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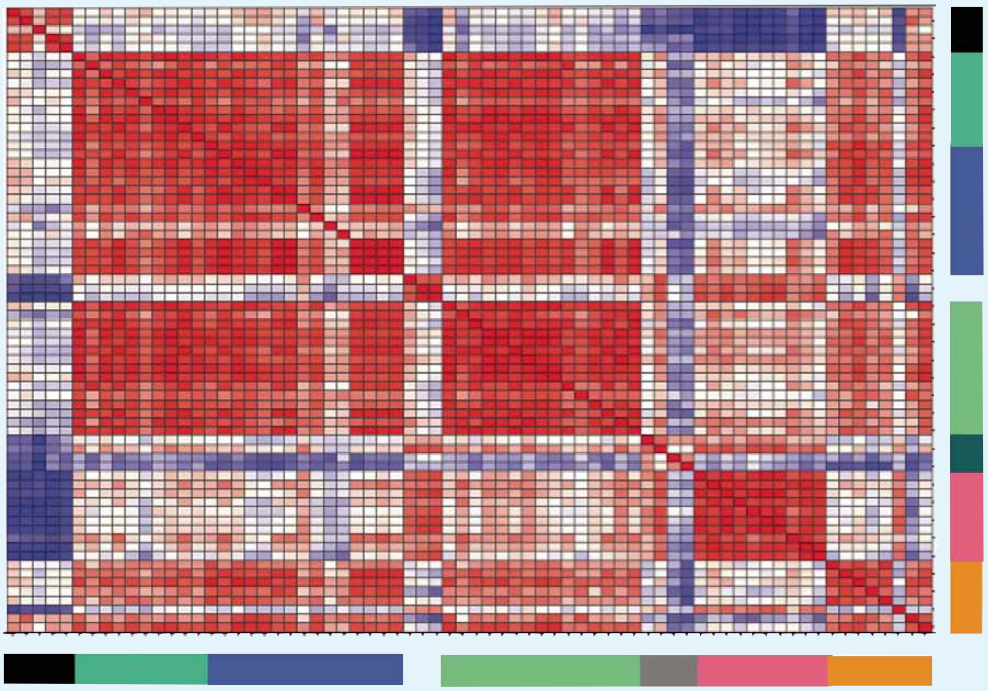
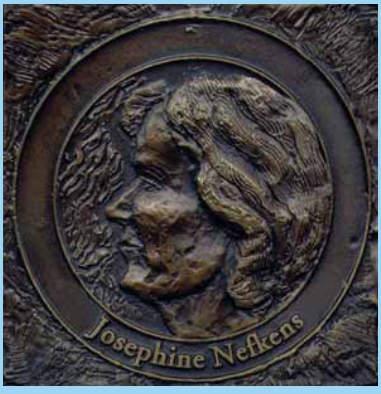


Daniel den Hoed Cancer News

What's new in tumor biology: novel concepts and dogmas



Josephine Nefkens Symposium,
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Editorial

With great pleasure we offer this Special Issue of Daniel den Hoed Cancer News. It is a scholarly and entertaining reflection on the first Josephine Nefkens Symposium by Jan Hein van Dierendonck.

The event itself was very rewarding with excellent presentations by scientists who are leaders in their fields addressing key developments in cancer research. The introduction to this issue puts their topics in a broad historical context underscoring the timeliness of the presented data. The stimulating enthusiasm of our big audience, some 400 people, spoke for itself.

I take this opportunity to thank the people who did most the work: Hans Groen, Karin de Beer, Margot van den Akker and the coordinator of the team, Gert Jan van Steenbrugge. Putting together the program was a combined effort from several leading people in cancer research in Rotterdam, but the biggest input was undoubtedly from Riccardo Fodde. Our sponsors are gratefully acknowledged.

I conclude by reminding you that this symposium is the first in a cycle of three. The second is the Daniel den Hoed symposium on November 30 this year. It's emphasis will be on clinical cancer research. Molecular targeted therapy will be the topic. The third and last Symposium in this cycle is the Josephine Nefkens Prize Symposium, planned on November 28, 2008, ten years to the day after the opening of the Josephine Nefkens Institute. The theme of this Symposium depends on the third winner of the Josephine Nefkens Prize after Jan Hoeijmakers in 2001, and René Bernards in 2004. Early next year the call for nominations for the prize will be circulated. It might be wise to already give thought to who might be suitable nominees for the Josephine Nefkens Prize of 2008.

We are confident that the high standard of the Josephine Nefkens Symposium can be maintained, and that we will meet again on both occasions.

On behalf of the organizing committee,

Wolter Oosterhuis

Cover: Correlation plot (Peason's coefficent) of the various types of germ cell tumors and normal testis (indicated in different colours) based on the expression of 157 miRNAs. Note the specific clustering of the various entities (Looijenga, page 21)

Introduction
Changing concepts in tumor biology

J.H. van Dierendonck, Ph.D; freelance Science writer

Over time, our view on the origin and dissemination of cancer has experienced many paradigm shifts. Today, with the rise of powerful high through-put genetic screening techniques, concepts are being proposed that are either novel or, when placed in an historical context, could possibly evoke a *déjà vu*. As an introduction to the summaries of the lectures presented on the 2006 Josephine Nefkens Symposium about the state of the art in cancer biology, I will here briefly present an overview of some concepts from the past.

