrophage Antigen CD68

CD68 is a highly glycosylated type I transmembrane glycoprotein that belongs to the lamp (lysosomal-associated membrane protein) – family of glycoproteins. It is mainly localized within late endosomes but it is found to less extend also on the cell surface. The function of CD68 is not known but it is suggested to take part in antigen processing or in the protection of lysosomal membranes against lysosomal hydrolases. Surface CD68 binds and internalizes oxidized LDL (low-density lipoprotein) in monocytes [34–38]. CD68 was previously described to be selectively expressed by a monocytemacrophage cell lineage and it was widely used in diagnostics and research as monocytemacrophage specific markers. Several groups have confirmed, however, that CD68 is expressed also in non-myeloid cells such as adipocytes, fibroblast, endothelial cells, giant cells and malignant melanomas [39–44]. The clinical and prognostic value of CD68 expression in tumor cells is not clear. CD68 expression in cancer cells is examined in few studies. In breast cancer, none of tumor samples from 139 patients expression of CD68in tumor cells [25]. Strojnik and coworkers could show that CD68 expression in human glioma was significantly more frequent in the malignant than in benign tumors and high CD68 staining of tumor cells correlated to poor prognosis [45].

7.4 Macrophage Antigen MAC387

MAC387 is a calprotectin molecule, an intracytoplasmic antigen, consisting of a 12 kD alpha chain and a 14 kD beta chain. It is expressed by granulocytes, monocytes and by tissue macrophages. Mac387 was reported to be expressed in soft tissue sarcomas, carcinomas keratinocytes, malignant melanomas, breast cancer and fibrohistiocytic lesions like dermal dendrocytes and dermatofibroma [25, 46, 47]. Lopez-Beltran and coworkers suggest that Mac387 may be a reliable marker for examining squamous differentiation in the urothelium and its tumors [48]. Out of 127 patients with breast cancer 12% expressed MAC387 (Fig. 7.1) in tumor cells and it's expression was correlated with other macrophage antigens like CD163 and DAP12. Expression of MAC387 is more common in estrogen receptor negative breast cancers and histological (NHG grade) advanced cancers.

7.5 Macrophage Antigen CD163

CD163 is a transmembrane receptor earlier known as M130 or RM3/1. It is encoded by a gene on chromosome 12, location p 13.3. It is a scavenger receptor for the haptoglobin-hemoglobin (Hp-Hb) complex and is expressed by monocytes/macrophages and by neoplasms with monocytic/histiocytic differentiation [49–52]. CD163 is a 130 kDa glycoprotein with an amino-terminal signal element, nine scavenger receptor cysteine-rich (SRCR) domains, one transmembrane element and a short cystoplasmic tail. Stable Hp-Hb complexes are delivered to the reticuloendothelial system by CD163 receptor mediated endocystosis followed by lysosomal preoteolysis of globin and conversion of haem to iron and bilirubin-ligand complex. The expression of CD163 is up regulated by the acute phase mediator IL-6, glucocorticoids and IL-10 and is down regulated by IL-4, TGF-beta, interferone-gamma and by

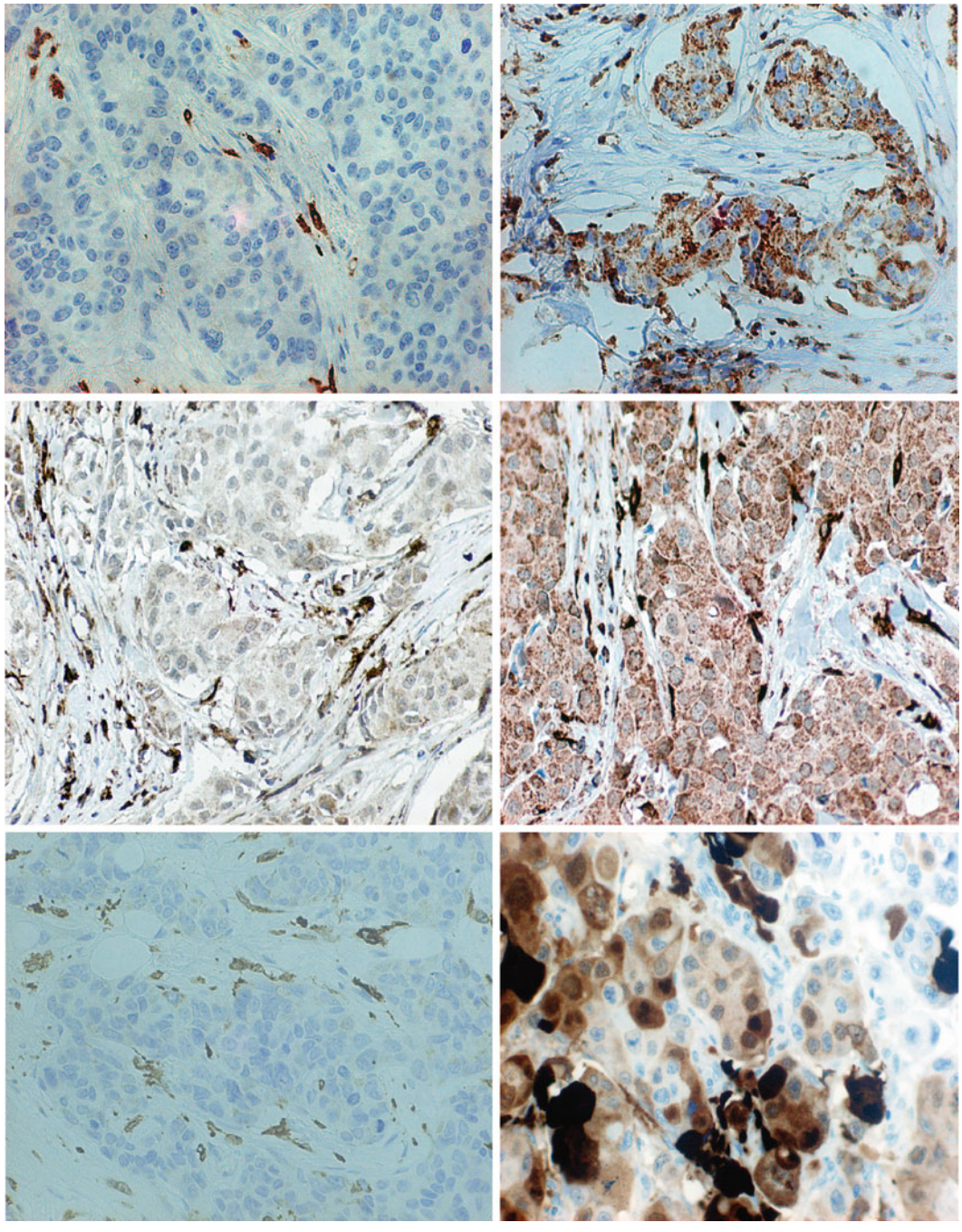

Fig. 7.1 Immunohistochemical staining of breast cancer with macrophage antigens CD163, MAC387 and DAP12. Note that staining of CD163, MAC387 and DAP12 is characterized by granular cytoplasmic, or cytoplasmic and membrane staining patterns. The tumor cells, which are pleomorphic and atypical with large nuclei and nucleoli, are easy to distinguish from macrophages

the proinflammatory liposaccharide (LPS) [50, 53–55]. It has been suggested that CD163 is a differentiation antigen for monocyte/macrophage. Macrophages have a higher expression of CD163 than monocytes indicating a maturation process to phagocytic macrophages. CD163 is expressed in the M2 macrophages and in tumor associated macrophages (TAMs) [56, 57].

7.6 CD163 Expression in Breast Cancer

CD163 expression was studied in 127 cases with breast cancers and was found to be expressed by cancer cells in 48% of the cases. Macrophages also expressed CD163 but not normal ductular epithelium close to the cancers nor ductular epithelium in control cases without breast cancer (Fig. 7.1). CD163 expression was more common in histologically advanced breast cancers, estrogen receptor negative tumors and increased proportionally to NHG tumor grade. Patients with CD163 positive breast cancers had a shorter distant recurrence free survival than those with cancers that did not express CD163 (Fig. 7.2). Multivariate analysis shows that CD163 has a significant prognostic impact in relation to distant recurrence and breast cancer mortality rates [25].

7.7 CD163 Expression in Colorectal Cancer

The CD163 antigen was expressed in about 23% of cases with rectal cancer [58] and in 18% of cases with colon cancer. Morphologically normal mucosa in neither colon nor rectal tissue expressed CD163 (Fig. 7.3). CD163 expression is related to advanced stages of colorectal cancer and patients with CD163 positive cancers had an earlier local recurrence and a lower survival time (Fig. 7.4).

The patients who were included in the rectal cancer study were previously included in the Swedish rectal cancer trial [59] and were randomized to either preoperative radiotherapy (5×5 Gy delivered in 1 week), followed by surgery within the next week (radiotherapy group), or to surgery with no additional radiotherapy (non-radiotherapy group). CD163 expression was analyzed in both patient groups in order to examine the in vivo effects of radiation on macrophage traits in the rectal cancer cells. CD163 expression was inversely correlated to apoptosis in tumors from patients treated with preoperative radiotherapy. Apoptosis after radiotherapy is more common in CD163 negative than in CD163 positive tumors. It was remarkable that this correlation between CD163 expression and apoptosis in cancer cells was not found in tumors from patients who were not given preoperative radiotherapy. This might indicate that CD163 positive cancer cells are less apoptotic and more resistant to irradiation than CD163 negative cancer cells. Macrophage infiltration in tumor stroma was not correlated to CD163 expression in the tumor cells but to angiogenesis or lymphangiogenesis. The proliferation activity in cancer cells, expressed as Ki-67 expression and S-phase fraction, was significantly higher in CD163 positive tumors.

7.8 CD163 and CD68 Expression in Malignant Melanoma

Based on the cell fusion theory, the expression of macrophage markers in malignant melanoma was investigated by Jensen et al. [60]. Tumor cells in 35% of melanomas expressed CD163 and the corresponding figure for CD68 was 10%. Melanoma cell expression of CD163, together with tumor thickness, was an independent prognostic factor of relapse-free survival and melanoma-specific survival. The melanoma cell expression of CD68 was independently associated to relapse-free survival [60].

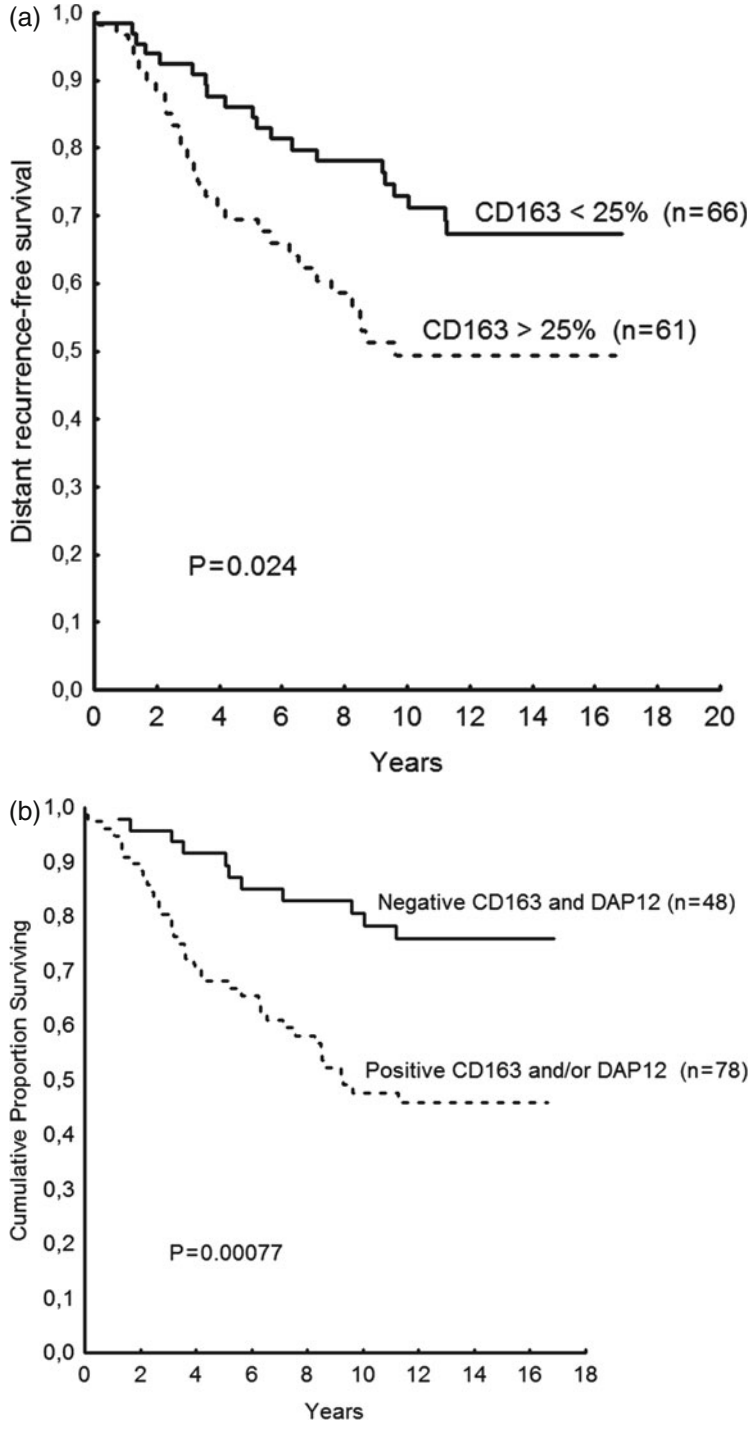

Fig. 7.2 Kaplan–Meier analysis of survival in patients with breast cancer in relation to DAP12 and CD163 expression. (**a**) DRFS in all patients according to the presence of CD163 expression. (**b**) Survival in patients with breast cancer expressing both or either CD163 or DAP12

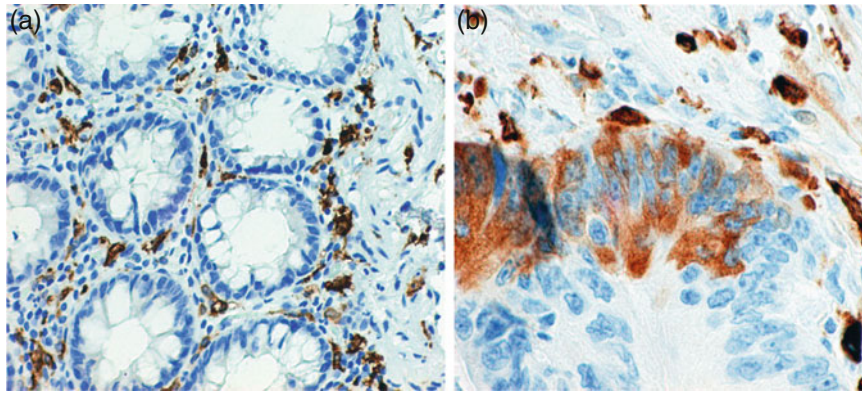

Fig. 7.3 Immunohistochemical staining of colon cancer with macrophage antigens CD163. (**a**) Norma colon mucosa section where macrophages are staining for CD163 but normal epithelial cells show no expression of CD163. (**b**) Colon cancer section where a group of cells show cytoplasmic and membrane staining patterns for CD163

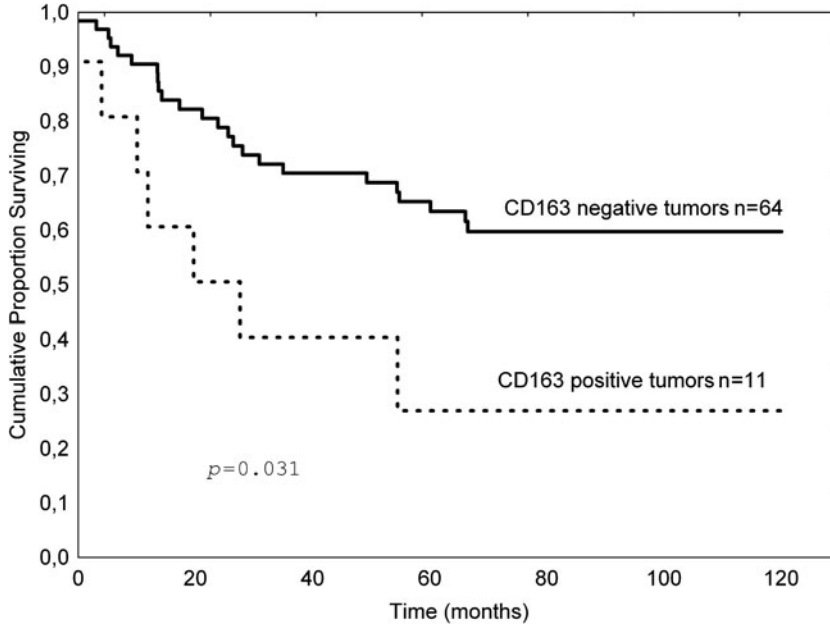

Fig. 7.4 Kaplan–Meier analysis of survival in patients with colon cancer in relation to CD163 expression in tumor cells

7.9 Macrophage Antigen DAP12

The signaling adaptor protein DAP12 (DNAX activating protein of 12 kD) is also known as KARAP (killer cell activating receptor-associated protein). It plays a crucial role in macrophage fusion during osteoclast formation [61, 62]. This protein may also be involved in macrophage fusion when leading to the formation of multinucleated giant cells and it constitutes a target to control the resolution of inflammatory disorders based on monocytes/macrophages and neutrophils [63]. The tyrosine residues in the DAP12-ITAM are phosphorylated by activating DAP12. This will result in recruitment and activation of the protein tyrosine kinases Syk and ZAP70, which in turn lead to the activation

of phosphatidylinositol 3-kinase (PI3K) [64, 65]. Several DAP12-associated receptors are presented on macrophages and other myeloid cells. In humans, mutations of DAP12 or TREM-2 lead to polycystic lipomembranous osteodysplasia with sclerosing leucoencephalopathy (PLOSL), which is associated with bone lesions and osteoporotic features. This phenotype is based on impaired osteoclast differentiation and function [61, 66].

Patients with tumors expressing DAP12 (Fig. 7.1) acquired (distance recurrence free survival – DRFS) skeletal and liver metastases earlier than patients with negative/low DAP12 expression. Interestingly, patients with lung metastases showed no differences in DRFS rates in relation to DAP12 expression. Patients with high DAP12 and/or CD163 expression had significantly lower survival than patients with breast cancer expressing neither CD163 nor low/negative DAP12 expression (Fig. 7.2). Multivariate analysis adjusted for clinicopathological variables revealed that DAP12 expression had a significant prognostic impact as an independent factor associated with skeletal metastases.

7.10 Conclusions

Macrophage traits in tumor cells and its clinical significance are investigated in a limited number of studies. CD163 is the most common macrophage specific marker expressed in cancer cells. The other markers used for identifying macrophages are not restricted to the myeloid cell lineage. Macrophage antigens are expressed by solid tumors but the clinical significance of this is not, so far, widely examined. The expression of CD163 is correlated to metastasis and poor survival in breast and colorectal cancers and CD163 has a significant prognostic impact in these cancers. Further, CD163 expression is inversely correlated to apoptosis after irradiation which might indicate that cancer cells with macrophage traits may increase or/and are more resistant.

The reason why cancer cells express macrophage antigens may be a heterotypic cell fusion between the primary cancer cells and tumor-associated macrophages. The cancer cells may be reprogrammed by the macrophages and transdifferentiate to a more mesenchymal phenotype that may be more aggressive. Another explanation is a genetic exchange between the cells by exosome-mediated transfer. It is recently shown that both mRNA and microRNA may be exchanged between cells by exosomes [67].

Further, the expression of macrophage antigens by cancer cells may indicate a more aggressive metastatic activity and may have a prognostic impact of clinical importance.