Black bile and blastema

Long before people were aware of the existence of cells, the humoral concept of black bile supported the idea that cancer was a generalized disease from its very inception. This early cancer concept was linked to elegant terms like 'dyscrasia', meaning an abnormal (imbalanced) condition of the body (especially the blood), and 'diathesis', meaning a predisposition or tendency of the body to develop cancers.[1] At the end of the sixteenth century, the French surgeon Nicolas de la Framboisière noted the spreading of cancerous disease to internal organs[2] and in 1757, his compatriot Henry Francois le Dran wrote that breast cancer is a local disease in its early, curable stage, but that, in a later stage, cancer 'juices' spread to local lymph nodes and into the blood stream, where they can affect the lungs.[3]

In fact, the prognostic significance of lymph node involvement was well-recognized before general acceptance of the concept of metastasis, but lymph was also regarded as a major cause of cancer itself, a theory elaborated by the philosopher Descartes. In 1773, the chemist and first experimental oncologist Bernard Peyrilhe wrote that experiments of Herman Boerhaave and others had sufficiently proven that lymph has a constant and exclusive communication with the cellular texture and, therefore, is the proximate cause of cancer.[4] Some years later the British surgeon John Hunter proclaimed in his lectures that 'coagulating lymph' (later identified as fibrin) represents the omnipotent 'blastema' from which normal and diseased organisms arise.[5]

Chronic irritation and aberrant cells

The term 'metastases' was first used in a book written by the French surgeon Joseph Claude Récamier and published in 1829, but the author thought that cancer was essentially an inflammatory process and that metastasis was brought about by nerves induced to provoke similar tumors at different sites.[6] By that time it was evident that cells are the fundamental basic units in animal tissues. Throughout the 1840s and early 1850s, both normal and pathological tissues were considered to consist of cells formed for the most part from a blood-derived amorphous blastema. This latter theory was challenged by the German embryologist Robert Remak, who in 1851 discovered cell division in embryonic cells ('I dare to suggest that pathological tissues, just like the normal ones, are not formed in an extracellular blastema, but are descendants or products of normal tissues of the organism').[7]

Also the 'father of cellular pathology',

J.H. van Dierendonck

Jan Hein van Dierendonck studied biology at the University of Leiden and graduated with a specialization in biochemistry. In 1983 he joined the group of Cees Cornelisse at the Department of Pathology of the Leiden University Medical Center. He worked on several projects involving hormonal regulation and metastatic progression of experimental breast cancer, endocrine-related tumor dormancy, and immunocytochemical detection of cell kinetic parameters. In 1990 he defended his thesis Cell Kinetic Aspects of Breast Cancer and continued his career as postdoc researcher and staff member of the department of Surgical Oncology. His group performed pioneering research on the regulation of apoptotic cell death in solid cancers and the in situ detection of dying cells and was also involved in basic studies on cytotoxic treatment of liver metastases of colorectal cancer.

In 2001, Van Dierendonck left scientific research and since then he has been working as a freelance science writer and illustrator. He produces texts, infographics, and cartoons for several magazines in the fields of medicine, chemistry and life sciences and has a special interest in the histories of medicine, science, and technology.

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Rudolph Virchow, became increasingly convinced of this concept, although he was comparatively late in reaching these conclusions. Having observed tumor formation at the site of chronic inflammation, Virchow suggested that the cause of cancer was local irritation[8]; he repeatedly stated that cancer arises from amorphous material within the connective tissue. Interestingly, to some extent his idea is being revisited in a recent proposal that inflammatory cells release potent pro-survival factors and positively influence tissue remodelling and angiogenesis; tissues in which inflammatory pathways are chronically engaged indeed seem to exhibit an increased risk of tumor development.[9] It is also remarkable that Virchow was unconvinced that metastases arose from disseminated cells rather than from 'morbid juices', in which case, he reasoned, one would always find these metastases in the lungs – which was a sharp contrast with actual findings.[10]

Virchow's pupil Julius Cohnheim proposed in 1867 that cancer results from the activation of dormant embryonic-tissue remnants.[11] The 'embryonal-rest hypothesis' of cancer was based on the histological similarities between the developing foetus and certain types of cancer, such as teratocarcinomas. In that same period, Karl Thiersch (the surgeon who was the first on the Continent to introduce Lister's antiseptic technique as a standard procedure), provided data indicating that skin cancers originate from abnormal cell division.[12] Soon thereafter, Heinrich Waldeyer-Hartz (the anatomist who coined the term 'chromosome' and founded the neuron theory) came to similar conclusions; his description of tumor initiation, growth, local spread and metastasis through lymphatics and blood vessels remains as relevant today as it was in 1867.[13]

Seed, soil, and progression

In 1889, the British surgeon Stephen Paget published his famous study on 900 autopsy records of patients with different primary tumors, demonstrating non-random patterns of metastasis to visceral organs and bones; on the basis of this data he formulated the 'seed and soil' hypothesis.[14] It was a concept that was later challenged by James Ewing, who in 1929 proposed purely mechanical factors resulting from the anatomical structure of the vascular system.[15]

During the 1950s, researchers started experiments with rodents, injecting them intravascularly with tumor cells and quantifying metastases in vascular organs – most often the lungs. Subsequent labeling studies with radioactive iododeoxyuridine prompted the question of whether development of metastases represents selective growth of unique subpopulations of malignant cells endowed with special properties.[16] It was gradually recognized that initially benign neoplasms gradually undergo a series of changes during the course of the disease, leading to a malignant, potentially lethal state. The British pathologist Leslie Foulds in 1954 described this phenomenon of tumor evolution as 'neoplastic progression', defined as the acquisition of irreversible qualitative changes in one or more characteristics of a neoplasm. As this progression could occur over periods of years, the behavior of cancers could vary at different stages.[17]

Tumor cell heterogeneity

The American pathologist Peter Nowell (discoverer of the 'Philadelphia chromosome') predicted that tumor cells that progress to advanced stages of malignancy would be less stable genetically.[18] Studies by his colleague and country fellow Isaiah Fidler showed that cells with high metastatic potential had, indeed, a much higher increase in the rate of mutation compared to low metastatic variants, indicating that progression could be a consequence of acquired genetic instability.[19] Work with the mouse B16 melanoma revealed that different tumor cell clones, each derived from

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individual cells isolated from a parent tumor, varied markedly in their ability to form pulmonary nodules after intravenous injections.[20] The finding that cell subpopulations with heterogeneous potential pre-exist within the same tumor was confirmed by many other investigators. Subsequent experiments also demonstrated that metastases originated from single proliferating cells, but that the microenvironment of the host tissue was in part responsible for determining the site of metastatic outgrowth.[21] The latter was also strikingly demonstrated in patients treated for ovarian cancer ascites by applying peritoneovenous shunts; despite continuous entry of millions of tumor cells into the circulation, metastases to the first vascular bed encountered, the lungs, were extremely rare.[22]

Genes

Soon after the Danish botanist Wilhelm Johannsen coined the word 'gene' to describe the fundamental physical and functional units of heredity, the German biologist Theodor Boveri postulated in 1914 that 'tumor cells with unlimited growth would arise if those "inhibiting chromosomes" were eliminated... and the unlimited tendency to rapid proliferation in malignant tumor cells would be deduced from a permanent predominance of the chromosomes which promote division'.[23] This prophetic prediction was confirmed in the early 70s with the discovery of the first viral oncogenes and with the research of the American epidemiologist Alfred Knudson on the relationship between hereditary and non-hereditary forms of retinoblastoma, which formed the basis for understanding loss of heterozygosity and the role of tumor suppressor genes.[24]

The elucidation of the human genome sequence in 2001 has now made it possible to identify genetic alterations in cancers in unprecedented detail. Last year, an international effort to analyze 13,000 genes in 11 breast and 11 colorectal cancers revealed that individual tumors accumulate an average of ~90 mutations, but that only a subset (average of 11 per tumor) were mutated at significant frequency, and thus could be considered to be 'drivers' of the neoplastic process.[25]

Another ongoing international study, in which the Josephine Nefkens Institute is also involved, has now provided data from 518 protein kinase genes in 210 diverse human cancers. A substantial variation was found in the number and pattern of mutations in individual cancers, reflecting different exposures, DNA repair defects and cellular origins. In no less than approximately 120 genes there was evidence for 'driver' mutations, indicating a much larger repertoire of cancer genes than hitherto anticipated.[26]

As outlined in the lectures by Carlo Croce and Leendert Looijenga (pages 17 and 21 respectively), the level of complexity has also been substantially increased by the discovery of genes encoding for cancer-linked microRNAs. Moreover, Kent Hunter (page 14) strongly advocated the role of genetic polymorphisms in the germ line; the concept that susceptibly to cancer and metastasis is, like eye color or body length, a quantitative (and therefore predictable) trait. It is difficult to resist the thought that the genetic paradigm increasingly resembles the humoral concepts prior to the discovery of (cancer) cells; susceptibility to cancer development and/or metastasis as a constitutional 'dyscrasia', not determined by one's composition of the blood, phlegm, and bile, but by subtle genetic variations. Just as the balance of humors thought to be affected by seasons, diets, and other factors, so may subtle effects of some genetic variants be magnified in the presence of certain environmental exposures.

Stem cells

In the 1950s, bone marrow reconstitu-

sults from pre-existing variant cells within a malignant tumour. Science 1977; 197: 893-895

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tion experiments, following lethal irradiation in mice, first indicated the existence of hematopoietic stem cells and soon these cells were suspected in other cell systems as well. In 1961, Chester Southam and Alexander Brunschwig showed that tumor cells harvested from patients with disseminated cancer and then injected subcutaneously into the same patients (!) led to a low frequency of tumor formation; the bottom line of tumor induction was injection of over one million cells.[27] Fifteen years later, Anne Hamburger and Sydney Salmon published data showing that only 1 in 1000 to 1 in 5000 cells isolated from solid tumors were capable of forming colonies in standard soft agar assays.[28]

These findings led to the concept that the entire population of a tumor's cells might arise from a few so-called 'cancer stem cells'. This concept didn't become readily accepted, until in 1994 a Canadian research group identified such cells in certain types of leukaemia.[29] These cancer stem cells were much harder to spot in solid tumors though, but in 2003, researchers in the laboratory of Michael Clarke in Michigan claimed to have characterized *bona fide* cancer stem cells in breast tumors.[30]

As outlined by René Bernards (page 10), the finding in his research group by Laura 't Veer and her colleagues that gene-expression profiles of breast cancer cells within primary tumors predict with 90% accuracy whether the tumor will remain localized or not (see cover illustration),[31] has cast doubt upon the idea that metastatic tumor cells emerge from the gradual selection of cell clones enforced with increasing metastatic capabilities. Further, the work presented by Paolo Comoglio (chapter 5) on genes involved in the embryonal development and epithelial-mesenchymal transition provides a basis for the concept that certain neoplasms tend to be malignant from the very start. Interestingly, in Austria, Martin Widschwendter and his coworkers have recently found that within embryonic stem cells, the gene targets of special proteins, which reversibly repress genes required for differentiation, are up to 12-fold more likely to have cancer-specific promoter DNA hypermethylation than non-targets. This strongly supports the concept that cancers might originate from stem cells in which reversible gene expression is replaced by permanent silencing, locking the cells into a perpetual state of self renewal and thereby predisposing to subsequent malignant transformation.[32]