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Chapter 8
Leukocyte-Cancer Cell Fusion: Initiator of the Warburg Effect in Malignancy?

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Abstract The causes of metastasis remain unknown, however it has been proposed for nearly a century that metastatic cells are generated by fusion of tumor cells with tumor-associated leukocytes such as macrophages. Indeed, regardless of cell or tissue origin, when cancer cells in the original in situ tumor transform to malignant, invasive cells, they generally become aneuploid and begin to express molecules and traits characteristic of activated macrophages. This includes two key features of malignancy: chemotactic motility and the use of aerobic glycolysis as a metabolic energy source (the Warburg effect). Here we review evidence that these phenomena can be well-explained by macrophage-cancer cell fusion, as evidenced by studies of experimental macrophage-melanoma hybrids generated in vitro and spontaneous host-tumor hybrids in animals and more recently humans. A key finding to emerge is that experimental and spontaneous cancer cell hybrids alike displayed a high degree of constitutive autophagy, a macrophage trait that is expressed under hypoxia and nutrient deprivation as part of the Warburg effect. Subsequent surveys of 21 different human cancers from nearly 2,000 cases recently revealed that the vast majority (~85%) exhibited autophagy and that this was associated with tumor proliferation and metastasis. While much work needs to be done, we posit that these findings with human cancers could be a reflection of widespread leukocyte-cancer cell fusion as an initiator of metastasis. Such fusions would generate hybrids that express the macrophage capabilities for motility and survival under adverse conditions of hypoxia and nutrient deprivation, while at the same time maintaining the deregulated mitotic cycle of the cancer cell fusion partner.

8.1 Introduction and Background

The concept of leukocyte-tumor hybridization as a mechanism for tumor metastasis was first put forth in remarkable detail a century ago by pathologist Otto Aichel [1]. In the past 40 years, host-tumor hybrid cancer cells have been documented in numerous animal tumor models where they can be found in metastatic tumors [2–5]. Animal and cell culture studies on cancer cell fusion, coupled with observations that malignant cancer cells routinely express macrophage traits, add experimental support to the Aichel proposal and have led to the hypothesis that leukocyte-tumor cell fusion represents a unifying mechanism for metastasis [2–5]. However, while it has been demonstrated that cancer cell-host cell fusion occurs in animals and more recently in humans [6–9] little is known regarding key questions such as the mechanisms through which leukocyte-cancer cell fusion and subsequent genomic

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Table 8.1 Model for leukocyte-cancer cell hybrid formation and the generation of metastatic cells

1. Tumor cells attract bone marrow-derived cells such as macrophages, lymphocytes, neutrophils and perhaps stem cells
2. In some cases leukocyte-cancer cell fusions occur with tumor and leukocyte chromosomes becoming pooled in a single nucleus, usually resulting in aneuploidy
3. Epigenomic gene expression patterns from both fusion partners are retained in hybrids. Some hybrids combine the migratory capabilities of the leukocyte with the de-regulated cell cycle of the cancer cell. Thus new mitotically active cells emerge with high invasive and metastatic potential
4. Hybrid cells express aerobic glycolysis (the Warburg effect), a metabolic energy pathway characteristic of macrophages and other inflammatory cells. Aerobic glycolysis is advantageous for survival in hypoxic environments and also for proliferating cells [47]
5. As part of the Warburg effect, hybrids use the autophagy-lysosomal pathway for sequestration and digestion of external food sources that are internalized via phagocytosis or other endocytic processes

hybridization occurs in vivo; the frequency at which hybrids are generated; how gene expression is regulated by the hybrid epigenomes from two different cell lineages; potential survival advantages of hybrids; the role of fusion in metastasis; and metabolic energy sources for hybrids. These questions and many more remain for future research and point to the difficulties of studying fusion and hybridization in vivo. A working model for in vivo cancer cell fusion and genomic hybridization is presented in Table 8.1.

Here we summarize evidence supporting this model, and focus on how aerobic glycolysis – a trait of activated macrophages that is expressed in macrophage-tumor hybrids and malignant cancer cells alike – may perform a central role in the regulation of hybrid metabolic energy balance following fusion.

8.2 Macrophage-Tumor Cell Associations: The Scene of the Crime?

Tumor associated macrophages (TAMs) facilitate all aspects of cancer initiation and progression [10]. Macrophages are attracted through chemotactic signals to tumors where they exert their abilities for matrix degradation, tissue remodeling, stroma deposition, neoangiogenesis and migration to distant tissues. These functions are normally employed in many physiological functions such as embryogenesis, osteogenesis, and wound healing. Macrophages accumulate in hypoxic regions of tumors at least in part through HIF-1-mediated upregulation of the chemokine receptor [11, 12].

However, tumor progression is not completely explained by the presence of TAMs. During transition to a metastatic phenotype, tumor cells notoriously co-opt leukocytic traits [2, 3]. Malignant cells are chemotactic, responding to chemokines and exhibiting their own matrix-degrading and angiogenic capabilities. Like migratory leukocytes, metastatic cells exhibit loss of homotypic adhesion, and the ability to transverse a basement membrane, migrate through the mesodermal matrix, intravasate into lymphatics or the blood circulatory system, extravasate from these vessels, and colonize lymph nodes and distant organs. But unlike normal leukocytes, cancer cells have deregulated mitotic cycles and their numbers continually increase, killing the host if left unchecked. It has been recently proposed that expression of leukocytic traits by malignant cells can be explained by leukocyte-cancer cell fusion and genomic hybridization [2–5]. Below are considerations of this process with examples of many macrophage or other inflammatory cell traits of malignant cells that could be explained by fusion.

In order for macrophage-tumor cell fusion to occur, the process must be initiated by close contact between the two fusion partners. Such contacts are readily seen when experimental tumor xenografts are dissociated and placed into culture. Shown in Fig. 8.1 are cultured cells obtained from a Cloudman S91 mouse melanoma tumor implanted into a DBA/2J mouse. The cells were fixed and stained for non-specific esterase (red), a macrophage marker. At the far right is seen a red-staining macrophage

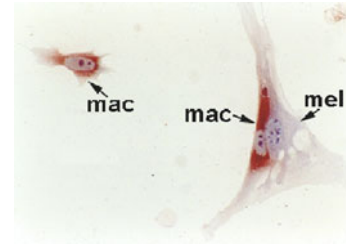

Fig. 8.1 A Cloudman S91 tumor was dissociated into individual cells and placed in culture. Non-specific esterase stain (*red*) revealed macrophages in contact with tumor cells

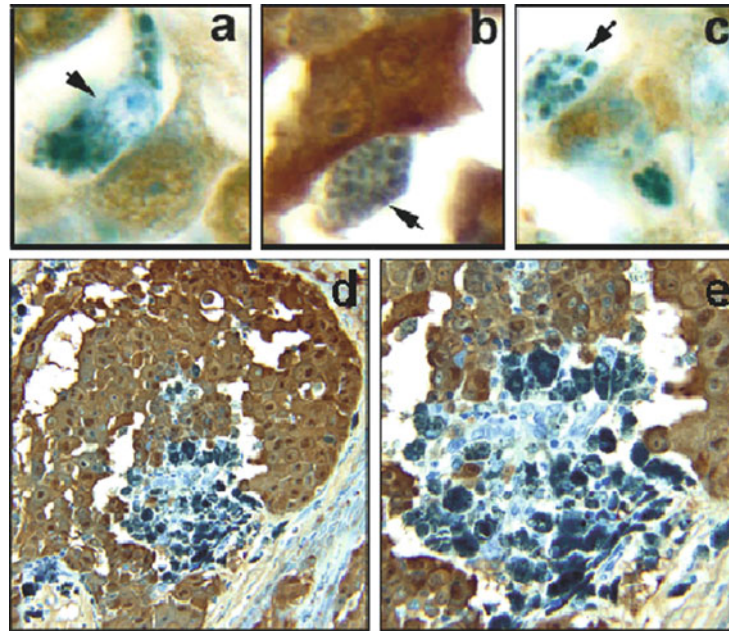

Fig. 8.2 Tumor associated macrophages (TAMs). (**a**–**c**) TAMs (*arrows*) staining with azure blue are seen in close contact with melanoma cells in three cases of primary cutaneous malignant melanoma. (**d**) A low power view of TAMs, (azure blue stain), are seen infiltrating into a nest of melanoma cells (S100 stain with brown chromogen) (**e**) Higher power view of (**d**). Note the highly vesicular nature of the macrophages due to the presence of autophagosomes and autolysosomes (*panels **a**–**c***) (from [13])

in close contact with a non-staining Cloudman melanoma cell. The melanoma cell is distinguished by its abundant nucleoli compared to those of the macrophage. On the far left is another macrophage staining red with a characteristic migratory orientation.

In pathology specimens of malignant melanomas, tumors are usually infiltrated with or surrounded by macrophages, most frequently in hyperpigmented areas of tumors [13] (Fig. 8.2a–e). Melanoma TAMs are referred to as "melanophages" due to their injestion of melanized melanoma cells and retention of incompletely digested melanin in autophagosomes or autolysosomes. High power views reveal that melanophages (azure blue) are found in close association with melanoma cells, with long segments of plasma membranes of the two cell types in apposition to one another (Fig. 8.2a, b), in some cases with macrophages engulfing melanoma cells (Fig. 8.2c). Thus macrophage-tumor cell contact – a prerequisite for cell-cell fusion – is common in human melanomas in vivo.

8.2.1 Fusion-Induced Aneuploidy

In his 1911 article, "About cell fusion with qualitatively abnormal chromosome distribution as cause for tumor formation" (from the German), Aichel first proposed the fusion theory of tumor progression and in order to experimentally verify this idea implored future scientists to "... *study chromosomes*

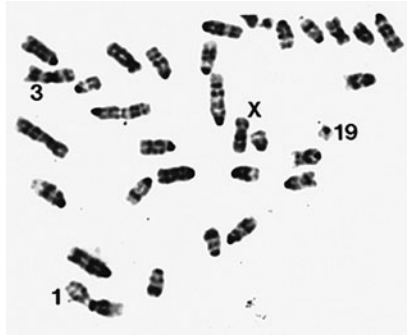

Fig. 8.3 Aneuploidy in human macrophage-mouse melanoma fusion hybrids created via polyethylene glycol-induced fusion in vitro. The photograph demonstrates the presence of human and mouse chromosomes in a metaphase spread of human macrophage-mouse melanoma hybrid 96–HJP1. Human chromosomes 1, 3, 19, and X are indicated and identified by high resolution Giemsa banding. Numerous acrocentric mouse chromosomes from the melanoma fusion partner are also seen (from [18])

from all angles." [1]. He proposed that the source of aneuploidy could stem from fusion of tumor-invading leukocytes with cancer cells, suggesting that a combination of extra chromosomes and the "qualitative differences" (now termed "epigenetic differences") in chromosomes from the two cell types could lead to the metastatic phenotype. With remarkably prescience he wrote, *Not only would the capacity for cell division increase, but after the mixing of the qualitatively different chromosomes the different traits and capabilities of the different cells depending on the type of cells fused would become obvious in different ways in the daughter cells. In this way the daughter cell may maintain some of the specialized function of the somatic [tumor] cell so that the tumor cell is capable to an extent to remain functional. On the other hand the traits of the leukocytes would also be incorporated in the daughter cell, so that a new cell with new traits and capabilities would emerge, a cell that has been thrown out of the path of the normal mother cell. The end product would be what we have learned to understand as a malignant cell.*

Decades later, the same hypothesis – that metastasis is caused by leukocyte-tumor cell fusion – was proposed independently by Meckler [14, 15] and by Goldenberg [16, 17]. Several laboratories have now reported that hybrids produced by fusion in vitro or in vivo were aneuploid and of higher metastatic potential (reviewed in [2–5]). As a demonstration of hybrid aneuploidy, shown in Fig. 8.3 is a metaphase spread from a human macrophage-mouse melanoma hybrid 96–HJP1 experimentally fused in vitro [18]. Seen are human chromosomes 1, 3, 19 and X along with numerous acrocentric mouse chromosomes. As discussed below, cells from this hybrid clone produced both human and mouse versions of the metastasis-related protein SPARC, indicating that the epigenomes from both fusion partners were active (discussed below). This was in line with the above predictions of Aichel that the cells would be aneuploid, mitotically active, and express genes from "qualitatively different" chromosomes of the leukocyte and tumor cell fusion partners.

8.2.2 Macrophage-Melanoma Fusion In Vitro Generates Altered Gene Expression and a Metastatic Phenotype In Vivo

Tumor-BMDC fusions might explain how common gene expression patterns emerge for different tumor types. We, and others, have found that when BMDC-tumor cell hybrids were isolated in vitro with no selective pressure other than for growth in drug-containing media, remarkably high numbers of them exhibited a metastatic phenotype in mice. Curiously, in melanoma the most metastatic clones tended to be highly melanized compared to parental melanoma cells or weakly metastatic hybrids as

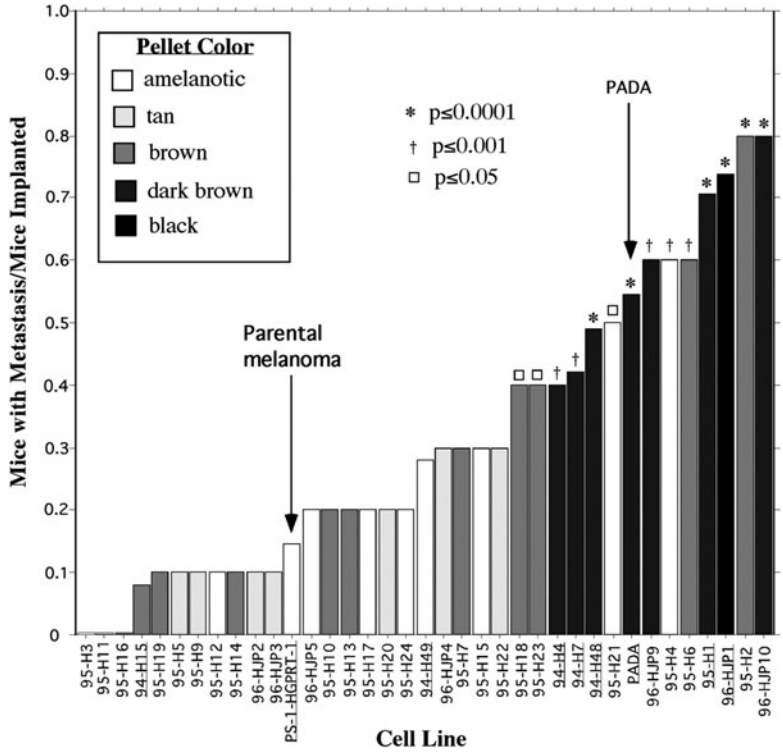

Fig. 8.4 Metastatic potential of macrophage-melanoma hybrid cell lines compared to parental Cloudman S91 melanoma cells. Results are shown for in vitro-generated hybrids and one spontaneous in vivo hybrid (PADA). Melanin was estimated in pelleted cells from clones on their first passage in culture and before metastatic potential was determined. A minimum of 10–20 animals was tested for each clone. In addition representative clones were tested repeatedly during continuous passage in culture for up to 4 years where 30–90 animals were tested for each clone with similar results as above. Statistical analyses of metastatic potential revealed that *p* values for significance vs. parental melanoma cells were < 0.0001 (*); < 0.01 (*dagger*); < 0.05 (*square*) (from [18])

described below (Fig. 8.4) [19, 20]. This was subsequently explained by the acquisition of macrophage glycosylation patterns involving β1,6-branched oligosaccharides that were associated with many phenotypic changes, including pigmentation [21] In two separate rounds of isolation, a total of 75 clones of PEG-fused macrophage-melanoma hybrids were isolated in vitro. About half showed increased chemotaxis in vitro and metastasis in mice [22]. Similar results were obtained in T-cell hybridomas from fusion of healthy T-lymphocytes with T-lymphoma cells [23] and in hybrids between mouse T-cell lymphoma cells and bone marrow-derived macrophages or spleen lymphocytes [24–26]. High frequency emergence of a common metastatic phenotype in vitro without host selective pressure was surprising, particularly in view of the apparently chaotic nature of aneuploidy.

8.2.3 Macrophage-Melanoma Fusion Hybrids Exhibit Up-Regulated Chemotaxis In Vitro

Increased metastatic potential in macrophage-melanoma hybrids was mirrored in increased chemotaxis in vitro (Table 8.2) [22]. In two-chambered assay systems comparing parental melanoma cells to low and high metastatic hybrids, the hybrids with high metastatic potential showed 5- to 100-fold increases in the rate of migration to 3T3- and lung fibroblast-conditioned media, primary lung slices, fibronectin (FN), and a 120 kDa FN fragment compared to parental melanoma cells. Unlike parental

Table 8.2 Metastatic potential in vivo vs. chemotactic migration in vitro of macrophage-melanoma hybrids

Cell line	Metastatic potential	Cells migrated		
		– MSH	+ MSH	p-value
Parental melanoma	14	2 ± 12	± 0	>0.050
Hybrid 95-H3	0	73 ± 16	32 ± 5	>0.050
Hybrid 95-H11	0	23 ± 5	42 ± 8	>0.050
Hybrid 95-H19	10	50 ± 3	70 ± 2	<0.020
PADA	54	87 ± 12	266 ± 20	<0.001
Hybrid 95-H1	71	142 ± 14	436 ± 21	<0.001
Hybrid 95-H2	78	143 ± 5	364 ± 42	<0.001

Migration to the underside of a Costar Transwell apparatus of parental Cloudman melanoma cells, low and high metastatic macrophage-melanoma fusion hybrids, and PADA, a spontaneous in vivo hybrid, in response to 3T3 cell-conditioned medium (33%, vol/vol) in the lower chambers. For migration, results represent mean+S.E. for triplicate assays of cells migrated/4 h. Where noted, cells were pretreated for 72 h in culture with MSH and the cAMP phosphodiesterase inhibitor isobutylmethylxanthine to raise cyclic AMP levels. p values are for differences between control vs. MSH/IBMX treatment [22].

cells, metastatic hybrids were further stimulated by pretreatment with melanocortin-1 (melanocyte stimulating hormone; MSH) [22].

Shown in Fig. 8.5 are fixed and stained cells from highly metastatic hybrid H95-H1 on the underside of a 2-chambered migration assay system. In a 4-h assay period, the cells traveled from the upper chamber through 12 μm diameter pores in response to a 120 amino acid fibronectin fragment in the lower chamber. The cells had been pretreated with MSH (Table 8.2).

From these results it seems likely that increased chemotaxis of macrophage-melanoma hybrids was due to expression of the macrophage epigenome. However, little is known of the regulation of gene expression in hybrids at the molecular genetic level. Evidence that BMDC-tumor hybrids express many of the same genes associated with invasive and metastatic cancers and that these genes are also expressed by macrophages and other migratory BMDCs is summarized below (reviewed in [2–5]).

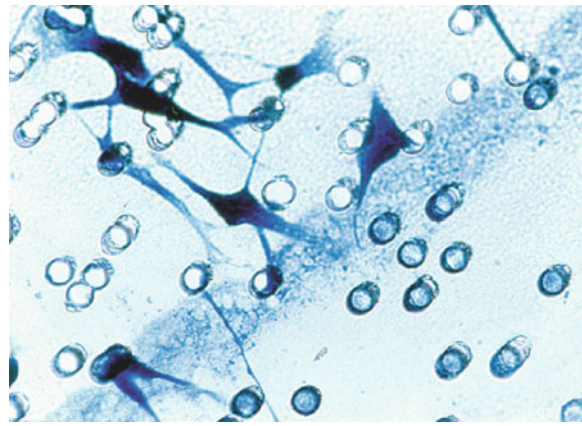

Fig. 8.5 A view of the underside of the partition between the upper and lower chamber of a two-chambered migration assay system with 12 μm pores in the partition between the chambers. Cells of macrophage-melanoma hybrid 95-H1 were pretreated for 72 h with MSH in monolayer culture and then seeded into the upper chamber in culture medium supplemented with MSH and IBMX. The lower chamber contained the same medium but further supplemented with a 120 amino acid fibronectin fragment. After 4 h in a gassed, humidified incubator (37°C) the cells were fixed and stained and cells on the under surface of the upper chamber were counted and photographed (from [22])

8.2.4 SPARC

The SPARC (secreted protein acidic and rich in cysteine; osteonectin; BM40) gene provides an example of gene regulation in leukocyte-tumor fusion. SPARC is expressed in macrophages, experimental macrophage-melanoma hybrid and is associated with tumor progression and poor outcome in melanoma and a number of carcinomas including breast, colorectal, ovarian and lung [2, 3, 27]. SPARC acts as a regulator of melanoma EMT by downregulating melanoma E-cadherin with loss of homotypic adhesion, stimulates motility, and increases expression of mesenchymal markers such as matrix metalloproteinase MMP-9 [28]. In tissue macrophages SPARC is expressed in regions of neovascularization, for example in wound repair [29] and degenerative aortic stenosis [30]. In fusions between mouse macrophages or human blood monocytes and weakly metastatic mouse Cloudman S91 melanoma cells, the total levels of SPARC mRNA were three- to four-fold higher per μg total RNA in metastatic hybrids compared to weakly metastatic hybrids and parental melanoma cells [31]. Notably, hybrids between human monocytes and mouse melanoma cells expressed both human and mouse SPARC mRNA [31]. This indicated that at least for SPARC, the epigenomes from the two different developmental lineages of the fusion partners were both active. In summary, SPARC gene expression was enhanced by hybridization of tumor cells with macrophages; high expression was correlated with high metastatic potential; and SPARC mRNA was produced in hybrids from the genomes of both parental fusion partners. That elevated SPARC expression was a characteristic of macrophage-melanoma hybrids provides a possible explanation for elevated SPARC and SPARC-mediated pathways in human melanoma and other cancers.

In addition to SPARC, macrophage-melanoma hybrids of high metastatic potential also showed marked elevations of other macromolecules that are characteristic of macrophages and known indicators of metastasis, including cMet, the melanocortin 1 receptor, and the integrin subunits α_3, α_5, α_6, α_v, β_1, β_3. We thus hypothesize that such gene expression patterns in cancer may be generated through fusion with macrophages (reviewed in [2, 3]).

8.2.5 GnT-V and β1,6-Branched Oligosaccharides

N-acetylglucosaminyltransferase V (GnT-V; Mgat5; E.C.2.4.1.155) is a Golgi complex enzyme that is highly expressed in myeloid cells and metastatic cancer cells. GnT-V and its enzymatic products, β1,6-branched oligosaccharides conjugated to N-glycoproteins, are associated with poor outcome in a number of cancers [32, 33]. β1,6-branched oligosaccharides were first purified from granulocytes [34]. From structural analyses they are composed of poly-N-acetyllactose amines that are carriers of sialyl lewis^x antigen (sialyl le^x) and therein used by both leukocytes and metastatic cancer cells for binding to E-selectin and/or galectin-3 on endothelial cells during systemic migration [35, 36].

GnT-V mRNA, protein, and/or enzymatic activity were elevated in high metastatic macrophage-melanoma hybrids formed in vitro [37], and following spontaneous host-tumor fusions in both lymphomas and melanomas in mice [24, 37, 38]. Multiple pathways in invasion and metastasis that are regulated by GnT-V were elevated in macrophage-melanoma hybrids – as seen below with motility-associated integrin subunits, cell surface expression of LAMP-1, and autophagy.

8.2.6 Cell Surface Expression of Lysosome Associated Protein-1 (LAMP-1)

LAMP-1 is a preferred substrate for GnT-V and a major carrier of sialyl le^x and poly-N-acetyl-lactose amines that bind to E-selectins and galectins [35, 36]. Cell surface LAMP-1 thus mediates binding to endothelial cells by both leukocytes and cancer cells [35, 36, 39]. Macrophage-melanoma hybrids showed elevated expression of cell surface LAMP-1 [37]. This was seen in high metastatic

macrophage-melanoma hybrids as well as peritoneal macrophages compared to that in parental melanoma cells and low metastatic hybrids.

8.3 Coarse Melanin and Autophagy in Experimental Macrophage-Melanoma Hybrids

As mentioned above, the parental melanoma cells used in the production of experimental macrophage-melanoma hybrids in vitro produced little or no melanin (Fig. 8.6, left), however many hybrid clones – particularly those of high metastatic potential – were heavily pigmented with coarse, dark melanin throughout the cytoplasm as shown for metastatic hybrid 94-H48 (Fig. 8.6, right) [40].

Electron micrographs revealed that although some melanosomes existed freely in the cytoplasm, the coarse granular appearance of melanin in hybrid 94-H48 was due to melanosome-filled autophagasomes in various stages of maturation (Fig. 8.7) [40].

Staining for β1,6-branched oligosaccharides with the plant lectin LPHA (leukocytic phytohemagglutinin) revealed that the coarse, melanin – containing autophagosomes contained these glycan structures in abundance (brown stain). The granular LPHA staining pattern (white arrows) encompassed large portions of the cytoplasm, often obscuring the nucleus ("n") (Fig. 8.8) [40].

Co-localization of β1,6-branched oligosaccharides to coarse melanin is shown in Fig. 8.9. A single cell of macrophage-melanoma hybrid H48-94 is seen in culture. The left panel shows an unstained culture allowing for the visualization of coarse melanin. The right panel shows the same cell after bleaching to decolorize the melanin and staining for β1,6-branched oligosaccharides with the lectin LPHA. The results demonstrate an exact correspondence between LPHA staining and coarse melanin autophagosomes [40].

8.3.1 Spontaneous Fusion In Vivo and Autophagy

There are numerous reports in animal cancer models of tumor cell fusion with host cells and many of these implicate macrophages or other BMDC's as host fusion partners [2–5]. An example from our lab is seen in the development of a spontaneous melanoma metastasis to the lungs in a Balb/c nude mouse (Fig. 8.10) [41]. Balb/c mice are albino due to a homozygous mutation in tyrosinase (c/c), the rate-limiting enzyme in melanogenesis. The parental melanoma cells implanted into this

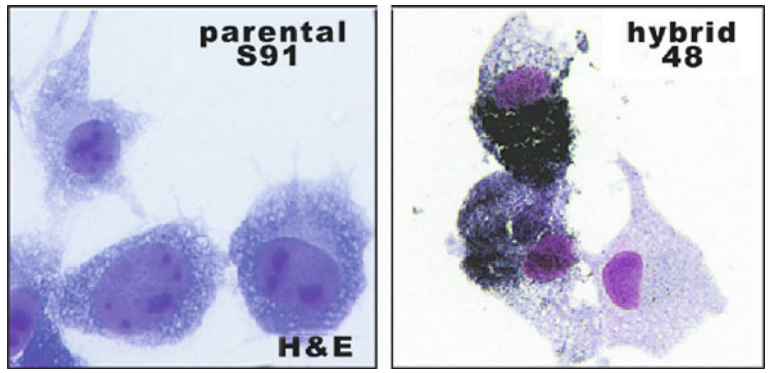

Fig. 8.6 Comparison of cultured parental Cloudman S91 melanoma cells and high metastatic macrophage-melanoma hybrid 94-H48 following staining with H&E. *Left*: parental Cloudman S91 cells showing lack of pigment; *right*: hybrid 94-H48 with abundant coarse melanin (from [40])

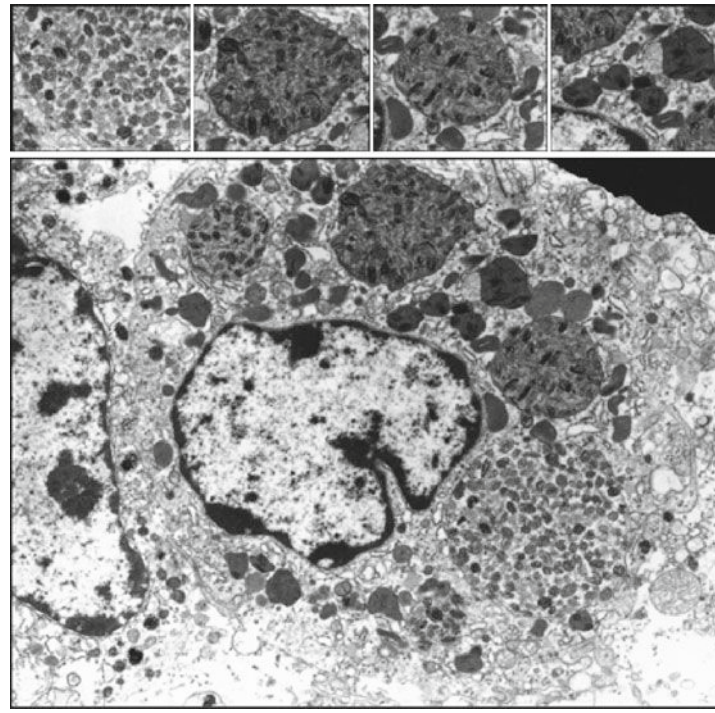

Fig. 8.7 Electron micrograph of high metastatic macrophage-melanoma hybrid 94-H48 showing cytoplasmic autophagosomes in the *lower panel.* The *upper* insets show individual autophagosomes and autolysosomes in various stages of maturation (early to late maturation stages are shown from *left to right*) (from [40])

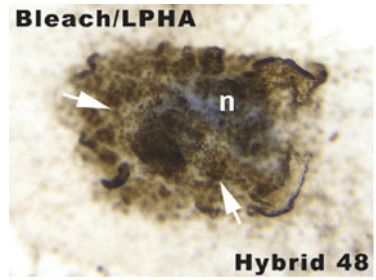

Fig. 8.8 A single cell of hybrid 94-H48 in culture. The culture was fixed and decolorized of melanin by bleaching. It was then stained with the lectin LPHA for β1,6-branched oligosaccharides (*brown stain*) and counterstained with hematoxylin. *Arrows* denote the granular staining pattern characteristic of autophagosomes. The nucleus (*n*) is partially obscured by the autophagosomes (from [40])

mouse were the same clone used as the melanoma fusion partners in the in vitro fusion experiments described above. Although the melanoma clone implanted into these mice was genetically wild type for tyrosinase (C/C), the cells produced little or no melanin in culture and formed amelanotic tumors in mice. Metastases, though infrequent, were generally small, amelanotic tumors in the lung, and were well tolerated by the mice [41]. However, in one experiment a mouse developed a melanin-producing *in transit* metastasis near the site of implantation in the tail dermis. The tail was amputated and the mouse was followed to see if distant metastases developed. After 5 weeks the mouse became moribund with a massive, highly pigmented pulmonary metastasis.

DNA analyses showed that cells from the metastasis had a genotype of C/c, indicating they were hybrids formed from fusion of the implanted tumor cells (C/C) with host cells (c/c). Cells from the

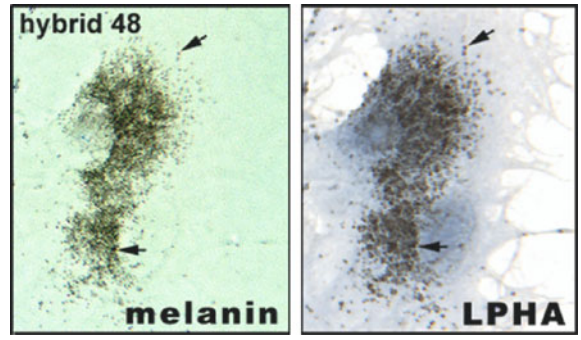

Fig. 8.9 Co-localization of coarse melanin with β1,6-branched oligosaccharides in highly metastatic macrophage-melanoma hybrid 94-H48. *Left*: unstained sections; *right*: the same sections bleached and stained for β1,6-branched oligosaccharides with the lectin LPHA (from [40])

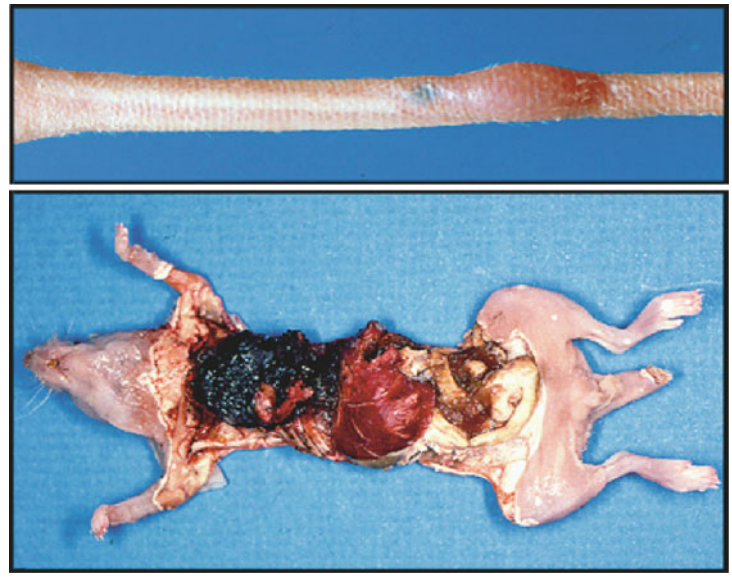

Fig. 8.10 A spontaneous host-tumor pulmonary metastasis. Cloudman S91 mouse melanoma cells were implanted s.c. into the tail of a Balb/c *nu/nu* mouse. *Upper*: an amelanotic primary tumor arising after 4 weeks. *Lower*: The same mouse 7 weeks after the tail was removed. The mouse died and was found to have a large melanotic pulmonary metastasis. Note that the tail had been previously transected as described in the text (from [41])

metastasis showed an average 30–40% increase in DNA content, increased chemotaxis in vitro, activation of the glycosyltransferase GnT-V, and production of β1,6-branched oligosaccharides (see below). They also produced the same "coarse melanin" granules seen in the in vitro macrophage-melanoma fusions above (Fig. 8.11, arrows). That the course melanin granules were indeed autophagosomes was verified by electron microscopy (Fig. 8.12) [41].

Small numbers of highly melanized, coarse melanin-producing cells were found within the original implanted tumor (Fig. 8.13a, b). These were not present in the cultured parental melanoma cells and were thus generated in vivo [41]. Morphologically identical cells were cultured from the metastasis and determined to be C/c hybrids with host cells, indicating that fusion and hybridization had occurred in the original implant (Fig. 8.13c, d). Moreover, histopathology studies of the original implant revealed that it was infiltrated with macrophages (not shown), indicating that macrophage-tumor fusion could have occurred in the tail tumor where the hybrid cells metastasized to the lung. In summary, the host-tumor cell hybrid lung metastasis showed the same characteristics as macrophage-melanoma hybrids experimentally fused in vitro, regarding aneuploidy, chemotaxis, enhanced pigmentation, and high levels of autophagy. This was also a characteristic of another spontaneous melanoma-host hybrid described previously ("PADA") [18–20]

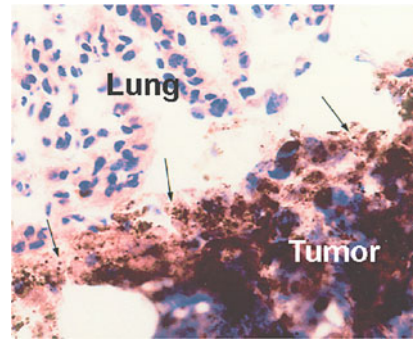

Fig. 8.11 Histological section of the pulmonoary metastasis shown in Fig. 8.10 and stained with H&E. Arrows show coarse melanin-containing cells in the tumor (from [41])

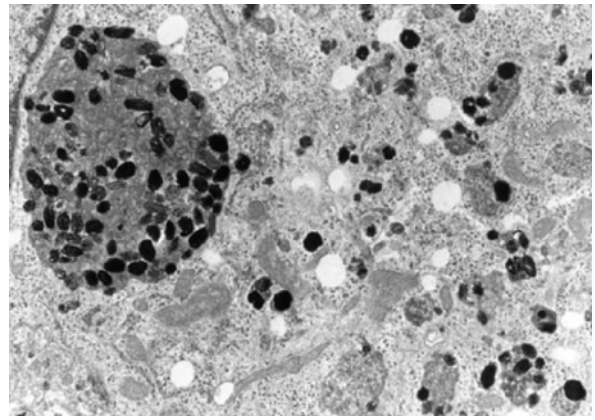

Fig. 8.12 Electron microphage of a cultured cell from the spontaneous host-melanoma hybrid shown in Fig. 8.10. Shown is a prominent autophagosome (from [41])

Thus, experimental macrophage-melanoma hybrids in cell culture and spontaneous host-melanoma hybrids arising in vivo and adapted to culture all appeared to show constitutive autophagy without autophagic death. It is important to note that this occurred in cell culture under normoxic conditions and ample nutrients indicating that hybrids expressed the aerobic glycolysis pathway (the Warburg effect) that includes autophagy [42–48]. Below it is discussed that macrophages also exhibit autophagy and aerobic glycolysis in vivo and in vitro, suggesting that perhaps these traits in macrophage-melanoma hybrids shown above may be an expression of the macrophage epigenome in the hybrid cells.

8.3.2 Autophagy and Aerobic Glycolysis in Macrophages and Cancers

Under hypoxic conditions most cells go through apoptotic death in a mitochondrial Bcl-2 – mediated fashion [49–53]. However inflammatory cells and cancer cells alike survive through utilization of the glycolytic pathway for ATP production, independent of mitochondria [54–56]. High densities of macrophages are found in tumors, wounds, atherosclerotic plaques, bone fractures, rheumatoid arthritis, and ischemic areas in diabetes (reviewed in [54]). Due to their reliance on glycolysis, macrophages need large amounts of glucose for ATP production. Thus, much like the vast majority (~90%) of malignant solid tumors [57], macrophages in hypoxic areas can be visualized by Positron Emission Tomography (PET) due to their avid uptake of the glucose analog fluorodeoxyglucose (^{18}F) [58–61]. Further, like cancer cells, activated macrophages exhibit glycolysis even under aerobic conditions, a state known as aerobic glycolysis or the Warburg effect [51, 52, 62]. Glycolysis in

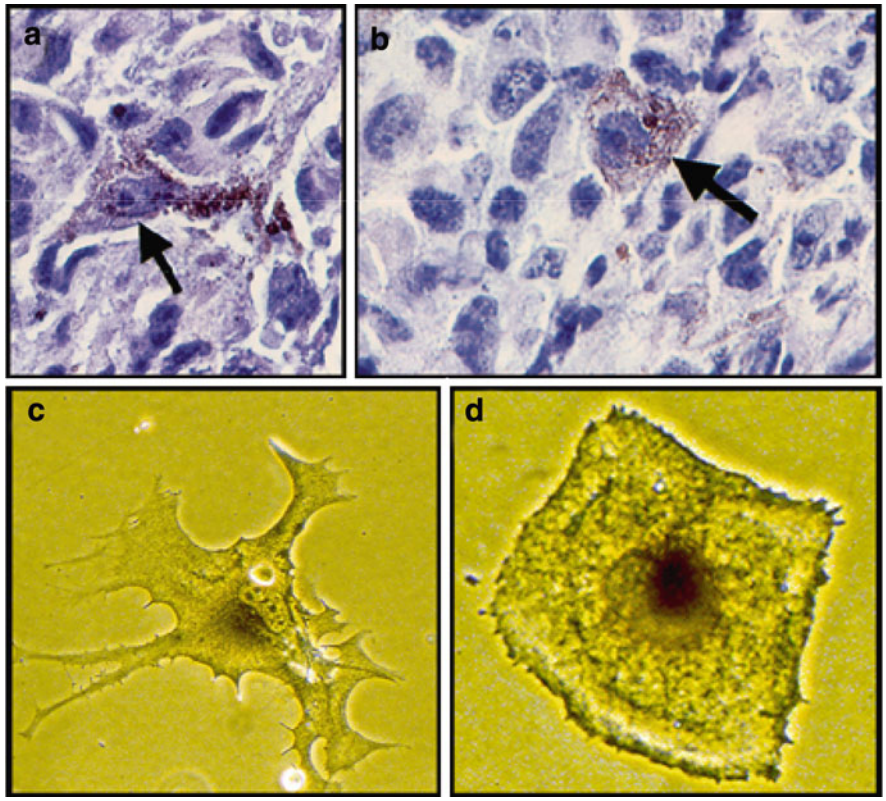

Fig. 8.13 *Panels a and b.* Histological sections of the tail implant of Cloudman S91 melanoma cells shown in Fig. 8.10 (H&E stain). Arrows denote coarse melanin containing melanoma cells. *Panels c and d*. Phase contrast photos of coarse melanin-containing cells cultured from the pulmonary metastasis seen in Fig. 8.10. The original clone of Cloudman cells used in this study was amelanotic in culture even though it was wild type for tyrosinase (C/C) Thus, the pigmented melanoma cells were generated in the mouse (c/c), potentially through macrophage-tumor cell fusion as seen in experimentally fused hybrids (NRC). As predicted, cells cloned from the metastasis were found to be C/c fusion hybrids (from [41])

macrophages is accompanied by dramatic changes in gene expression patterns and phenotype, including activation of pathways for motility and phagocytosis and markedly increased cell viability [52, 54, 63–67]. The transcription factor HIF-1α is a master regulator of the hypoxic phenotype [55, 68, 69]. HIF-1α is regulated by the serine/threonine kinase Akt, which also mediates the switch to aerobic glycolysis [70].