Niches

In 1861, the French physician Armand Trousseau stated that if the diagnosis of a suspected carcinoma of an internal organ could *not* be verified, the sudden and spontaneous appearance of thrombophlebitis in a large vein afforded necessary proof for diagnosis. An interesting observation from Comoglio's laboratory is that the proto-oncogene *met* also causes intravenous thrombosis, even prior to expansion of *met*-transformed cell clones.[33] Like Virchow and Cohnheim, they suspect that a crucial key to cancer development may lie in extracellular fibrin – not as an origin of cancer cells, but as a provisional matrix that attracts blood vessels and provides anchorage for cell growth and migration. It appears that the hypoxia-driven expression of the *met* gene not only is involved in processes of the early embryo, but also in tissue repair and regeneration; the concept that tumors are wounds that never heal.

In that context, Kari Alitalo's lecture (page 29) was focused on the question how tumor cells manipulate surrounding lymphatics; 350 years after discovery of the lymphatic system by the Danish physician Thomas Bartholin, Aliralo's research group has been describing the genes responsible for the spread of 'cancer juices'.

Jeffrey Rosen (page 33) has devoted his (scientific) life to the mammary gland, searching for stem cell niches within the normal gland and for factors that alter the regulatory mechanisms and lead to tumor initiation. And finally, Riccardo Fodde (page 37) has found 'his' niche in the gut: Fodde's research group has been unravelling the role of Wnt-signalling in the onset and malignant behaviour of intestine cancer stem cells.

In his classic book, *Spread of Tumors in the Human Body* the surgeon R.A.Willis introduced in 1952 the term 'dormancy' to denote long latency periods between apparent curative resection of a primary tumor and detection of recurrent disease.[34] The concept of very slowly proliferating cancer stem cells that, as a result of changes in their niche, after many years suddenly start to give rise to more rapidly growing cell populations, seems a powerful one. However, as Bernards will ask in the next article: if slowly cycling stem cells are the culprits of malignancy, how do these cells acquire sufficient numbers and genetic changes? Enter the concept of dedifferentiation: could it be possible that differentiated tumor cells, either by genetic or epigenetic mechanism, are able to de-differentiate into cancer stem cells?

From progression puzzle to stem cell paradox

R. Bernards, Ph.D., Netherlands Cancer Institute, Amsterdam, The Netherlands

The classical model of progression of tumors to a metastatic phenotype is based on the concept that rare subpopulations of cells within the primary tumor acquire advantageous genetic alterations over time, which enables these cells to mobilize, invade blood and lymph vessels, and successfully invade and colonize distant tissues. Though in the past a variety of studies have challenged this 'genetic selection model', only recent data obtained by gene-expression profiling of human breast carcinomas received broader attention. This data imply that the metastatic capacity of poor-prognosis breast tumors might be acquired by mutations at much earlier stages of tumor development than was previously assumed.

Classic experiments

In the late 70's, Fidler injected cultured B16 melanoma cells intravenously into mice and considered the outgrowth of cells struck in the vascular bed of the lungs as a proper model for real metastases. These lung nodules were harvested and disaggregated, and cells were subsequently injected into new mice. The latter developed significantly more lung nodules than the first group, pointing to the selection of cells with enhanced metastatic potential. In addition it was observed that, after cloning of B16 cells in cell culture, different B16 variant cell lines greatly differed in their ability to produce experimental metastases.[1]