The advantage of cancer cells for utilizing glycolysis over oxidative respiration has long been unclear, since the Krebs cycle is far more efficient than glycolysis in ATP production. However a recent proposal argues that the glycolysis pathway is best suited to proliferating cells which constantly need to increase biomass and thus depend on the uptake and/or production of glucose, nucleotides, amino acids, and lipids [49].

8.3.3 Autophagy in Macrophages

Melanophages are macrophages filled with melanized vesicles are similar under light microscopic examination to coarse melanin seen in melanoma cells nearby in the same tissue (Figs. 8.2 and 8.14). These vesicles are presumably generated from phagocytic engulfment by the macrophage of melanoma cells containing melanin, followed by transfer of the engulfed cellular debris into

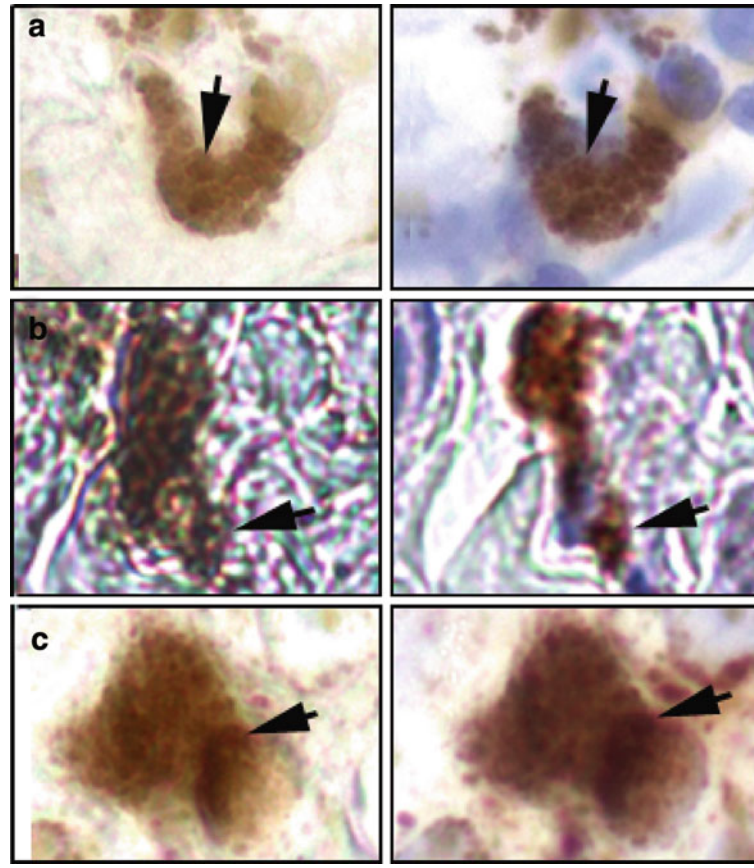

Fig. 8.14 Co-localization of granular melanin with heavily melanized vesicles in melanophages. Melanin in unstained sections is displayed in the *left column* and the corresponding stained fields are shown in the *right column*. *Arrows* denote examples of co-localized vesicles. (**a**) A section stained for LC3B. (**b**) A section stained for the Golgi 58k protein. (**c**) A section stained for β1,6-branched oligosaccharides with biotinylated LPHA (from [71])

autophagosomes. Co-localization studies showed that the autophagosome marker LC3B (Fig. 8.14a), the Golgi 58k protein (Fig. 8.14b), and β1,6-branched oligosaccharides (Fig. 8.14c) were all constituents of the melanized vesicles in melanophages, indicative of autophagy [71].

Electron micrographs of melanophages confirmed that the vesicles were autophagosomes (Fig. 8.15) [71]. These autophagosomes in the melanophages are limited by double membranes and, similar to autophagosomes in macrophage-melanoma hybrids, contain what appears to be partially digested, melanized melanosomes, and other cellular debris. The melanophages were surrounded by collagen bundles (labeled "c") confirming their dermal location beneath a melanoma tumor in the epidermis (melanoma in situ) seen under the light microscope in pathology specimens.

8.3.4 Autophagy in Human Cancers

The above findings of autophagy in macrophages and in macrophage-melanoma hybrids motivated us to launch a larger pathology survey of melanomas and other human cancers as summarized below.

8.3.5 Normal Epidermis

In regions of normal epidermis, melanocytes and keratinocytes did not stain for LC3B or β1,6-branched oligosaccharides and showed no signs of autophagy confirming previous findings that these cells do not appear to produce β1,6-branched oligosaccharides [32, 71, 72].

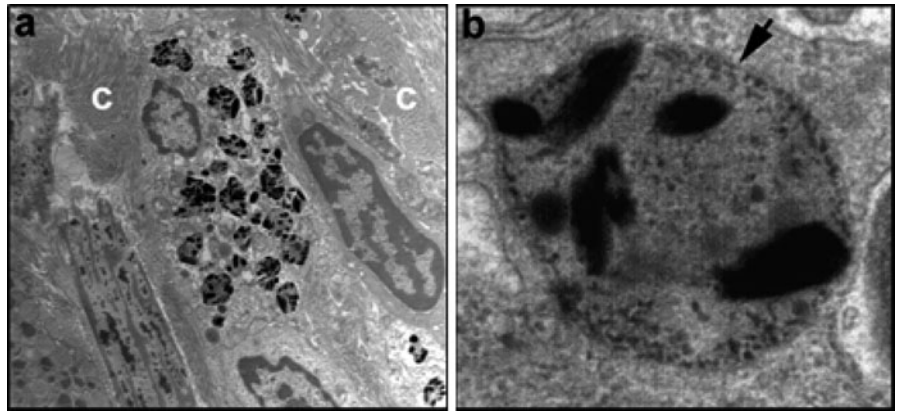

Fig. 8.15 Electron micrographs of a dermal melanophage. (**a**) Low power view showing numerous melanin-containing vesicles within the cytoplasm of a melanophage. Collagen bundles in the adjacent dermis are labeled by "c". (**b**) High power view of a vesicle, which is an autophagosome, enveloped by a double membrane (*arrow*), and containing partially digested, heavily melanized melanosomes (from [71])

8.3.6 Early Melanoma In Situ (MIS)

Early MIS presents as a subtle proliferation of atypical melanocytes (melanoma cells) disposed as single units as well as in a few small nests at the dermal epidermal junction and above it (Fig. 8.16a–d). Similar to normal melanocytes, melanoma cells in regions of early MIS did not show prominent melanin in their cytoplasm as seen with H&E staining (Fig. 8.16a). Cells of early MIS did not stain or stained only weakly for LC3B (Fig. 8.16b) and for β1,6-branched oligosaccharides (Fig. 8.3d). However, similar to normal melanocytes they did stain for the Golgi 58k protein in a globular perinuclear pattern (Fig. 8.16c) [71].

8.3.7 Autophagy in Florid MIS

In florid MIS there is an irregular, asymmetric, and poorly circumscribed proliferation of melanoma cells. There are nests of melanoma cells that vary markedly in size and shape, which are not equidistant from one another. Single melanoma cells predominate over nests in some high power fields and there are individual melanoma cells as well as melanocytic nests above the dermal-epidermal junction. Melanoma cells are also seen down adnexal structures. In our study melanoma cells in florid MIS of all 13 cases produced coarse melanin to at least some extent (Fig. 8.17a). In addition, in all cases most if not all of the cells of florid MIS stained for the autophagosome marker LC3B with a heterogeneous vesicular pattern in the cytoplasm, indicating the presence of autophagosomes (Fig. 8.17b). Surprisingly, the Golgi 58k protein was distributed not in a globular perinuclear pattern, characteristic of normal cells and early MIS (above), but in a heterogeneous vesicular pattern, similar to that of coarse melanin and LC3B (Fig. 8.17c). In previous studies such a vesicular pattern for Golgi staining has been described as Golgi "fragmentation" or "vesiculation" [27, 34–36]. Unlike melanocytes in normal epidermis and melanoma cells in early MIS, the nested melanoma cells in florid MIS produced β1,6-branched oligosaccharides, which, like the LC3B and Golgi 58k protein, also stained in a heterogeneous vesicular pattern (Fig. 8.4d). In summary of florid MIS, most if not all of the

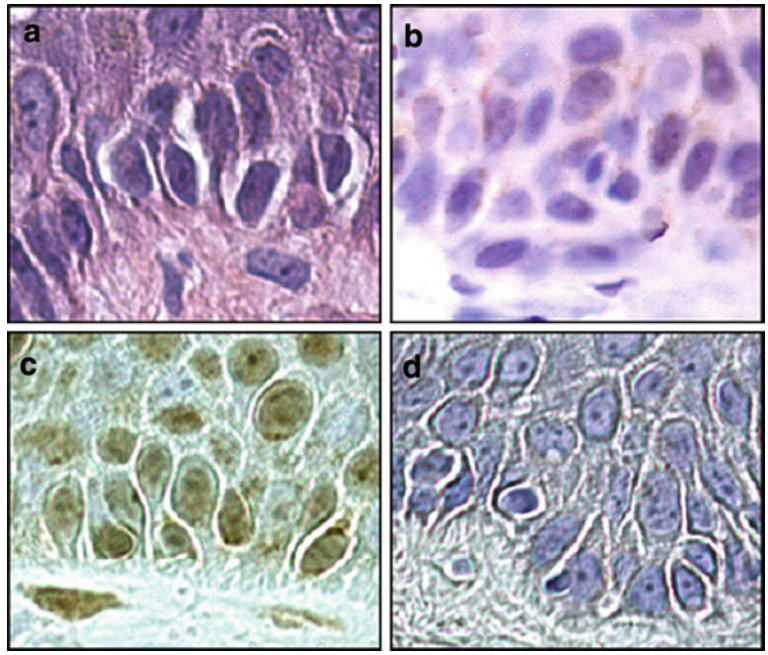

Fig. 8.16 A region of early melanoma in situ in a case of cutaneous malignant melanoma showing crowded melanocytes (melanoma cells) near the dermal epidermal junction. (**a**) A section stained with H&E. (**b**) A section bleached to decolorize melanin, subsequently stained with anti-LC3B, and counterstained with hematoxylin, showing faint staining for LC3B. (**c**) A section bleached, subsequently stained with anti-Golgi 58k protein, and counterstained with azure blue with a positive perinuclear staining. (**d**) A section bleached, subsequently stained with biotinylated LPHA, and counterstained with hematoxylin showing negative staining (from [71])

melanoma cells had autophagosomes. The autophagosomes contained LC3B, the Golgi 58k protein, and β1,6-branched oligosaccharides [71].

A region of florid MIS was analyzed by electron microscopy. Numerous vesicles surrounded by double membrane and filled with heavily melanized melanosome-like structures and other debris were seen (Fig. 8.18) [71]. The melanosomes seemed to be partially digested and might have represented only residual, undigested melanin; however, that remains to be further elucidated. Such double-membraned vesicles containing cytoplasmic organelles are defined as autophagosomes [73]. Thus, we conclude that coarse melanin vesicles are autophagosomes containing LC3B, the Golgi 58k protein, and β1,6-branched oligosaccharides.

While coarse melanin in florid MIS was present in all cases to some extent, only 4 of the 12 cases showed coarse melanin in the invasive component (cases 4, 5, 7, and 11). The invasive components of the remaining cases were only lightly pigmented or amelanotic. Regardless of the presence or not of coarse melanin, melanoma cells in the invasive component of all 12 cases showed positive staining, for the autophagosome marker LC3B (Fig. 8.19) [71]. Staining patterns for both markers were heterogeneous and vesicular. The same pattern was also seen for β1,6-branched oligosaccharides in all 12 cases of invasive melanoma (not shown). We have since examined an additional 19 individual cases of primary invasive melanomas and again obtained the same results – nearly uniform LCB3 staining in a granular fashion in all the invasive components, indicative of autophagosomes and/or autophagolysosomes. Thus, in 31 of 31 individual cases examined, invasive components of primary cutaneous malignant melanomas exhibited an autophagic phenotype.

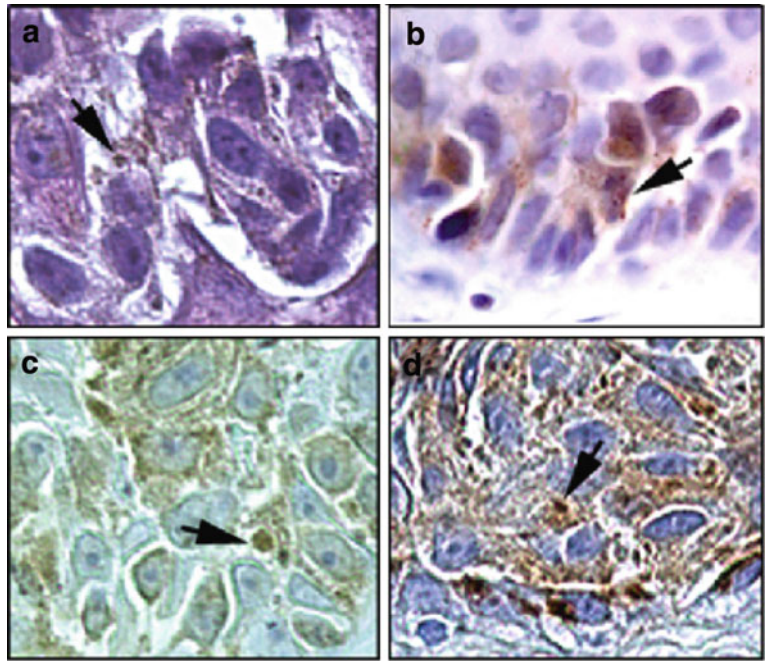

Fig. 8.17 Florid MIS in malignant melanoma. Small nests of melanoma cells were bleached, stained with antibody or lectin, and counterstained with hematoxylin. *Section c* was not bleached, but directly stained with the antibody and counterstained with azure blue. *Arrows* denote coarse melanin (**a**) and vesicular staining pattern (**b–d**). (**a**) A section stained with H&E. (**b**) A section stained for LC3B. (**c**) A section stained for the Golgi 58k protein. (**d**) A section stained for β1,6-branched oligosaccharides with biotinylated LPHA (from [71])

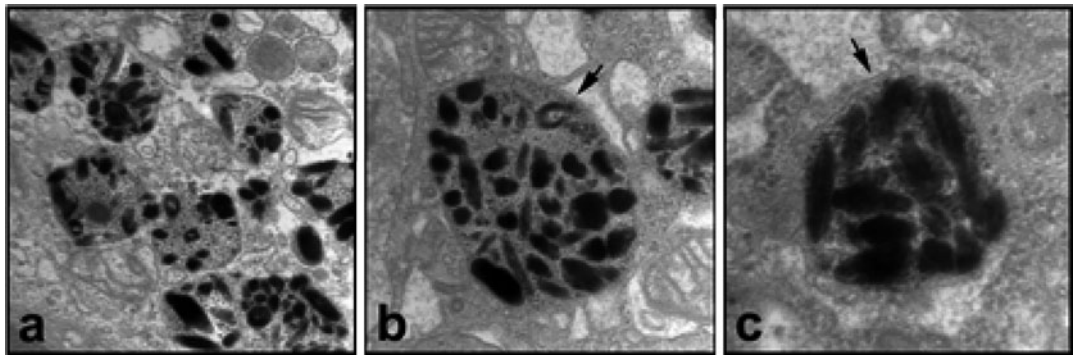

Fig. 8.18 Electron micrographs of coarse melanin vesicles in a region of florid MIS. (**a**) Low power view showing vesicles with heavily melanized melanosome-like structures. (**b**) and (**c**) High power views of individual vesicles. The arrows show that the vesicles are bordered by double membranes and by definition represent autophagosomes (from [71])

8.3.8 Autophagy in Tumor Progression

The above pathology studies on autophagosomes in primary melanomas has recently been expanded to a larger study including nearly 2,000 solid tumor pathology specimens from 20 different cancers [71]. In summary, regardless of tumor type a large majority of cancer cells contained autophagosomes, as assessed by LC3B granular staining and, in the case of melanoma, by electron microscopy (Fig. 8.18).

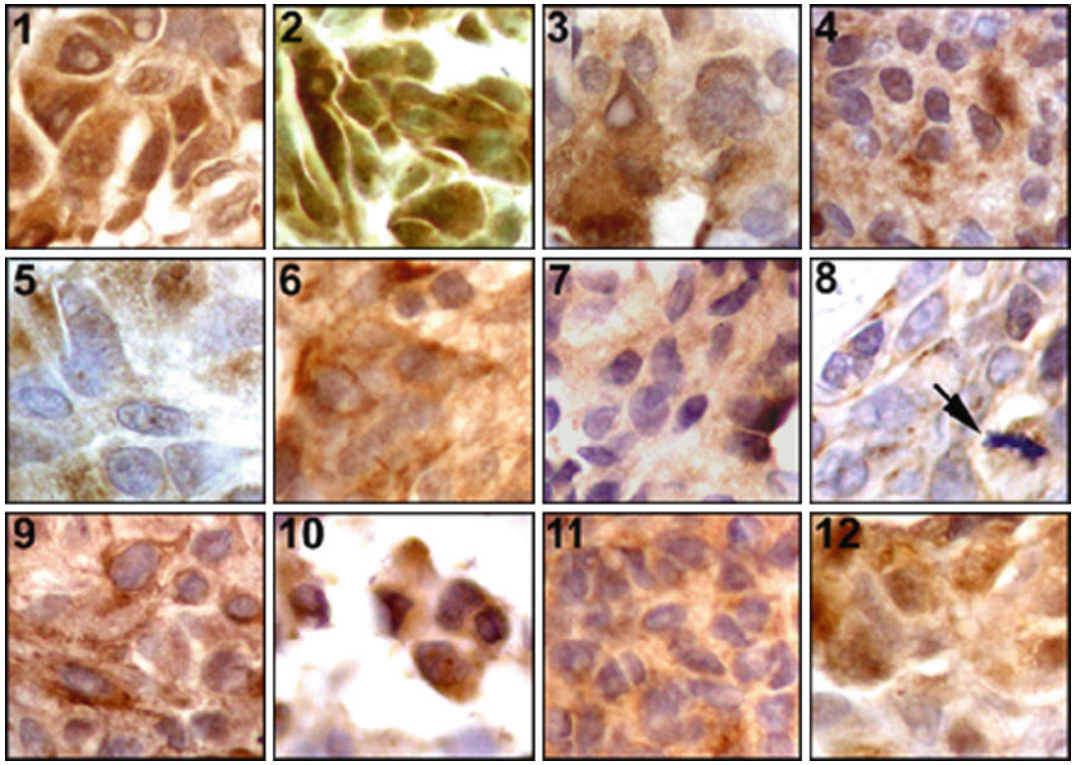

Fig. 8.19 LC3B staining in the invasive components of 12 malignant melanoma cases. The panel numbers indicate different cases. *Case 2* was stained with anti-LC3B and counterstained with azure blue. The remaining cases were first bleached to decolorize any melanin, stained with anti-LC3B, and counterstained with hematoxylin. The *arrow* in case 8 denotes a mitotic figure in an autophagic cell (from [71])

The presence of autophagosomes was associated with tumor proliferation, spread, and worse patient outcome. Further supporting a role for autophagy in proliferation, mitoses were widely present in autophagosome-containing cells. For example a mitotic figure in an autophagic cell is seen in Fig. 8.19 (case 8, arrow). The findings support the recent proposal by Vander Heiden et al. that the Warburg effect provides a metabolic advantage for proliferating cancer cells since they have a constant need for anabolic precursors to support cell division [47]. Since autophagy is an evolutionarily-conserved mechanism for providing such precursors [44, 74], it would appear that it plays an important role in this process by providing fuel for cell division and ultimately an increase in tumor mass. However, this could not be sustained by self cannibalism alone and we propose that in order to maintain a positive energy balance autophagy is likely to be linked to phagocytosis or other endocytic processes as it is in macrophages, providing a mechanism for ingesting and digesting exogenous food sources.

8.4 Conclusions and Future Directions

Could autophagy in human cancer result from fusions between cancer cells and macrophages or other phagocytes? In fact, macrophages express active autophagy as a part of the pathway for digestion of phagocytosed microorganisms and cells and autophagy in macrophages is linked to phagocytosis, another characteristic of metastatic cancers [75–80]. Therefore, activation of phagocytic and autophagic pathways in human cancers could reflect expression of imprinted genes of myeloid lineage

in macrophage-tumor cell fusion hybrids. We propose that should cancer cell autophagy be linked to phagocytosis or other endocytic processes as it is in macrophages, nutrients could be continuously internalized from external sources and digested through the autophagy-lysosome pathways, providing an exogenous food source for proliferation and migration.

In summary, this chapter has reviewed evidence for the leukocyte-cancer cell theory of metastasis and how leukocyte-cancer cell fusion could be an underlying initiator of aerobic glycolysis or the Warburg effect in cancer. Results from several laboratories support the points of the model for cancer cell fusion presented in Table 8.1. A cartoon model for this is presented below (Fig. 8.20) [2].

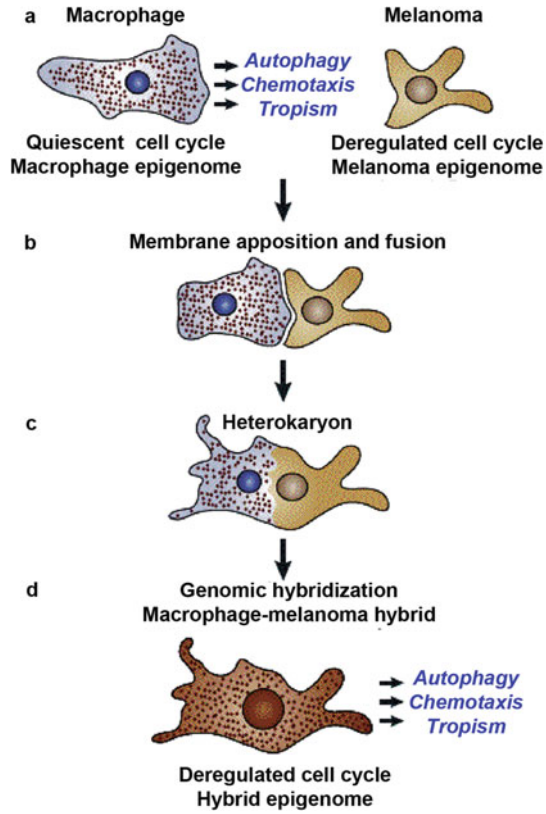

Fig. 8.20 A model for generation of a metastatic phenotype following fusion of a melanoma cell with a macrophage. (**a**) A macrophage is attracted to a non-migratory melanoma cell in situ. The epigenomes of the two cells reflect their myeloid and melanocytic lineages respectively. The melanoma cell produces "fine" or "dusty" melanin – individual melanosomes in the cytoplasm, generally with a golden-*brown color*. Melanoma-associated macrophages are known as melanophages because they are laden with autophagolysosomal vesicles containing melanin from injested melanoma cells, and thus at times difficult to distinguish from melanoma cells at the light microscope level. (**b**) The macrophage and melanoma plasma membranes form close appositional contacts, normally as a prelude to injestion and destruction of the melanoma cell. However in some cases rather than the macrophage digesting the melanoma cell, the two cells fuse. (**c**) Following fusion a heterokaryon is formed with the two nuclei separate in the cytoplasm. (**d**) Genomic hybridization occurs and a mononuclear macrophage/melanoma hybrid emerges. From studies of macrophage/melanoma hybrids generated experimentally in vitro and of melanoma/host hybrids generated spontaneously in mice, such hybrids have a deregulated cell cycle, are aneuploid and exhibit epigenomes of both parental lineages. Some exhibit the myeloid capability for chemotaxis in vitro and tropism in vivo, common characteristics of metastatic cells (from [2])

It should be noted that we did not attempt to incorporate concepts of cancer stem cells and fusion into this review. While we have used macrophages as examples for cancer cell fusion partners in vivo, there is no reason to rule out other leukocytes or stem cells that have fusion and hybridization capabilities [81]. It is interesting in this regard that, like tissue macrophages, hematopoietic stem cells in the bone marrow exhibit constitutive aerobic glycolysis [82].

Many more studies are needed to establish a role for cell fusion in human cancer. However it is interesting that the studies of cell fusion in animals and cell culture lead us to the findings of extensive autophagy in human cancers. Whether or not this was caused by leukocyte-cancer cell fusion, the results suggest that autophagy may play a key role in fueling cell division, and thus may represent an area of great vulnerability to cancer cells, underlining the emerging therapeutic importance of this metabolic pathway. We hope that the observations and speculations presented herein will stimulate new research in these most interesting areas.

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Chapter 9
Cell Fusion, Drug Resistance and Recurrence CSCs

Christa Nagler, Kurt S. Zänker, and Thomas Dittmar

Abstract Cancer stem cells (CSCs) are a rare population of cancer cells exhibiting stem cell properties, such as self-renewal, differentiation and tissue restoration. Beside the initiation of the primary tumor, CSCs have also been associated with metastasis formation and cancer relapses. In the context of cancer relapses, we have recently postulated the existence of so-called recurrence CSCs (rCSCs). These specific CSC subtype will initiate relapses exhibiting an "oncogenic resistance" phenotype, which are characterized by a markedly increased malignancy concomitant with a drug resistance towards first line therapy. In the present chapter we will discuss the necessity of rCSCs as a distinct CSC subtype and that cell fusion could be one mechanism how rCSCs could originate.

9.1 Introduction

Within the past 10–15 years our knowledge about cancer and how cancer cells originate has changed dramatically. What was once believed as a disease that has its origin in fully differentiated somatic cells, which have undergone malignant transformation due to accumulation of genetic aberrations (e.g., activation or loss of tumor suppressor genes and oncogenes [1], or aneuploidy [2–4]) is now believed to have its origin in undifferentiated adult stem and/or progenitor cells [5, 6]. Because of their inherent cell cycle activity, both adult stem cells and progenitor cells can accumulate genetic aberrations, which ultimately can give rise to so-called cancer stem cells (CSCs) [7, 8]. Evidences exist for both possibilities. Findings of Houghton et al. that gastric cancer can originate from bone marrow-derived stem cells (BMDCs) support the hypothesis that CSCs may originate directly from adult stem cells [9, 10]. By contrast, data being published in the past years strongly suggest that progenitor cells, which have regained self-renewal activity, are most likely the precursors to CSCs [11–13]. A third hypothesis exists postulating that CSCs might originate from rare cell fusion events [7, 14]. Here, cell fusion between somatic cells and stem cells might create genetic instability, which ultimately might lead to the origin of CSCs [7, 14]. Likewise, fusion between mutated stem cells or somatic cells might also give rise to CSCs, whereby mutations could occur in the stem cells, in the somatic cells or in the hybrid cells [7, 14]. However, experimental data are still lacking supporting this hypothesis.

The knowledge that a tumor is organized hierarchically like normal tissues, namely comprising of a small number of stem cells, which give rise to differentiated cells, thereby maintaining tumor tissue

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integrity and tumor organ function is of crucial interest for our understanding how to treat cancer in future times. The dilemma of current cancer therapies (conventional chemotherapy, radiation therapy, hormonal therapy, humanized monoclonal antibodies, and/or inhibitors) is that although most cancer patients respond to therapy, only few are definitely cured [15]; a matter, which applies to both solid tumors as well as hematological disorders. This phenomenon, which has been entitled as "the paradox of response and survival in cancer therapeutics" [15] has been compared to "cutting a dandelion off at ground level" [15, 16]. Current cancer therapies are designed to target highly proliferating tumor cells. Determination of tumor shrinking concomitant with mean disease free survival of patients are commonly used as read-outs for the efficacy of the appropriate therapy. While such strategies eliminates the visible portion of the tumor, namely the tumor mass, they mostly fail to eliminate the unseen root of cancer, namely CSCs. CSCs, like normal stem cells, possess an inherent resistance towards cytotoxic compounds and radiation [17–19], thus being capable to survive therapy. These surviving CSCs are then the seed of relapses that could occur months to years later after therapy.

Within the past 15 years, CSCs have been identified in a variety of cancers including acute myeloma leukemia (AML) [20], chronic myeloid leukemia (CML) [21], brain [22], melanoma [23], colon [24], colorectal [25], pancreatic [26], prostate [27], and lung cancer [28]. Due to the knowledge that cancer originates from rare CSCs, which may also survive cancer therapies, different strategies are currently developed to specifically eliminate CSCs. Ito and colleagues reported recently that the promyelocytic leukemia protein (PML) tumor suppressor, which expression levels are inversely correlated to the clinical outcome in CML, might be a putative target molecule for the eradication of quiescent leukemia-initiating cells (LICs) [29]. Arsenic trioxide (As_2O_3) treatment yielded in reduced PML expression levels, thereby decreasing significantly the number of quiescent LICs [29]. Moreover, As_2O_3 treatment followed by arabinoside (Ara-C) exposure significantly increased the efficacy of Ara-C-mediated induction of apoptosis in LICs, resulting in eradication of these cells even 4 weeks after treatment discontinuation [29]. Likewise, Interferon-α (IFN-α) has been shown to activate dormant hematopoietic stem cells in vivo, which might be an explanation for the so far unexplained positive clinical effects of IFN-α on leukemic cells [30]. If IFN-α would be able to induce leukemic stem cells to enter into an active cell cycle these cells would become susceptible towards conventional cancer therapy.

In any case, the connection between cancer, CSCs and the possible outcome of this disease nicely illustrates why the CSC hypothesis is at the center of a rapidly evolving field that may play a pivotal role in changing how basic cancer researchers, clinical investigators, physicians, and cancer patients view cancer [5].

9.2 Why Are Different CSC Subtypes Required in Cancer Progression?

Because of their capacities to initiate tumor growth and, most likely, to survive conventional cancer therapy, CSCs have not only been linked to primary tumor formation, but also to metastasis formation and induction of cancer relapses. This poses the question whether a single CSC population exist, being capable to induce the primary tumor, its metastases as well as initiate tumor regrowth after cancer therapy, or whether the primary tumor, its metastases as well as recurrences can be attributed to distinct CSC subtypes.

Recently, Wright et al. demonstrated that hereditary breast cancer harbors different CSC populations [31, 32]. The authors isolated cell lines from five distinct Brca1-deficient mouse mammary tumor and were able to show that all cell lines derived from one tumor included increased number of $CD44^+/CD24^-$ cells, whereas cell lines derived from another mammary tumor exhibited low levels of $CD44^+/CD24^-$ cells, but 2–6% of $CD133^+$ cells [32]. Both, $CD44^+CD24^-$ or $CD133^+$ sorted cells induced tumor formation in NOD/SCID mice at low cell numbers (50–100 cells were implanted) clearly indicating the cells tumorigenic capacity and their putative CSC phenotype [32].

Likewise, Hermann and colleagues identified two distinct populations of CSCs in pancreatic cancer: $CD133^+$ pancreatic CSCs were exclusively tumorigenic and highly resistant to standard chemotherapy, whereas $CD133^+$ $CXCR4^+$ pancreatic CSCs, being present in the invasive front of the tumor, determined the metastatic phenotype [33]. Transplantation of both CSC populations into athymic mice revealed that both groups showed a similar tumor development [33]. However, only $CD133^+$ $CXCR4^+$ cells, but not $CD133^+$ $CXCR4^-$ pancreatic CSCs could be identified in the circulation of transplanted mice. Moreover, removal of the $CD133^+$ $CXCR4^+$ and $CD133^+$ $CXCR4^-$, respectively, tumors revealed that only the $CXCR4^+$ group, but not the $CXCR4^-$ group, developed liver metastases [33]. These data indicate that both pancreatic CSC populations were capable to induce tumor formation, but that only the $CXCR4^+$ population induced secondary lesions. Additionally, because implantation of $CD133^+$ $CXCR4^-$ pancreatic CSCs did not lead to metastasis formation the authors concluded that $CD133^+$ $CXCR4^+$ pancreatic CSCs did not derive from $CD133^+$ $CXCR4^-$ pancreatic CSCs [33].

In summary, these data strengthen the hypothesis that distinct CSCs (sub)populations exist exhibiting different properties. In case of pancreatic cancer, a CSC subpopulation was identified being capable to induce metastasis formation. These findings are in agreement with a recent hypothesis of Li and colleagues who postulated that the existence of primary tumor CSCs (pCSCs) and metastatic CSCs (mCSCs) [8]. The first CSC subpopulation – pCSCs – induce primary tumor formation, whereas metastases originate from circulating mCSCs [8].

The finding that only $CD133^+$ $CXCR4^+$ pancreatic CSCs were capable to induce metastasis formation is further in agreement with several data indicating that the organ-specific metastatic spreading of various cancers, including pancreatic cancer, is directed by chemokines, which act as a navigation system of circulating cancer (stem) cells [34].

9.3 Recurrence CSCs

9.3.1 CSCs Exhibit an Inherent Resistant Towards Radiation and Cytotoxic Compounds

Because of their inherent resistance towards radiation and chemotherapeutic compounds, such as doxorubicin, 5-fluorouracil, cyclophosphamide, etc., CSCs have been associated with cancer relapses [17, 19, 35, 36]. For $CD133^+$ glioblastoma (stem) cells it was demonstrated that these cells preferentially survived radiation treatment at increased rates as compared to $CD133^-$ cells [18]. Likewise, radiation treated $CD133^+$ cells were also able to induce tumors in xenografts with nearly the same efficiency as non-irradiated $CD133^+$ cells [18]. Analysis on a chromosomal pattern revealed that radiation consistently induced DNA damage to similar degrees in both $CD133^+$ and $CD133^-$ cells, whereby the DNA damage was more efficiently repaired in $CD133^+$ cells [18]. Since $CD133^+$ cells often showed a basal activation of rad17, a component of the DNA damage checkpoint, it might be assumed that $CD133^+$ CSCs, like normal stem cells, are primed to genotoxic stresses [18].

Further mechanisms that may play a role in CSC radiation resistance include the Notch-signaling [37] and the Wnt/β-catenin signaling [38], respectively. Philips and co-workers demonstrated that fractionated doses of irradiation led to an increased Notch-1 activation level concomitant with an enhanced percentage of cancer stem/initiating cells in the nonadherent population of MCF-7 breast cancer cells [37]. Radiation of mouse mammary epithelial cells with clinically relevant doses resulted in an enrichment in both $Sca\text{-}1^+$ and side population (SP) cells [38]. Compared to irradiated $Sca\text{-}1^-$ cells, irradiated $Sca\text{-}1^+$ cells showed a selective increase in active β-catenin expression as well as an unaffected colony formation ability, which suggest an active role of Wnt/β-catenin signaling in radiation resistance [38].

Analysis of SP cells, representing a cell population that efficiently effluxes Hoechst dyes (including Hoechst Blue and Hoechst Red) via various ATP-binding cassette (ABC) multidrug transporters, such as ABCG2 (also named breast cancer resistance protein (BCRP)), ABCB1 (also named multidrug resistance transporter 1 (MDR1)), and ABCC3 [39], showed that these cells were positive for Notch-1 and β-catenin [40]. SP cells are more tumorigenic than non-SP cells and possess some intrinsic stem cell properties [40, 41]. The finding of Notch-1 and β-catenin expression may therefore indicate the "stemness" of these cells as well as suggesting that SP cells might also be resistant towards radiation. However, at present it remains unknown whether SP cells are true CSCs or solely a CSC-like population.

The enzyme aldehyde dehydrogenase 1 (ALDH1) mediates resistance towards cyclophosphamide in normal stem cells [42]. Because of this connection it is assumed that increased ALDH1 expression levels correlate to a resistance of CSCs towards the chemotherapeutic compound cyclophosphamide [42]. Increased ALDH1 expression levels and activity have been identified in leukemic CSCs [43] and breast carcinomas [44]. In the latter, ALDH1 is a suitable marker to identify the tumorigenic cell fraction. Moreover, high ALDH1 expression levels in primary breast cancer patient samples were correlated with a poor prognosis of the afflicted patients [44], whereby it remains to be elucidated whether this correlation is attributed to an enhanced chemoresistance of tumor cells or due to a higher amount of tumor initiating cells.

Another possibility how CSCs survive might be attributed to their putative low cell cycle activity, which is a characteristic property of stem cells. However, considerably less is known about the cell cycle activity of CSCs. Under physiologically conditions the cell cycle activity of stem cells is regulated by their specific niche. Normally, the niche provides an environment that predominantly retains stem cells in a quiescent state. Thus, both proliferation and differentiation processes are inhibited [45]. In case of e.g., tissue injury, transient proliferating signals from the niche are directed to the stem cell, thereby inducing cell cycle activity [45]. However, at present it remains unknown whether CSCs are niche-dependent or even in-dependent [46]. Acute myelogenous leukemia CSCs as well as chronic myeloid leukemia CSCs exhibit a low cell cycle activity, which may contribute to therapeutic resistance [47, 48]. On the other hand, a rapid proliferation rate of solid tumor CSCs have been demonstrated in in-vitro assays [17]. However, it must be taken into account that in-vitro culture conditions are always artificial, e.g., due to the use of high serum and growth factor levels. Thus, the high proliferation rate of solid tumor CSCs under in-vitro conditions might by an artifact.

In summary, CSCs are capable to survive conventional cancer therapy (chemotherapy/radiation) due to various resistance mechanisms. While this suggest that CSCs, which have survived first line cancer therapy, can re-initiate tumor growth month to years after cancer therapy, the question remains how CSC survival correlates to the phenomenon of "oncogenic resistance".