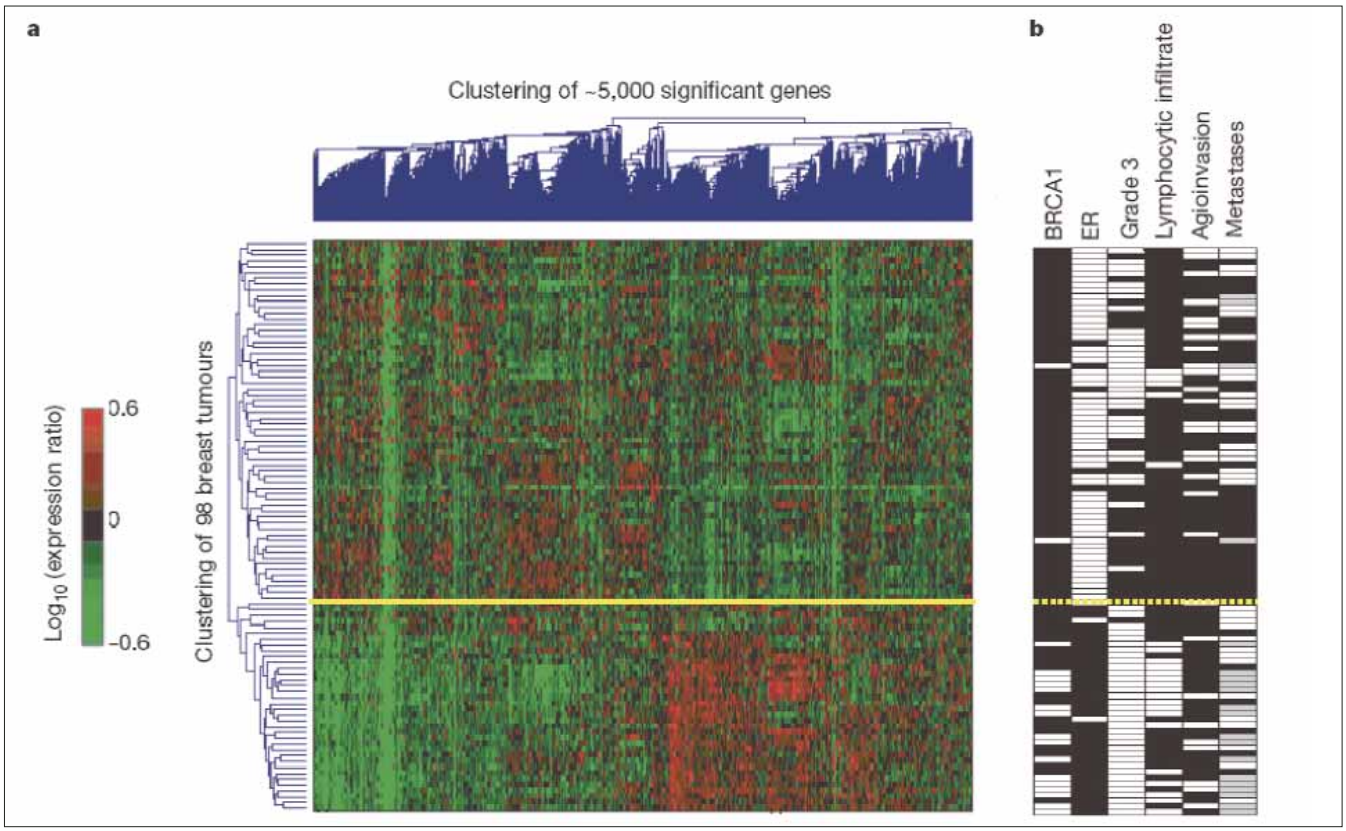
These kind of studies contributed to the strong metaphor of Darwinian selection of cells with increased malignancy; through genetic or epigenetic changes, cells acquire characteristics favoring survival and rapid cell division and become the founder of a cell population that starts to dominate the tumor mass. Within this population subsequently new rare variants emerge, some of which even better fitted, i.e. dividing more rapidly and/or resisting apoptosis. They may display invasive growth patterns, have learned to induce blood vessel growth and allow the tumor mass to expand beyond its limits. Between six to ten such clonal successions, with accumulating genetic en epigenetic changes, may suffice to reach the final stage of tumor evolution: possessing all requirements to disseminate and survive and expand at distant sites.

Invasiveness does not select for growth

At first sight this concept looks straightforward. But think twice, it contains a striking inconsistency; the genes that specify the final steps would not seem to confer increased proliferative benefit at the primary site. That is, there is no reason to think that a metastatic phenotype enables cells to proliferate more effectively within the tumor mass, thereby increasing their representation in the overall tumor cell population. Rare metastastic cells remain rare and as the success rate of individual cells undertaking dissemination and formation of new tumors at distant sites is extremely low, it is difficult to imagine how this would ever proceed. This reasoning forces us to think of an alternative mechanism: could it be possible that metastasis is a property tightly associated with increased cell prolifer-

R. Bernards

René Bernards received his PhD in 1984 from the University of Leiden, where he studied cell transformation by adenovirus. For his postdoctoral training, he joined the group of Robert Weinberg at the Whitehead Institute in Cambridge, USA, where he was involved in identifying the rb gene and investigated the role of myc in tumor progression. He joined the Massachusetts General Hospital Cancer Center as an assistant professor in 1988 to study oncogenes and tumor suppressor genes. From 1992, he has led the Department of Molecular Carcinogenesis at the Netherlands Cancer Institute, and studies mammalian cell cycle regulation. In addition, he has been a part time professor at Utrecht University since 1994. In the past few years, his group has focused on the development of tools to carry out genome-wide genetic screens for identification of cancer-related genes and has recently completed the construction of a collection of 24,000 RNAi vectors to perform large-scale loss-of-function genetic screens. Many of Bernard's papers appeared in Nature, Science, and Cell and he received both the NWO Spinoza Award and the Josephine Nefkens Award. In 2003, he co-founded a genomics-based diagnostic company (Agendia) that started offering the first microarray-based diagnostic test for the clinical management of breast cancer.



ation early in tumorigenesis? In other words: do malignant tumors start out on the wrong foot and do cells from very small tumors already have the ability to colonize distant sites? Are the culprits in fact the oncogenes and tumor suppressor genes cancer biologists have studied now for decades?

This different paradigm seems consistent with longstanding observations in certain breast cancer patients that, despite small and well localized breast tumors, carcinoma cells are clearly detectable in the bone marrow. More recently, DNA microarray-analysis has provided another clue: gene expression patterns of metastatic cells are often strikingly similar to that of cells confined to the primary tumor mass from which they were derived. Moreover, gene-expression profiles of breast cancer cells within primary tumors have been used to predict with 90% accuracy whether the tumor will remain localized or not (Fig. 1) [2] and in certain mouse models of tumorigenesis, oncogenes involved in proliferative power, such as *myc* and *ras*, have been proven to be involved in metastasis too.[3] Could it be that, whereas we searched far and wide in the genomes for metastasis genes, these were staring us in the face?