9.3.2 Oncogenic Resistance Demands a Novel Type of CSCs: Recurrence CSCs

The term "oncogenic resistance" describes the phenomenon that most regrown tumors have become resistant to first line therapy and generally exhibit a more aggressive phenotype than the original cancer [16]. But how does the phenomenon of "oncogenic resistance" correlates to the hypothesis that first line therapy surviving CSCs will be the seed of the regrowing tumor? If we agree with the assumption that CSCs, which had survived conventional cancer therapy, will be capable to re-initiate tumor growth months to years later than we have to conclude that the regrowing tumor should be phenotypically similar to the original tumor. Thus the recurrent cancer should be treatable with the same chemotherapeutic drugs and/or radiation, which have been used for the treatment of the original tumor. This, however, is not the fact in case of "oncogenic resistance". Here, all cells of the regrowing tumor, and not only the regrowing tumor initiating CSCs, are resistant towards first line

therapy medics. Thus, the only rationale is that regrowing cancer initiating CSCs must be different from those CSCs that caused the original tumor.

In this context, we recently postulated the existence of recurrence CSCs (rCSCs), which defines the specific CSC population that re-initiates tumor growth after cancer therapy [49]. How rCSCs will originate is not clear, but it can be assumed that first line therapy may act as a driving force (or a selection pressure) in this process. That such scenario is possible was recently demonstrated by Shafee et al. [50]. In their work the authors reported that CSCs contribute to cisplatin resistance in Brca1/p53-mediated mouse mammary tumors [50]. After 2–3 months of complete remission following platinum treatment, tumors relapsed and became refractory to successive rounds of treatment, which was associated with an increase in the amount of $CD24^{med}CD29^{hi}$ mouse mammary tumor stem cells (5.9% in primary tumors, 8.8% in partially platinum-responsive transplants, and 22.8% in platinum-refractory secondary transplants) [50]. Interestingly, the expression levels of the normal stem cell marker Nanog as well as of Top2A were decreased in platinum-refractory secondary transplant $CD24^{med}CD29^{hi}$ mouse mammary tumor stem cells as compared to controls [50]. Moreover, in one case Top2A down-regulation was accompanied by genomic deletion of Top2A [50]. These data clearly indicate that the selection for cisplatin resistant mouse mammary tumor cells went along with genetic alterations, which is in agreement with our proposed rCSC hypothesis.

9.3.3 Cell Fusion and the Origin of rCSCs

Because the phenomenon of "oncogenic resistance" only occurs after cancer therapy it can be concluded that the therapy itself is the driving force in the origin of rCSCs. In this context, the origin of rCSCs resembles Darwinian evolution: a selection pressure (first line cancer therapy) is exerted to the system "cancer" and only those cells that can resist to the selection pressure will survive. Moreover, as stated out above, during this selection process a novel type of CSCs has to emerge: rCSCs.

Thus the question remains how rCSCs may originate. Shafee and colleagues demonstrated that platinum-refractory secondary transplant $CD24^{med}CD29^{hi}$ mouse mammary tumor stem cells revealed lower Nanog and Top2A expression levels, whereby in one case Top2A down-regulation was attributed to a genomic deletion of Top2A [50]. These data indicate that genetic/epigenetic variations can occur in CSCs during cancer therapy, which ultimately give rise to a new CSC phenotype. Moreover, these data let assume that, despite an enhanced resistance towards cytotoxic compounds and radiation, CSCs are influenced by the conventional cancer therapy treatment.

Another mechanism that may promote the origin of rCSCs could be cell fusion, which has been associated with several characteristics of tumor progression, such as an increased malignancy [51], enhanced resistance to apoptosis, and drug resistance [52]. Moreover, an increased malignancy as well as an enhanced drug resistance are also hallmarks of "oncogenic resistance". For instance, hybrid cells, originated from spontaneous cell fusion events between two mouse cell lines, grew faster than their parents [53]. Likewise, the polyethylene glycol (PEG)-mediated fusion between primary mouse mesenchymal stem cells (MSCs) and mouse fibroblasts gave rise to hybrid cells with an increased proliferation and an altered differentiation capacity [54]. Finally, we have recently shown that breast stem cell/breast cancer cell hybrids exhibit an increased proliferation rate as compared to the parental cells [49]. Fusion of 5-fluorouracil resistant tumor cells with methotrexate resistant cancer cells gave rise to hybrid cells being resistant to both compounds [55]. Interestingly, hybrid cells became also resistant to mephalan, a drug to which both parental tumor cell lines were sensitive [55]. Likewise, fusion of etoposide sensitive E1A expressing human fibroblasts with parental primary fibroblasts gave rise to etoposide (and apoptosis) resistant heterokaryons [56]. Similar results were achieved when primary fibroblasts were fused with etoposide sensitive cancer cell lines (HeLa and Jurkat). Those fibroblast/cancer cell line hybrids were resistant to both etoposide and apoptosis [56]. These data

indicate that cell fusion can give rise to hybrid cells exhibiting an increased proliferation rate, an increased drug resistance as well as an enhanced resistance to undergo apoptosis.

It can be concluded that cell fusion should be a common event in tumor tissues, particularly in tumor tissues being treated by cancer therapy. It is well recognized for several decades that tumor cells are highly fusogenic. In fact, some tumor cell lines are so fusogenic that they fuse spontaneously more efficiently than in the presence of polyethylenglycol [52, 57]. Cancer (stem) cells can either fuse with other cancer (stem) cells or with tumor tissue-associated cells, such as macrophages [58], bone marrow-derived cells [59, 60] or tissue stem cells [49, 61].

Likewise, both chemotherapy and radiation treatment will lead to an environment that may promote cell fusion. Cancer therapy kills rapidly proliferating tumor cells, thereby leading to massive tumor tissue destruction, which in turn results in local inflammatory conditions and the subsequent recruitment of immunocompetent cells and BMDCs. For both BMDCs and cells of the myelomonocytic lineage it was demonstrated that they restore tissue integrity by cell fusion [62, 63]. Moreover, Rizvi et al. already showed that BMDCs can fuse with neoplastic intestinal epithelium, thereby giving rise to stable heterokaryons [60]. Thus it is conceivable that due to cancer therapy mediated tumor tissue destruction BMDCs as well as cells of the myelomonocytic lineage will be recruited to restore the degenerated tumor tissue by cell fusion. Thereby, BMDCs as well as cells of the myelomonocytic lineage can fuse with both tumor cells and CSCs.

However, it should be emphasized that the phenotype of such stem cell/tumor (stem) cell hybrids can not be predicted. In general, three scenarios are possible: (i) the emerging hybrid cell(s) exhibit a weaker malignancy than the parental tumor (stem) cells, (ii) the malignancy of the emerging hybrid cell(s) is similar to the parental tumor (stem) cells, and (iii) the emerging hybrid cell(s) a more malignant than the parental tumor (stem) cells. Rizvi et al. demonstrated that hybrid cells, derived from fusion events between BMDCs and intestinal adenomas, did not initiate cancer of the small intestine [60], suggesting that stem cell/tumor cell hybrids did not promote tumor progression. On the other hand, the used mouse model (Min mice) does not live long enough to evaluate the impact of fusion on tumor progression. Thus the authors concluded that the fusion between BMDCs and tumor cells may be a common, but late event in intestinal tumorigenesis [60].

An in vivo mouse model was applied by Rachkowsky et al. to study the metastatic capacity of macrophage×melanoma hybrids [64]. Thereby, a few macrophage×melanoma hybrids displayed lower metastatic capacities than the parental melanoma cell line, whereas the majority of hybrid cells possessed a (partially) markedly increased metastatic capacity [64]. These data nicely illustrate that the phenotype of a hybrid cell can not be predicted, particularly if tumor cells will be one fusion partners. Tumor cells are extremely heterogeneous and thus each tumor cell exhibits a unique genetic/epigenetic phenotype. Moreover, even the fusion partner, e.g., BMDCs, does not exhibit a stable genetic/epigenetic background. Genetic/epigenetic variations among BMDCs might be attributed to different stages of the cell cycle or different activation states.

Further variation possibilities occur after the fusion process since the genetic/epigenetic profile of the parental cells will be merged. In early heterokaryons the nuclei of the fusion partners can be distinguished [49], whereas in older heterokaryons only one nucleus is detectable indicating that nuclei fusion must have occurred. Due to the (now) aneuploid karyotype the cellular homeostasis is unbalanced and chromosomes will be lost or segregated in a non-uniform manner during subsequent cell divisions, which again has an impact on the genetic/epigenetic background of the cell. In summary, also cell fusion resembles “Darwinian evolution”. Only those hybrid cells will survive, which exhibit a suitable genetic/epigenetic background. This suitable genetic/epigenetic background of hybrid cells might allow some of the heterokaryons to e.g., spread out of the primary tumor, thereby contributing to tumor progression, whereas other surviving heterokaryons will remain in the tumor tissue without any impact on tumor progression. However, it has to be noticed that cell fusion is an event, which occurs not only once in tumor tissue, but which rather is a common phenomenon in this disease. Thus, even if hybrid cells will originate that do not contribute to the progression of the disease, it will be

those hybrid cells exhibiting a more aggressive phenotype, which will be important players in tumor progression.

9.3.4 From Which Cell Types May rCSCs Originate?

From which cell types rCSCs most likely originate remains unclear. Cells that possess fusogenic capacities include tumor cells, macrophages, BMDCs and tissue stem cells (e.g., breast stem cells) [52, 65–67]. We presume that also CSCs should be fusogenic and thus could act as a fusion partner for other cells.

If we agree with the hypothesis that cell fusion could be one mechanism how CSCs originate, whereby one fusion partner is a stem cell [7, 14], then rCSCs could either originate from a CSC and a cell fusion partner (e.g., tumor cell or a recruited BMDC or immunocompetent cell) or a recruited BMDC and a cell fusion partner (e.g., tumor cell, CSC) or a tissue resident adult stem cells and a cell fusion partner (e.g., tumor cell, CSC). It might be speculated that one fusion partner should be a CSC, which brings the repertoire of cancer stem cell characteristics into the hybrid cell.

In addition to the potential cell types, which may give rise to rCSCs (possibly by cell fusion), the question remains in which tumor tissues rCSCs most likely originate. Since we presume that rCSCs most likely originates from CSCs we can conclude that the generation of rCSCs should take place in tissues harboring CSCs. This could be the primary tumor with its pCSCs as well as the metastases including their mCSCs. Since the primary tumor (and its pCSCs) is generally removed completely in cancer therapy we conclude that rCSCs should mainly originate from mCSCs. However, it is also conceivable that rCSCs originate from pCSCs if the primary tumor was only partially removed or if surgically resection was not feasible.

In summary, cell fusion can give rise to cellular phenotypes exhibiting properties, such as increased malignancy, decreased apoptosis rate, and enhanced drug resistance, which are commonly associated with “oncogenic resistance” in recurrent cancers. Thus, cell fusion might be one mechanism how rCSCs could originate.

9.4 Conclusions

In the present chapter we discussed the existence of rCSCs and the need for this specific CSC subtype with regard to the initiation of cancer relapses exhibiting an “oncogenic resistance” phenotype. Such regrowing cancers are characterized by an increased malignancy and a resistance towards first line therapy. Thus, the phenomenon of “oncogenic resistance” is not compatible with the hypothesis that CSCs can withstand cancer therapy (chemotherapy and/or radiation). If so, the regrowing cancer should be phenotypically similar to the original cancer, which, however, is not the case in the context of “oncogenic resistance” and because of that a new type of CSC, the so-called rCSCs, is mandatory.

As one possible mechanism how CSCs may originate, we discussed the phenomenon of cell fusion. In fact, hybrid cells may share several characteristics of “oncogenic resistance”, such as increased drug resistance and malignancy as well as enhanced resistance to apoptosis, which are all hallmarks of “oncogenic resistance”. The phenomenon of cell fusion is further favored since cancer therapy results in a massive tumor tissue destruction concomitant with markedly inflammatory conditions and recruitment of immunocompetent cells as well as BMDCs. For the latter, and for cells of myelomonocytic origin, it was demonstrated that these cell types restore tissue function by cell fusion [62, 68, 69]. Thus, cell fusion of tumor (stem) cells with other cells, e.g., recruited immunocompetent cells and/or BMDCs or tissue resident cells such as tissue stem cells, should be a common event in tumor tissues. Another aspect that may favor cell fusion as the mechanism how rCSCs likely originate is

the fact that cell fusion contributes to aneuploidy [14], which in turn has been associated with cancer progression [70] and the origin of multidrug resistance [71]. Thus, due to an aneuploid karyotype, hybrid cells may possess an enhanced capability to withstand the selection pressure "cancer therapy". However, the fatal side effect of this selection process is the evolution of a novel CSC subtype, the rCSCs.

The dilemma of current cancer therapies is that they eliminate the visible portion of the tumor mass, but that they spare the root of cancer, namely CSCs. It is now generally accepted that the eradication of CSCs is a mandatory prerequisite for a successful cancer therapy. Eradication of this particular tumor cell type do not only sear the tumor itself, but will also eliminate a potential cell fusion partner, which may give rise to a more malignant CSC subtype.

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Chapter 10
The Role of Microvesicles in Malignancies

Erna Pap

Abstract Microvesicles are membrane-covered cell fragments whose size varies between 30 and 1,000 nm. They are generated by all cell types, constituvely and in response to activation signals. Their importance in intercellular communication has been only recently discovered. They seem to enhance the potential of information transfer between cells, displaying a large number of proteins and lipids as membrane constituents and as components of the inner vesicular content. The content reflects the phenotype of the donor cell and allows the identification of the microvesicular origine as well. Complex "packets" of molecules are transmitted to the target cells this way, modifying their cellular physiology. Additionally, epigenetic changes may be induced by transmitted DNA and RNAs, that have also been identified in these vesicles. The vesicles can act in close and far distances as well. Microvesicles have been implicated in several physiological and pathological processes. There is an increasing evidence, that they play a pivotal role in tumorigenesis. Vesicles shedding from tumor cells reflect the special potential of the tumor for survival and expansion, independently from cell-to-cell contact. Tumor derived vesicles are fully equipped to facilitate the escape of tumor cells from immune surveillance through their protein and RNA content, at the same time they are involved in the establishment of an optimal environment for newly formed and metastatic tumor cells, influencing angiogenesis and the reorganization of the extracellular matrix. As immune cells, endothels, platelets and stem cells also release microvesicles, a multilevel communication network draws up, allowing a complex interplay between the cells. The concentration of tumor derived vesicles increases in blood plasma and other body fluids with the progression of the disease; therefor they may serve as prognostic markers. The microvesicular approach can offer new perspectives: interfering with the formation, release and propagation of these vesicles, they can be considered as new targets in tumor therapy.

10.1 Introduction

The fate of a cell depends on its inner gene set, whose expression is orchestrated by epigenetic regulation and by outer signals. Depending on the timing and the duration of the different signals, the life of the cell reaches subsequent stages of differentiation, division and finally apoptosis or cell death. The large majority of cells follows this pathway in a healthy organism. However, some cells "loose control" and slip out of normal regulation. The result will be the formation of malignant cells. The causes can be retrieved to genetic mutations, to epigenetic changes or/and to altered signalization. The chance to overcome the tumor cells with the help of immunological defense responses is often defeated by the escape strategies developed by the malignant cells themselves.

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The communication between cells is a basic characteristic of living systems. It is attained through the classical signalization pathways when the soluble or the membrane-bound ligand binds to the receptor of the target cell, initiating a cascade in the intracellular reactions and provoking a response from the target cell (Fig. 10.1). Another special communication form is via gap junctions. In addition to these well-known and well-described mechanisms, a new form of communication system has been revealed – underappreciated until about a decade ago – the intercellular communication via membrane vesicles.

Membrane vesicles are membrane-covered cell fragments shedding from practically all cell types. They are considered to play a pivotal role in the information transfer between cells, as they display a variable number of molecules enclosed in their cytoplasmic fraction and in their membrane. The mechanism of the membrane vesicular action is quite different from the up till now known communication modes. Whole "packages" of information can reach the target cell, since membrane domains and cytoplasmic components travel in the vesicles. The vesicular content delivered to the target cell contains a wide range of molecules: proteins – signal proteins and receptors as well, just as much as cytoskeleton or effector proteins –, lipids, DNA and RNA. (Furthermore, prions and viruses have also been shown to infect the host cell through membrane vesicles.) Depending on the vesicular content, simultaneously more stimuli and more information will be delivered to the cells, allowing a more complex and more effective cellular response. As a consequence, after having received new sets of proteins, the nature of the target cell may be altered, resulting in new responses, in new signalization pathways and further vesicle release can be elicited. Thereby membrane vesicles represent an "enhanced potential" in the information flow between cells and they open a new era in the study of intercellular communication [1–5] (Fig. 10.1).

Besides several physiological and pathological processes, membrane vesicles have been described to participate in tumorigenesis [1, 2, 6–8]. They were found in an increased quantity in the body fluids of patients suffering from different kind of tumor. They can be identified as vesicles originating from tumor cells, since the phenotype of the donor cell is reflected in the vesicular protein content. The vesicles reflect not only the special protein set characteristic to a certain tumor cell, but they also bear the special potential of tumor cells for survival and for the expansion of the tumor, independently from cell-to-cell contact. Tumor derived vesicles have the potential to facilitate the escape of tumor cells from immune surveillance through their protein and RNA content, at the same time they are involved in the establishment of an optimal environment for new and spread tumor cells, influencing angiogenesis and the reorganization of the extracellular matrix. Revealing the role of tumor-derived vesicles allows a new approach for the understanding of the nature of the tumors and opens new perspectives in their treatment.

Since tumor derived vesicles are involved in tumorigenesis at multiple level and drugs themselves can be expulsed from tumor cells via vesicles [9], interfering with the formation, release and propagation of these vesicles can be a novel and alternative issue in cancer therapy.

The aim of this overview is to show and to discuss those, recently described different mechanisms, which are thought to participate in creating malignancies and to present those potentials that lie in vesicle-related tumor therapy.