The real culprits of malignancy

What then could be a driving force both for cellular proliferation and dissemination? René Bernards is convinced we can find the answers in embryology, in the way tissues are built and maintained, i.e., in the nature of stem cells.[4] After stem cell division, one daughter cell is a perfect replica, while the other is the progenitor of so called transient amplifying cells, cells that rapidly divide, thereby generating the tissue volume, and in the end differentiate into different cell types that no longer are able to divide, but have a limited life span. As will be outlined in chapter five, invasive growth and cell migration of stem cells and stem cell derived progenitor cells are part of a morphogenic program in the embryo. In adult tissues these cells play a crucial role in tissue repair and the mobility of these cells is likely driven by the

Figure 1. A:
Two-dimensional presentation of transcript ratios for 98 breast tumours. There were 4,968 significant genes across the group. Each row represents a tumour and each column a single gene. As shown in the colour bar, red indicates upregulation, green downregulation, black no change, and grey no data available. The yellow line marks the subdivision into two dominant tumour clusters.
B: *Selected clinical data for the 98 patients in* **A:** *BRCA1 germline mutation carrier (or sporadic patient), ER expression, tumour grade 3 (versus grade 1 and 2), lymphocytic infiltrate, angioinvasion, and metastasis status. White indicates positive, black negative and grey denotes tumours derived from BRCA1 germline carriers who were excluded from the metastasis evaluation. The cluster below the yellow line consists of 36 tumours, of which 34 are ER negative (total 39 ER-negative) and 16 are carriers of the BRCA1 mutation (total 18).(Nature 415, 530, 2002)*

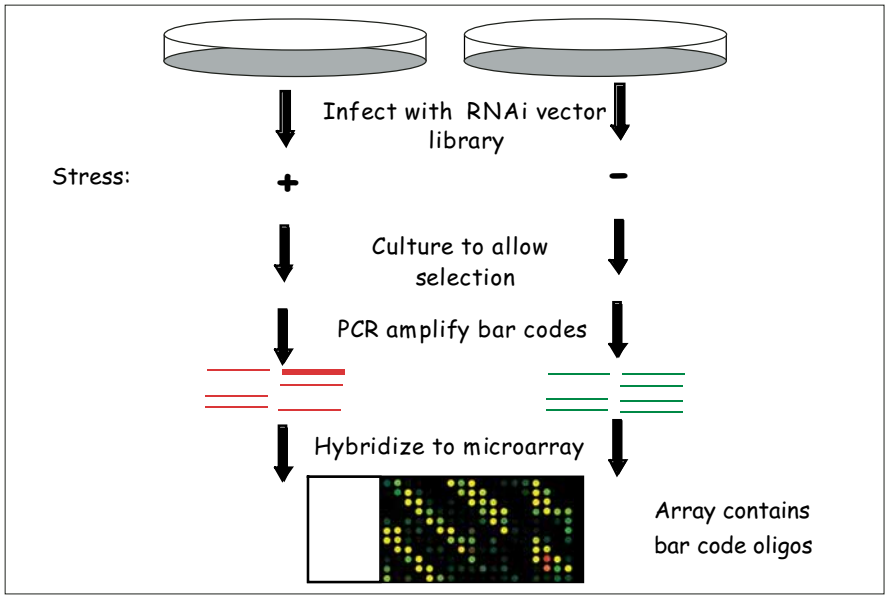


Figure 2.
SiRNA barcode screens

nature of the surrounding stroma and by adverse micro-environmental conditions, such as hypoxia.

By definition, an adult stem cell is a cell that comes from a given organ, has long term replicative potential, and possesses the ability of both self-renewal and to differentiate into the cellular components of that organ. A recent study using cell surface markers to identify cell populations in mouse mammary glands showed that the combination Lin-CD29^{hi}CD24^{+} identified cells highly enriched for mammary stem cells. Even a single cell was able to reconstitute a complete mammary gland *in vivo*.[5] In normal organogenesis, this self-renewal is tightly regulated. In cancer, these control mechanisms fail. It has been estimated that less than 0.1% of the breast cancer cells that have entered the blood circulation are able to establish a metastatic lesion, i.e., are able to generate cells that proliferative indefinitely.[1] This accords with the idea that, while the majority of the cancer cells have a limited ability to divide, few cancer cells actually harbor tumor-initiating capacity and could be considered as 'cancer stem cells'. In human breast carcinoma, cancer-initiating cells have been identified as CD44^{+}/CD24$^{-/low}$ cells; only these cells could be cultured as non-adherent mammospheres.[6]

Differentiation in reverse?

Growing evidence suggests that pathways that regulate the self-renewal of normal stem cells, involving genes like *wnt, shh* and *notch*, are deregulated in cancer stem cells and make them the driving force of both tumor formation and metastasis. In this picture, mutations in transit amplifying cells (descendants from stem cells) lead to non-metastatic tumors with 'good prognosis'; only mutations in the undifferentiated stem cells may lead to a metastatic phenotype. However, there is a major paradox involved in this concept. Dividing cells are extremely vulnerable to DNA damage. As stem cells give rise to whole tissues, it is essential they keep their cell division rate at a minimum and DNA damage control mechanisms red alert. It is therefore extremely unlikely that oncogenic mutations accumulate in the slowly proliferating stem cells.

Until recently it was believed that the process of cellular differentiation culminating in terminally differentiated mammalian cells is irreversible, but a recent study showed that by forced expression of the gene *msx1*, terminally differentiated murine myotubes can be induced to dedifferentiate.[8] Moreover, pluripotent stem cells can be induced from adult mouse fibroblasts by introducing the factors oct3/4, Sox2,

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c-Myc, and Klf4. Introduction of these cells into nude mice results in tumors containing a variety of tissues from all tree germ layers.[9] Thus, differentiation is not a one way street, and only a few factors suffice to change terminally differentiated cells into tumor stem cells.

Bernards therefore speculates that stem cell-like programs could be initiated by genetic changes in rapidly proliferating progenitor cells. Such mutations should prevent these cells from terminal differentiation and combine the mobility of stem cells with rapid outgrowth. Of course, oncogenic mutations in relatively undifferentiated 'early' progenitor cells will have a worse prognosis compared to mutations in more differentiated 'late' progenitor cells.

Barcode strategy

The million dollar question is: which genes control the stem cell phenotype? Only if the signaling pathways involved in differentiation and self-renewal are identified in detail, will it be possible to identify drug targets specifically eliminating cancer stem(-like) cells.

A potentially powerful approach to identify these targets has emerged from he discovery of RNA interference; short double stranded RNA molecules (small interference RNA, siRNA) that recognize and bind to specific mRNAs and, by simultaneously activating the RNA-induced silencing complex, cause the degradation of these mRNAs. In principle, it is possible to design siRNA sequences for every mRNA and by introducing these into cells, temporarily block the synthesis of any specific protein. This interference with protein production can be made permanent by insertion of siRNA-encoding DNA fragments into the nuclear genome (using a viral vector). For that purpose, DNA oligonucleotides are designed encoding so called 'hairpin sequences': they contain 19 nucleotides for a specific mRNA and 19 RNAanticodons, separated and flanked by non-coding regions; after transcription these RNA sequences from doubled stranded hairpins (short hairpin RNAs, shRNAs). Within the cytoplasm, 'non-coding' (and thus not-annealing) single strand regions are removed by special enzymes, thereby transforming shRNAs into functional siRNAs. An additional advantage of this method is that the inserted DNA can be manipulated to function as a specific label, a barcode: the DNA-codes in the inserted flanking regions are chosen to function as a target of DNA-primers used in the polymerase chain reaction (PCR) technique. Each barcode can then be detected by labeling the PCR product with a fluorescent dye and screened on microarrays consisting of barcode-complementary DNA-fragments (Fig. 2).

Searching for stem cell targets

A very useful application of this approach is to compare two cell populations, both of which harbor a collection of shRNA vectors, but only one of which is exposed to a biological signal of interest, for example DNA damage, apoptosis inducing agents, cytotoxic drugs or inactivation of a tumor-suppressor gene. When a barcode appears to be lost specifically in the treated cells, but not in the untreated cells, this indicates that shRNA is lethal with the stimulus used in the assay.

In the research group of Bernards at The Netherlands Cancer Institute, a set of 55.000 shRNA vectors has been generated, targeting 23.000 human and mouse genes. Using the barcode technique, it was demonstrated that cells having lost expression of SMAD4 (a protein known to be involved in the TGFβ-pathway) failed to respond to 'cellular stress', induced by treatment with TGFβ. Thus validated, the technique is now being used by Linda Smit on cancer cells with stem cell characteristics, for instance the human breast cancer cell line MCF7. Preliminary experiments indicate that indeed genes can be identified whose inactivation enhances stem cell phenotype of breast cancer cells. The ultimate goal of these experiments is to find drug targets for stem cells. But as outlined above, one should be aware of the possibility that mere killing of *bona fide* cancer stem cells may not suffice, as dedifferentiation of progenitor cells may generate new stem-like cells. It is expected that in near future, the barcode method will provide relevant data on this matter.

Hunting for insidious polymorphisms

K. Hunter, Ph.D., NCI, National Institute of Health, Bethesda, MD, USA

The hallmark study by Van 't Veer and coworkers, who identified a 70-gene expression profile that predicted disease outcome in young patients with breast cancer with an 83% accuracy,[1] has opened a debate regarding the mechanism of metastasis: do metastatic cells originate from only a small subset of cells within the primary tumor, cells that through successive mutations progressively acquire metastatic potential (the conventional 'rare cell hypothesis')? Or is this insidious potential, as the gene expression profiling studies suggest, already determined by oncogenic mutations at the onset of primary tumour development, thus in principle being a feature shared by all tumor cells (the 'oncogenic combination hypothesis')? According to Kent Hunter, who extensively studied cancer development in mice, this debate should include a third hypothesis: the possibility that the metastasis-predictive gene expression profile, rather than being a product of somatic mutations in evolving tumors, primarily results from genetic polymorphism in our germ line, similar to traditional phenotypes, such as body morphology of disease susceptibility ('quantitative traits'). The existence of such inherited metastasis risk factors would have a significant impact on experimental models of metastasis, clinical prognosis and the development of tailored treatment.

Breeding mice

The best example that metastasis is also strongly influenced by genetic background came from Hunter's breeding experiments with the PyMT mouse (a transgenic mouse that expresses the mouse polyoma middle-T antigen). Within two months after birth, these animals invariably develop mammary tumors, and nine out of ten develop lung metastases by 100 days of age. Breeding male PyMT mice with female animals from various different homozygous, inbred mouse strains resulted in 28 different heterozygous F1 progeny animals. These mice still harbored the same number of copies of the tumor inducing PyMT locus at the same genomic site and showed the same frequency and time schedule in tumour development. But the development of lung metastases greatly depended on the particular outcross, some F1s developing tenfold fewer metastases, and others 3-fold more than the original mouse strain.[2] Subsequently, using a quantitative trait mapping and cloning strategy, numerous chromosomal loci were discovered that seemed to contribute to this difference in metastasis efficiency (Fig 1).

Genetic variations

If the efficiency of metastasis is indeed hidden in our genome, this would fit with the observation that all or most of the primary tumor cells express the predictive-gene signature, providing an alternative to the oncogenic combination hypothesis. In fact, studies in both mice and man have provided evidence that metastases do have a clonal origin,[3,4] indicating that the rare-cell hypothesis cannot be completely ruled out.