10.2 What are they, these Membrane Vesicles?

10.2.1 Nomenclature, Classification

Membrane vesicles are subcellular membrane-covered cell fragments, whose size varies between 30 and 1,000 nm. They originate from practically all types of eukaryotic cells in vivo and in vitro [6, 7, 10–13]. Their release is enhanced by activation or by the initiation of apoptosis. The nomenclature is

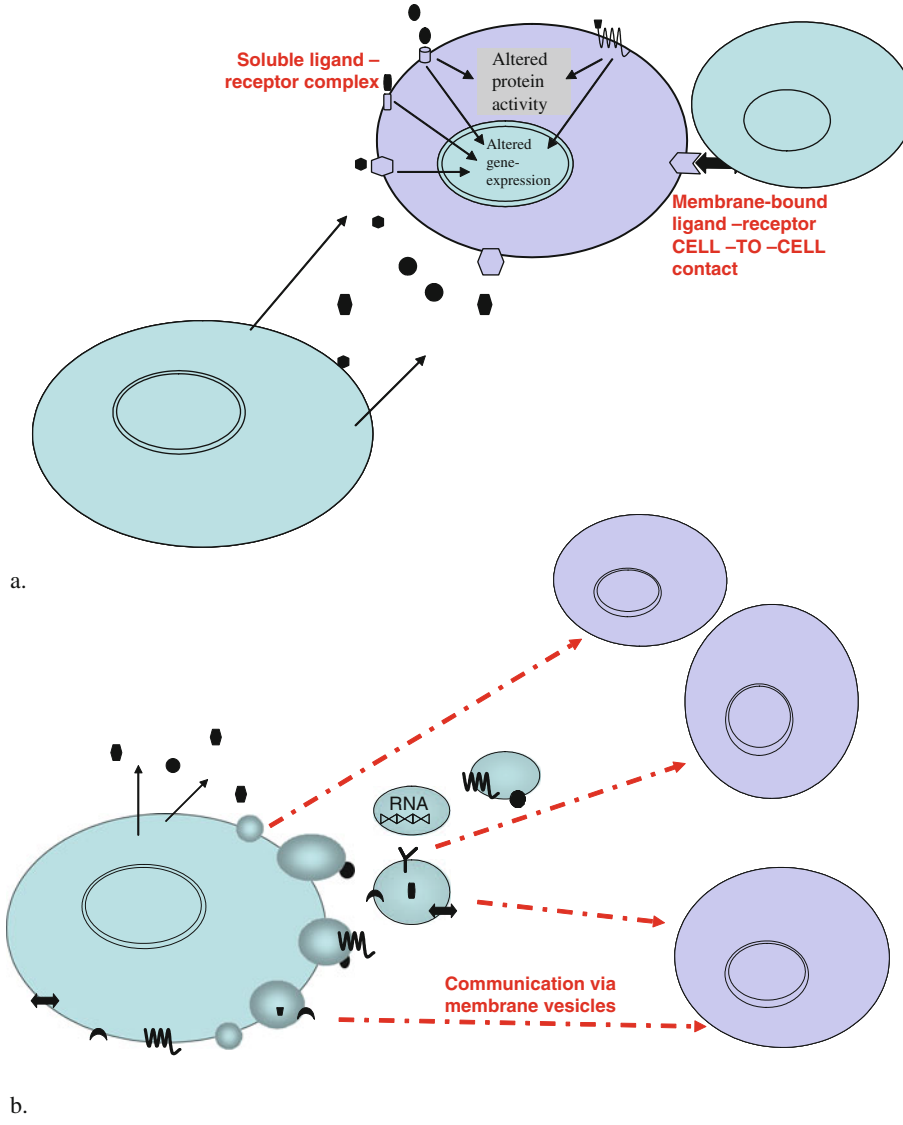

Fig. 10.1 The schematic drawing of the classical and of the membrane vesicular signalization pathways. (**a**) *Classical signalization pathway*. Cells secrete different molecules that serve as soluble ligands for the target cells. There is a "one- to- one" interaction between the ligand and its receptor. Cells can communicate with one another through membrane-bound ligands as well, which requires a close cell-to-cell contact. (**b**) M*embrane vesicular signalization pathway.* In case of membrane vesicular communication the vesicles serve as "packages" of information, as they display several signaling/altering molecules at the same time. They have a pleiotropic effect due to their membrane protein pattern which enables them to provide information to several types of target cell, via a very specific recognition, depending on the receptor/adhesion protein molecular set on the recipient cell's membrane. The nucleic acid content of the MVs allows the epigenetic regulation of the recipient cell. The MVs can exert their effect in close and far distances as well

still controversial: presently they are collectively called membrane vesicles. The term "exosome" comprises vesicles smaller than 100 nm, while the larger ones are commonly referred as "microvesicles", "ectosomes", "microparticles", "exovesicles" or "apoptotic vesicles" [1, 2, 14]. In the present review the collective term microvesicle (MV) will be used by us for subcellular fragments below 1,000 nm

and in those cases, when a distinction is needed, vesicles smaller than 100 nm will be referred to as exosomes. We do it so for the reason that most papers, discussing the relation between tumor and membrane vesicles use the term microvesicle and exosome.

In order to avoid confusion, we find it important to underline that the so-called apoptotic bodies differ from the MVs discussed in this present study, although apoptosis itself also elicits MV release. Yet, the apoptotic bodies are released in the final stages of the programmed cell death and their size is larger than 1,000 nm. In contrast, the apoptosis-induced MVs are formed at the beginning of the apoptosis with a size smaller than 1,000 nm and do not contain organelles.

10.2.2 Formation, Composition and Detection of MVs

The formation and the release of MVs is a constitutive process, but it is enhanced by activating signals [2]. The distinction between the strictly called exosomes and vesicles with size from 100 to 1,000 nm is based on their different formation [1, 7, 15–17]. The small vesicles are formed via exocytosis, which explains the term "exosome". They develop from endosomes that assemble to create multivesicular bodies. Instead of following the lysosomal pathway, these multivesicular bodies fuse with the plasma membrane and release their small vesicle – exosome – content. Cytoplasmic proteins can enter the future exosomes as well, which together with the proteins of the invaginated cell membrane represent the "donor cell". Thus, the origin of the exosomes can be identified through the specific protein set of the donor cell [3]. Vesicles, shedding from tumor cells, often fall into the exosome category [2] (Fig. 10.2).

The so-called reverse budding occurs at the release of most MVs between 100 and 1,000 nm. This kind of vesicle formation is strictly coupled to the reorganization of the cytoskeleton, which is the result of multiple intracellular interactions. The elevation of intracellular Ca^{2+} ion concentration plays a key role in the initiation of the disruption of the actin cortex. Budding also occurs when apoptotic signals reach the cell, and by the end of a series of interaction the cell membrane gets detached from the cytoskeleton. As the budding vesicles also contain cytoplasmic fragments and areas from the cell membrane, the origin of the cell type they derive from can be identified [2, 4, 18] (Fig. 10.2).

Considering that the way of MV formation follows common pathways independently of the nature of the donor cell, there are common similarities also in the content of both their membrane and

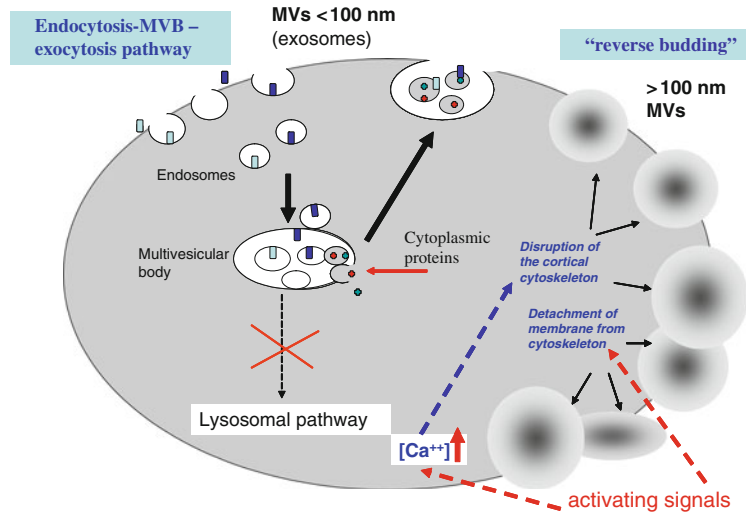

Fig. 10.2 The formation of MVs. The "exosomes" are formed via exocytosis. Vesicles larger than 100 nm are formed via reverse budding, which is the result of changes in the structure of the cytoskeleton and of the cell membrane (the exact mechanism is not presented here)

cytoplasmic fraction. The phospholipid asymmetry of the budding vesicles is due to an increased Ca^{2+} ion concentration. The AnnexinV binding capacity of the MVs is based on the negatively charged phosphatidylserine (PS) molecules on their outer membrane layer, which can promote the attraction of macrophages as well [14, 15]. Cytoskeletal and chaperone proteins are recurrent components of the budding vesicles. In accordance with their origin, exosomes contain endocytic markers, i.e. tetraspannins and hsp73 [10]. Besides the commonly displayed molecules, the specific protein and lipid composition of the MVs reflects the nature of the donor cell, inasmuch as their cell surface proteins, their membrane fusion and receptor proteins, their adhesion proteins and the lipid rafts are equal to those of the parental cell. The miRNA content serves as possible source of epigenetic factors for the recipient cell, meanwhile the mRNA molecules represent a new translational potential. DNA fragments have also been identified in MVs [19, 20].

Tumor-derived MVs (TMVs) can be characterized by specific molecular sets [11]. Besides the generally occurring proteins and lipids, they display a specific molecular pattern, mirroring the donor cancer cell. They contain tumor specific determinants, such as Her-2/neu, mesothelin, TSG101 (tumor susceptibility gene101), etc. They carry tumor antigens such as MelanA/Mart-1, carcinoembryonic antigen (CEA), HER-2 and MHC I, MHC II. (HLA-G, HLA-E) complexes for antigen presentation. They carry tumor necrosis factor-α (TNF-α), FasL and/or TNF-related apoptosis inducing factor (TRAIL) as apoptosis inducers and tumor growth factor-β (TGF-β) as immunosuppressive molecule. They were shown to contain mRNA for β-actin and adhesion proteins, for chemokine IL-8, for vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and for CD44, the cell surface receptor for hyaluronate. Drug transporters were also identified in their membrane [2, 21, 22]. The total phospholipid content of the TMV membrane is lower than that of the cell membrane, but the ratio of cholesterol and sphingomyelin is higher [23, 24]. Sphingomyelin is needed for angiogenesis. It plays an active role in inducing endothelial cell migration, as prerequisite for neovascularization [25]. Considering, that the sphingomyelin level of highly metastatic cancer cells are significantly increased compared to less metastatic cells [26], it is suggested that TMVs amplify the inducing effect of sphingomyelin. PS dominance in the outer phospholipid layer of the TMVs also contributes to shaping tumors' microenvironment. PS molecules draw macrophages to the site [14, 15], which can contribute to the induction of inflammation, a beneficial milieu for tumors. Complementary to this, phagocytes and T lymphocytes were found to expose PS binding receptors – TIM4 and TIM1 proteins respectively – which help in capturing TMVs [27]. Interestingly, exosomes are enriched in ceramide. This increased ceramide content of the membrane seems to be necessary for the sorting of vesicles into different endosomes at the very early stages of the endosomal- lysosomal pathway: to decide whether the endosomal content will be degraded in lysosomes or to choose those vesicles, which will be released as exosomes [28].

As mentioned above, the origin of the MVs can be determined. In order to obtain pure, isolated pool of MVs with size 100–1,000 nm from body fluids or from cell culture supernatants, a centrifugation between 10 and 15,000 g is recommended. As for the exosome size a sucrose gradient ultracentrifugation is needed at 100,000 g [2, 29–32]. Flow cytometry is the conventional method for the identification of MVs from platelet-free plasma or from supernatants. On the one hand the quantity and the size can be characterized, on the other hand the origin of the MV can be identified through immunophenotyping. The size and the integrity of the vesicles should be controlled with electron microscope.

It is to be mentioned, that different problematic questions arise in respect of the microvesicular studies. The purification methods represent the most critical and crucial step. Treatments with MVs should exclude the presence of all other molecules, which circulate in blood or are secreted into the supernatants, although washing and purifying techniques can not fully ensure pure MV pool, without damaging the integrity of the microvesicular membrane. Also larger vesicles can disperse to smaller ones, or smaller ones can fuse in response to mechanical forces. There is a problematic aspect standing up with flow cytometry identification as well: vesicles below

300 nm can not be detected with reliability with the conventional machines. Protein identification in these vesicles is restricted to western blot analysis and high performance liquid chromatography (HPLC), which bumps into another difficulty: the collection of significantly larger quantities of vesicles.

These difficulties may give an explanation for the controversies of some results between different research groups and show as well that the methods need to be further refined.

10.3 The Role and the Significance of MVs in the Development of Tumor

A large number of studies have already proven that tumor cells release MVs. The first observations about melanoma derived small particles were published in 1985 [33] which was then followed by a long silence. Gradually more and more papers appeared in the field, TMVs could be detected in plasma, sera and other body fluids in vivo and in supernatants in vitro [3, 4, 7, 16, 34]. Although different characteristics of these TMVs have been reported, all reflect the special potential of tumor cells for survival and for the growth of the tumor. TMVs are fully equipped with all those factors which facilitate the escape of tumor cells from immune surveillance, meantime they facilitate the propagation of the tumor, preparing a "niche" for new and spread tumor cells.

TMVs are constitutively released by tumor cells, but their blood concentration increases in vivo with the progress of the disease. An about five times increase is reported in cancer patients [35–39]. In healthy volunteers its concentration was measured to be lower than 0.5 μg protein/1 ml blood, while in cancer patients it increased to 2–5 μg protein/1 ml blood. The values are often used as diagnostic/prognostic markers.

Tumorigenesis can be studied from cell biological and immunological aspects at several levels. Mutations induce the formation of abnormal cells with uncontrolled cell division. Gap junctions, that had been modified by epigenetic factors, can lead to "communication problems" between cells, leading to tumor formation [40]. The survival of malignant cells is sustained by different escape mechanisms: the lack of adequate immune responses or the induction of immune tolerance as well as the potential to escape apoptosis. The expansion of the tumor is coupled to the remodeling of the extracellular matrix and to neoangiogenesis. Inflammation – an inflammatory milieu – seems to be the hotbed for tumor formation.

There is an increasing evidence that MVs participate in the development and in the spreading of tumors. In addition to the malignant cells, cancer stem cells and cells from healthy tissues – such as platelets, immune cells, endothelial cells, etc – release MVs. The special role of MVs leading to malignancies lies in the method of their action. They represent a "signaling complex" just as much as a "recipient cell modifying complex" through surface-expressed ligands, through the transfer of membrane proteins (receptors or/and receptor coupled proteins), through the delivery of cytosol proteins, lipids, siRNA, miRNA and mRNA. They provide a more stable conformational condition to the protein content, since the milieu of the proteins does not get altered, as it does when the proteins are simply secreted into the extracellular matrix. Thereby the bioactivity of the proteins will also be increased in a transmembrane form. Finally MVs improve the biological distribution of molecules, since they act in a paracrine way and as remote messengers. These attributes contribute to create a highly efficient information flow between cells through the "microvesicular network".

The following chapters attempt to introduce the role of MVs in the processes leading to the development, growth and propagation of a tumor. Revealing the importance of MVs allows a new approach to the understanding of the nature of the tumors, therefore new possibilities can be addressed for cancer therapy.

10.4 The Effects of MVs in Tumorigenesis

Shifting from healthy to malignant cells requires a multiple level of reaction series in the cell. Alterations in the genetic code may lead to the mutation of "control genes", such as tumor suppressor genes and proto-oncogenes, or genes of the DNA repair mechanism. Alterations in the epigenetic code may lead to altered gene expression. The transformed genome and epigenome result in uncontrolled division rate and in a new gene expression pattern, providing new phenotype and new behavior to the cell. The "acquired nature" of the malignant cells help them to fight against immune attacks. Once they survive, they can grow and metastasize through neoangiogenesis and the remodeling of the extracellular matrix.

Results published in an extensive literature in the last about 15 years support the concept on the role of MVs in tumorigenesis – as vehicles or "communicasomes" for intercellular communication (Table 10.1).

Table 10.1 Events leading to tumorigenesis and the role of MVs in the process

Events leading to tumorigenesis in the would-be tumor cells and in their environment	Result	Role of MVs
Gene mutations	Mutated proto-oncogenes, tumor suppressor genes, DNA repair enzymes	Horizontal transfer of the oncogenic form of EGFR to yet "healthy" cells
	Abnormal division rate	Possible horizontal transfer of mutated DNA – fragments???
	Abnormal control	
Epigenetic regulation – altered gene expression	Increased oncogenic activity	Transfer of miRNA and mRNA to other cells
Escape from immune cells – altered gene expression	Failure in tumor antigen presentation	MVs shed from the tumor cell, thus "loose" antigens
	HLA-G, HLA-E expression	MVs transfer HLA-G and HLA-A to other tumor cells
	Escape from T killer and NK cells	
	FasL expression	Transfer of FasL to other tumor cells
	Escape from apoptosis: killing T killer and NK cells	
	Synthesis of inhibitory cytokines (TGF-β)	Transfer of cytokines in a membrane-bound form
Insensitivity to anti-tumor drugs	Increased/altered MDR expression	Transfer of MDR to other tumor cells
Remodeling of the extracellular matrix	Increased extracellular matrix degrading activity	Transfer of MMP enzymes, of adhesion molecules
Neoangiogenesis	Increased angiogenic activity in the tumor cell	Transfer of VEGF, VEGF receptor, TF etc
Inflammation	Establishment of a tumor favoring milieu (in certain tumors anti-tumor milieu)	Platelet, endothelial –and immune cell derived MVs carry proinflammatory cytokines, their receptors and adhesion molecules

10.4.1 The Effects of MVs on the Survival and the Maintenance of the Tumor

In order to evade the protective machinery of the organism, the escape mechanisms of the tumor cells include failure in tumor antigen presentation, expression of proapoptotic signals and inhibitory cytokine secretion, possibility to induce immune tolerance and insensitivity to anti-tumor drugs. It seems that all these processes can be achieved with the help of MV release as well (Fig. 10.3).

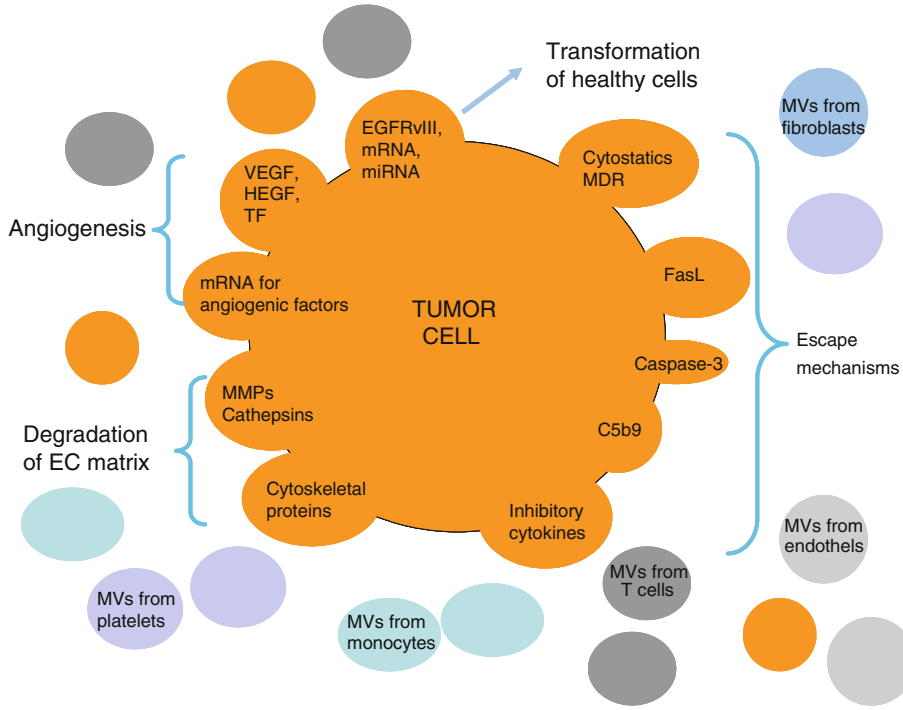

Fig. 10.3 The role of TMVs in tumorigenesis. TMVs, shedding from the tumor cells promote tumorigenesis, transporting and transferring all those factors – proteins and non-proteins – which are in some way responsible for the survival and for the expansion of the tumor. This figure summarizes the potentials that lie in TMVs. The tumor cells are surrounded by the MVs from other cell types, creating complex MV networks for signalization and molecule transfer

One theory implies that the tumor antigen presentation can be damaged by the increased rate of TMV shedding. This way the tumor cells loose the majority of their antigens, so cytotoxic T lymphocytes and NK cells do not recognize them. On the other hand, those self-protective non-classical MHC complexes like HLA-G and HLA-E, which are expressed and presented in tumor cells, can travel in the membrane of TMVs and fusing with neighboring or distant tumor cells they propagate the protection of less protected cells, thus they induce immune tolerance [41, 42]. It is interesting to note, that in our studies about the immunology of pregnancy, we also showed the presence of HLA-G on the trophoblast cell derived MVs which then targeted maternal T cells. This indicates that immune suppression seems to be mediated via MVs both in physiological and pathological processes: in case of the maintenance of pregnancy and in the maintenance of tumor, respectively [13].

The immunsuppressive effect of TMVs was partially revealed by the group of R. Valenti [6]. They performed studies on TMVs, deriving from cell lines from cancer patients with melanoma, colorectal, head and neck tumor and ovarian carcinoma. When these TMVs were added to monocytes, they skew the process of normal differentiation into defective dendritic cells which then expressed low levels of CD80, CD86, HLA-DR co-stimulatory molecules, or even lacked them. Furthermore, they started to secrete TGF-β spontaneously. Thereby T cell proliferation and functions have got blocked.

The ability of MVs to induce suppressive and "deviating" immune responses was proven by A. Clayton and his group as well. They established the so called "exosomes' double hit to cellular immunity" theory. They treated interleukin-2 (IL-2) induced NK, $CD8^+$ and $CD4^+/25^+/foxp3^+$ T cells with exosomes collected from mesothelioma tumor cell line from cancer patients and with exosomes from

Jurkat and leukemia cell lines. The ability of NK and $CD8^+$ cells to fight against tumor cells was demolished, they stopped proliferating and differentiating. On the other hand, the immunosuppressive effect of $CD4^+/25^+/foxp3^+$ regulatory T cells was enhanced, due to the presence of the membrane-associated form of TGF-β1 on the TMVs [22]. An about 1.4 times higher potency was found in the efficacy of the membrane-bound TGF-β, compared to the soluble form. It may arise by the sustained signaling through the membrane-bound contact of the TMV and the recipient cell [22, 43]. This effect sustains the idea of the high efficiency of MV communication, in as much as the conformation of the membrane-inserted proteins preserves a powerful bio-activity.

One further form of the escape machinery established by tumor cells is the induction of apoptosis of T cells by proapoptotic molecules expressed on tumor cells. Consequently, the question arose whether TMVs, bearing tumor antigens, can also induce T cell apoptosis. It has been shown by several research groups that TMVs express FasL and/or TRAIL, which implicate that the MVs themselves participate in the spread of the immune-tolerated status for the tumor cells [34, 44–46]. Valenti's group showed that when antitumor T cells were treated with TMVs, FasL and TRAIL sensitive tumor-specific T cells underwent apoptosis [6]. Similar results were found when $FasL^+$ TMVs, isolated from the sera of patients with active oral squamous cell carcinoma, induced apoptotic pathways in Jurkat and activated T cells from peripheral blood [47]. Taylor described the apoptotic effect of TMVs by downregulating the expression of CD3ξ and Janus-activated kinase-3 in T cells [48]. At this point it is also to be noted, that we identified FasL on the trophoblast derived MVs in our pregnancy studies. This is one further evidence of the immune suppressive role of MVs, independently of the physiological or pathological "needs" of the organism for immune suppression [13].

Tumor cells not only induce cell death but can escape it themselves. One way to escape cell death was described in K562 erythroleukemia cells. The membrane attack complex (MAC) accumulates in the cell membrane, but through vesicle shedding the cells can avoid complement-induced cell death [49]. Other strategies of the tumor cells are also supposed to be beneficial to them. The following mechanisms have not been proven directly on tumor cells yet, but results obtained on other cell types let suppose that similar strategies may exist in tumor cells as well. Platelets and endothelial cells form large amounts of MVs, in case they face internal stress. This way they can get rid off caspase-3 proteins, which event definitely prevents the cell from undergoing apoptosis. When MV formation was inhibited, the caspase-3 content remained high and the cells underwent apoptosis. Researchers suggest, that an increased TMV formation in tumor cells would lead to the loss of caspase-3, so cell death would be avoided [50–52]. In addition, caspase-3 may serve as an important factor for cytoskeletal reorganization, thus may have a role in MV budding. The lack of caspase-3 was found to interfere with apoptotic body release. MCF-7 breast cancer cell line does not contain functional caspase-3, due to a deletion in an exon. These cells do not display the typical morphological membrane changes prior to apoptosis. When transfected with a construct of caspase-3, they start to show membrane blebbing [53]. Although the mechanism is not understood, it may be associated with the influence of caspases on the integrity/desintegrity of the cytoskeleton. Also, one could speculate, that MV formation in general may be similarly related to caspase action, in an unknown way.

Finally, a broadly applied strategy of tumor cells to defend themselves is to "ignore" drug therapy. The effectiveness of drug therapy is dramatically reduced in some tumors in certain patients. One cause is that this resistance to drugs is due to multidrug resistant (MDR) drug-efflux pumps, such as P-glycoproteins. It has been shown that drug-sensitive (MDR^-) cells also acquire the MDR phenotype subsequently, not by an increased expression rate, rather by an intercellular transfer of P-glycoprotein. This transfer is suggested to be via TMVs [54, 55], thus TMVs would be capable to spread drug resistance to those tumor cells, which had not sustained high MDR expression earlier. Another option to ruin drug efficiency is sending them out in TMVs. Certain drugs were found to accumulate in vesicles, which then shed off the cells. Ovarian carcinoma cells eliminate doxorubicin via exosomes [9].

10.4.2 The Effects of MVs on Tumor Growth and Metastasis

TMVs not only facilitate the escape of tumors from immune surveillance, but promote tumor cell invasion as well (Fig. 10.3).

TMVs can facilitate the progression of the tumor through autocrine and paracrine signaling. They transfer growth factors and cognate receptors, mRNA and miRNA, thereby they can modify the phenotype and the nature of the cells, even in the lack of earlier mutagenic events. Several groups described the so-called horizontal transfer by TMVs between cells.

Skog and his group showed that glioblastoma TMVs, isolated from the serum of patients suffering from brain tumor, transport RNA and proteins that promote tumor growth [56]. In glioblastoma tumor, the truncated and oncogenic form of the epidermal growth factor receptor (EGFR)vIII induces several oncogenic activities: the activation of mitogen-activated protein kinase (MAPK) and Akt pathways, the change in expression of EGFRvIII-regulated genes (VEGF, Bcl-x (L), p27). Al-Nedawi and his group showed, that in mice, although EGFRvIII. is expressed only in a small percentage of the glioblastoma cells, it will be transferred through TMVs to other brain tumor cell subsets. Therefore this horizontal transfer leads to the spreading of oncogenic activity [57]. They obtained similar results later with human TMVs, wich displayed EGFR. These TMVs were taken up by endothelial cells and increased the expression of VEGF, which lanced furthermore an autocrine activation on the expression of VEGF receptor-2 [58]. Lima and her group reported that TMVs of the B16F10 melanoma cell line induce melanoma metastasis even in mice, which are normally resistant to this cell line [43]. The group of Janowska-Wieczorek found horizontal transfer among platelet-derived MVs and human lung and breast cancer cells, transferring platelet-derived adhesion molecules to them, which resulted in a strong metastatic potential [59, 60].

The “success” of tumor cells to form metastasis greatly depends on their ability to anchor to the extracellular matrix, to degrade it, to migrate and to get attached to a new surface. The process must be accompanied by the formation of new vessels. Adhesion proteins and receptors, extracellular matrix proteases and inducers of vascularization were found in TMVs of numerous cancer types as well. They contain extracellular matrix metalloproteinase inducer, metalloproteinases (MMP), tissue factor (TF), VEGF, HGF, CD44 β1-Integrin and intercellular adhesion molecule-1 (ICAM-1), etc [2, 21, 25, 61–63]. These TMVs can on the one hand stratify (amplify) the malignant abilities of other tumor cells through the horizontal transfer of effector molecules, on the other hand they can reach distant areas and prepare the favorable microenvironment for the migrating malignant cells.

Degradation of extracellular matrix is indispensable for tumor expansion. Cathepsin B, a cystein protease, has a dual effect on the remodeling of the matrix. It is present in the membrane of TMVs, degrades laminin and collagen. Most MMPs work at neutral pH, but cathepsin B works at an acidic one. This condition is created by the tumor cells themselves, as a result of their high glycolysis rate. In addition to its degradative function, cathepsin B activates further MMPs, therefore the degradation will be further escalated [64].

A series of complex interactions draws up between TMVs, stromal fibroblasts and endothelial cells. The players act mutually, creating an interacting network. Human and murine lung cancer cell line derived TMVs chemoattract endothelial cells directly, which then secrete more IL-8 and express more ICAM and (vascular cell adhesion molecule (VCAM). These TMVs promote angiogenesis through stroma cells indirectly as well, inducing them to express several pro-angiopoietic factors [65]. TF is extremely highly expressed in tumor cells. TMVs bearing TF may trigger systemic coagulopathy and transfer TF activity among various cells. TF bearing TMVs originate from cancer stem cells or already fully mature cancer cells [66]. TMVs obtained from prostate carcinoma cell lines promote MV shedding from activated fibroblasts, which in turn are able to increase migration and invasion of highly metastatic prostate cancer cells [67].

10.4.3 The Effects of MVs From Different Tissues on the Induction of Tumor

Immune cells are abundant cell types in the microenvironment of a tumor. There is an increasing awareness of the role of inflammation in cancer. Immune responses from both the innate and adaptive immunity are mediated by cytokines and chemokines. The growth of some tumors can be limited by some inflammatory responses, while it can be enhanced in some other tumors.

In inflammation macrophages help to create a beneficial microenvironment for tumor cells through their TGF-β1 secretion. TGF-β inhibits not only the proliferation/ differentiation of T and B cells, but also the induction of tumor cell apoptosis. Since tumor cells expose high levels of PS, they attract and stimulate macrophages. In case of inflammation, macrophages are already in the "neighborhood", so they can react with the tumor cells, inducing the tumor maintaining effect. As we mentioned in Section 10.2.2, phagocytes expose PS binding receptors, which can capture the PS displaying TMVs [14, 15, 27]. Thus further macrophages from longer distances can get recruited and activated, helping the establishment of a tumor favoring microenvironment. Studies on the highly metastatic B16F10 melanoma cell line suggest that melanoma cell derived TMVs exert anti-inflammatory and immunosuppressive activities [68]. Direct interactions between TMVs and human monocytes/macrophages were described by M. Baj-Krzyworzeka. The authors could show that TMVs, isolated from the cell lines of pancreatic, colorectal adenocarcinoma and lung carcinoma transferred CCR6 and CD44v7/8 to monocytes, which resulted in the alteration of their biological activity [21, 69]. These studies imply that TMVs affect not only other tumor cells or the "would-be" tumor cells, but different cell types in the "niche" of the developing tumor. By altering the biological activity of those cells, a tumor-favoring or/and an anti-tumor milieu will be set up. It is not clear though what kind of advantage could be obtained for the tumor cells by the release of TMVs provoking an anti-tumor effect in monocytes. It is to be noted that the above described results were obtained from cancer cell lines in vitro, and supposedly, in vivo, the monocyte-induced effects would lance themselves further interactions between the cells and the MVs from all kind of origin, present in a given tumor environment. Presently it is quite difficult to roll up all in vivo interactions in the cellular – microvesicular network.

Besides immune cells, platelets, fibroblasts and all other cells release MVs in the hematopoietic microenvironment of an inflammation. MVs secreted by activated platelets bind to endothelial cells, induce the production of pro-inflammatory cytokines and products of the arachidonic acid pathway. In turn, pro-inflammatory cytokines (TNF-α, IL-1β) or the terminal complement complex C5b-9 can induce MV shedding of inflammatory cells (like platelets and endothelial cells) which play an important role in the regulation of the process [70]. Furthermore, these MVs upregulate adhesion molecules on endothelium and leukocytes [71, 72], which results in increased extravasation and leukocyte adhesion to the endothelium [73, 74]. Platelets and platelet-derived MVs can interact with leukocyte, forming aggregates. Platelet adherence induces gene expression of chemokines and cytokines in inflammatory cells.

Taken together, the interplay between the different cells and MVs of different origin modulates cellular responses in inflammation and results in a highly complex communication network, which further influences the behavior of the tumor cells and of the "would-be" tumor cells.

10.5 MVs and Tumor Therapy

On the basis of the role of TMVs in tumor progression, another door has been opened toward a new field in cancer therapy.

Considering that TMVs are involved in tumorigenesis at multiple levels, and that drugs themselves can be expulsed from tumor cells via MVs, interfering with the formation, release and propagation of these vesicles can be a novel and alternative issue in cancer treatment.

The potentials in MV-related tumor therapy include classical cell biological possibilities and immunological strategies. By the former one TMV formation and release can be influenced, by the latter one more effective immune responses can be elicited by MVs serving as vaccines. A third attempt is the removal of TMVs by dialysis.

10.5.1 Cell Biological Approach: Inhibition of TMV Formation and Release

The process of MV formation is coupled to the components of the intracellular vesicular transport and of the cytoskeletal elements. Preventing the function of one of these components disturbs the exocytosis of exosomes and the budding of larger TMVs.

Taxol and vinca alkaloids, as conventional chemotherapeutic medicaments not only inhibit cell division, but additionally decrease exosome release, by inhibiting microtubule formation, needed for vesicle forwarding. Using proton pump inhibitors (PPIs), exosome formation can be blocked in lack of gradually decreasing acidic milieu [8]. In order to restrain ceramide accumulation needed for exosome sorting, the inhibition of the ceramide synthesizing enzyme sphingomyelinase leads to exosome release block [28]. A large number of proteins participate in the complex cell biological process of vesicle trafficking. Rab-GTP-ase proteins are involved in vesicle fission, fusion and transport. Out of these at least 21 have been implicated in tumorigenesis. Additional proteins, such as clathrin, sorting nexins and more have been also described to be overexpressed in different tumors [75–79].

The scale of proteins is extremely broad: which one to choose as therapeutic target in order to prevent TMV formation and release? But just because normal vesicle physiology is a prerequisite for life in all cells, the usability of such a drug would be uncertain, unless the most appropriate and tumor specific agent would be found. Broad effects may have adverse side effects in healthy cells.

10.5.2 Immunological Strategies: MVs as Vaccines

The idea of MV vaccination is based on the finding that exosomes efficiently transfer tumor antigens from tumor cells to other antigen presenting cells, – mostly to dendritic cells (DC) –, allowing initiation and amplification of antitumor immune responses [10, 80, 81].

Chaput and her group proposed two distinct methods in the treatment of melanoma and ovarian cancer patients [10]. The methods would result in a targeted anti-tumor cytotoxic T cell response. One method, the “active vaccination” comprises injection of dendritic cell derived exosomes (DEX) loaded with tumor peptides into the patients. These DEXs were purified from DCs, cultured after leukapheresis of melanoma patients, then they were loaded with tumor peptides and reinjected into the patients. The second “active vaccination” method involves isolation of ascites derived exosomes (ExAs) from patients with ovarian carcinoma, adjuvants are added to them, and then they are reinjected into the patients. In both cases the vaccines (the exosomes) are injected into the subcutaneous tissues at different sites, far from the already existing tumor’s immunosuppressive environment, with a strong antigen presenting potential. They provoke a strong and targeted anti-tumor T cell response. A tumor derived exosome mediated tumor antigen cross-presentation by DCs to T cells can be obtained with the so called “adoptive transfer”. ExAS would be first added to DCs, isolated from the patient’s blood, so the ExAS containing DCs can then present the tumor antigen to specific T cells, which after culturing, represent specific cytotoxic T cell clones against the tumor. In this case the cytotoxic T cells would be injected to the patient, ready to fight the cancer cells.

Some of the above listed methods have already successfully undergone first phase clinical trials [10, 82].

Notably the effect of tumor derived exosome vaccines alone, without DC injection, are at least questionable, if not counterproductive. An accelerated tumor growth has been shown through NK cell inhibition in mice [83] and in humans as well [22], in the absence of DCs as carriers for exosomes.

10.5.3 Removal of TMVs from the Patient's Blood

Ichim and his group proposed a physical approach in order to remove tumor exosomes from the body fluids of the patients. The method is an extracorporeal "dialysis" through a so called Hemopurifier^{TM}, which is a hollow-fiber cartridge, originally designed to eliminate heavily glycosylated surface proteins and viruses. Tumor cell membranes and shed immunosuppressive TMVs are also highly glycosylated and thus bind preferentially to the resin compared to non-malignant cells. Furthermore, attaching specific molecules or antibodies to the cartridge, the selective removal of targeted TMVs can be increased. This method combines dialysis and affinity chromatography. It has been used in clinical trials. The immunsuppressive activity, normally found in the ascites fluid of patients with ovarian cancer was completely removed by the Hemopurifier^{TM}. The authors propose that this method can be a novel, easily implemented approach in cancer therapy [44].

10.6 Conclusion

Cellular interactions play a crucial role in all physiological and pathological processes. The classical view comprises soluble (or plasma membrane-bound) ligand-receptor binding. Relatively recent findings suggest the existence and the importance of an additional communication system among cells, namely the microvesicular information transfer. With the advance of research, its role seems to be more and more complex in tumorigenesis, considering that TMVs help establish tumor escape mechanisms against the immune surveillance of the organism, and they are involved in the establishment of an optimal microenvironment for the tumor: survival, progression, angiogenesis and invasiveness are promoted by TMVs (Fig. 10.3).

The diverse effects of MVs have begun to be revealed, but the exact molecular mechanisms still remain to be elucidated. Either the effector molecule of the MV or the molecular process in the target cell is often not clarified. The protein set of the MV membrane can be relatively easily identified with flow cytometry in the case of 300–1,000 nm large vesicles, meanwhile several obstacles are faced during the analysis of the internal content of the vesicles. It has also not been understood so far how the appropriate "cargo" molecules are selected into vesicles. The broad range of membrane-bound signal, receptor and adhesion molecules explains the pleiotropic effect of the vesicles, parallelly the specific binding to the target cells seems to be well functioning. What decides the most appropriate selection of the target cell pauses further questions. The in vivo concentrations may exert an influence on it, but they are hard to measure presently.

Understanding the efficiency and action of TMVs is of great cell biological importance as well. Horizontal transfers of gene products between cells were until recently considered impossible. TMVs, containing numerous biologically active proteins and RNAs, dramatically change the phenotype of the recipient cell, which per se, can be considered as epigenetic influence. TMVs act in a paracrine/endocrine way, they transfer information which leads to altered "transcriptome" and "proteome" in the target cells. This suggests that their role goes much beyond simple molecule transfer, the reprogramming of numerous other cells ends up in creating an altered microenvironment (Fig. 10.4). This is the case when TMVs prepare the niche for growing or metastatic tumors. Their potential in shaping tumor microenvironment can not be neglected with regard to cancer therapy either: the insights could offer a novel and alternative view to cancer treatment. Considering that most of the

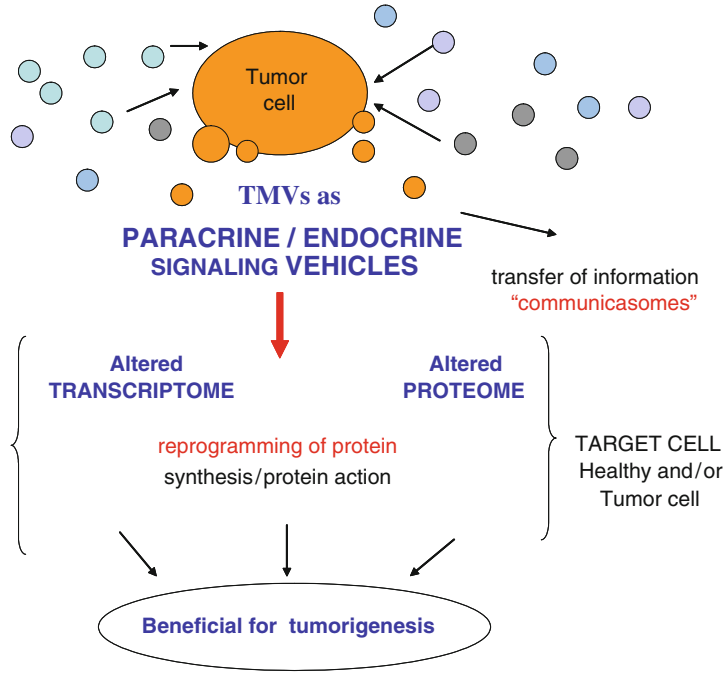

Fig. 10.4 TMVs serve for information transfer and for paracrine/endocrine signaling. TMVs, containing numerous biologically active proteins and RNAs dramatically change the phenotype of the recipient cell. They not only transfer information, but can reprogramme the recipient cells with the goal of favoring tumorigenesis. The release of TMVs is influenced by MVs from other cell origin as well

body cells, especially immune cells and platelets release MVs, multilevel microvesicular networks are established all over the tissues, whose fine tuning must be well orchestrated. TMVs dramatically and drastically disrupt this fine balance, as we attempted to demonstrate it in the present review.

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