In addition, the genetic background hypothesis may also explain a riddle that emerged from three independent

K. Hunter

Kent Hunter received a B.S. in biochemistry from the Pennsylvania State University in 1985 and his PhD in biology from the Massachusetts Institute of Technology in 1991. He was an associate member at the Fox Chase Cancer Center from 1996 to 1999, and now works as an investigator at the Laboratory for Population Genetics at the National Cancer Institute in Bethesda, Maryland. The major goal of his laboratory is to complement the existing and historic investigations of the tumor genome by characterizing the impact of constitutional genetic polymorphism on metastatic progression. Using the polyoma middle-T transgene-induced mouse mammary tumor model, he demonstrated that the genetic background upon which a tumor arose significantly influenced the ability of the tumor to form pulmonary metastases. Quantitative trait genetic mapping analysis revealed the presence of a metastasis efficiency locus, designated Mtes1, on a mouse chromosome region orthologous to human chromosome 11q12-13. Subsequently, he identified the signal transduction gene Sipa1 as a probable candidate for this locus. These results are the first evidence suggesting that constitutional polymorphism, in addition to somatic mutation within the tumour epithelium, plays a significant role in determining metastatic efficiency, having profound implications for cancer prognosis and clinical management.

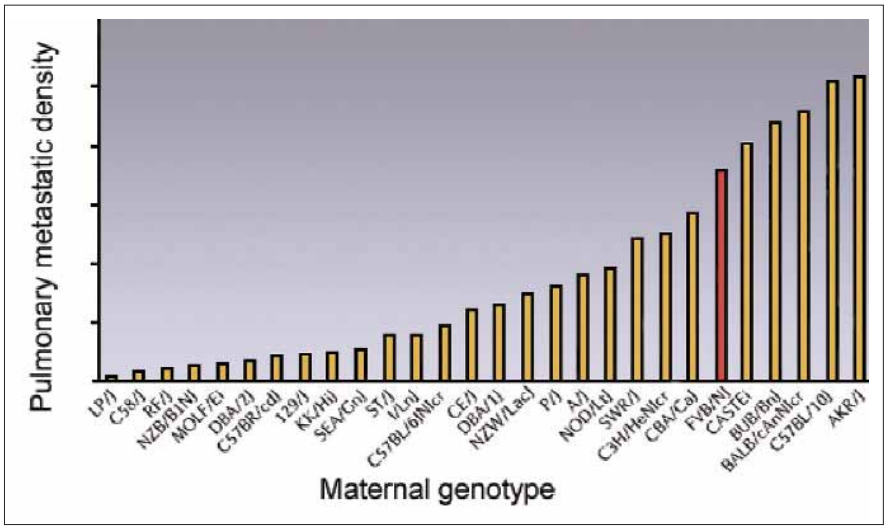

Figure 1.
The effect of maternal genotype on the metastatic capacity of a polyoma middle-T antigen-expressing tumor. *Male mice that express the polyoma middle-T antigen were bred to females from various different inbred strains (x axis), and the density of pulmonary metastases quantified (y axis) in the transgene-positive F1 female progeny. The red bar represents the metastatic efficiency of the polyoma middle-T antigen-expressing tumor in the original FVB/NJ homozygous inbred background.*

expression profile studies in human breast cancer.[1, 5-7] By comparing gene expression in tumors from a set of breast cancer patients with either poor or good prognosis, each study identified a set of genes that was successful in predicting survival in follow-up studies. However, the predictive success of these studies was frustrated by the fact that the different sets of survival-related genes had very little in common. In-depth analysis of the original Van 't Veer data-set demonstrated that, even with a single set population, using different subpopulations to derive the metastasis predictor resulted in entirely different sets of genes, each of which could be as sensitive as the original set in determining metastatic risk.[8] This suggests that gene expression patterns are not measuring just expression of individual genes, but reflect the single-nucleotide polymorphisms that underlie both the gene-expression changes and metastatic capacity. Polymorphisms that underlie metastatic capacity and/or gene expression patterns are probably modulating entire pathways or classes of genes and examination of the published metastasis predictive gene profiles does in fact reveal common classes of genes, e.g. genes involved in the composition of the extracellular matrix (ECM).

Dangerous genes

In the PyMT model, chronic exposure to high doses of caffeine prior to the appearance of palpable mammary tumors significantly reduced both tumour burden and the appearance of lung metastases. When caffeine exposure began after the appearance of frank tumors, caffeine suppressed metastasis without changing primary tumor burden. Gene and protein expression patterns resulting from caffeine treatment showed that metastasis suppression may be associated with up-regulation mRNA expression of multiple extracellular matrix genes.[9] Hunter discovered that in PyMT mice several relevant quantitative trait loci are involved in modification of metastasis efficiency. Of these, the locus Mtes1 was most extensively studied, ultimately resulting in identification of signal-induced proliferation-associated gene 1 *(sipa1)*.[10] Preliminary data show that polymorphisms in the human variants of this gene indeed appear to be associated with poor outcome of human breast cancer. Manipulation of sipa1 mRNA levels in PyMT mice using siRNA technology, revealed that subtle variations in cellular *Sipa1* protein have dramatic effects on the number of lung metastases.

Recently, a second interesting gene was discovered. Enforced expression of *anakin* gene downregulates expression of ECM gene *Col5a3* hundredfold en strongly suppresses tumor growth and metastasis. Unpublished data in-

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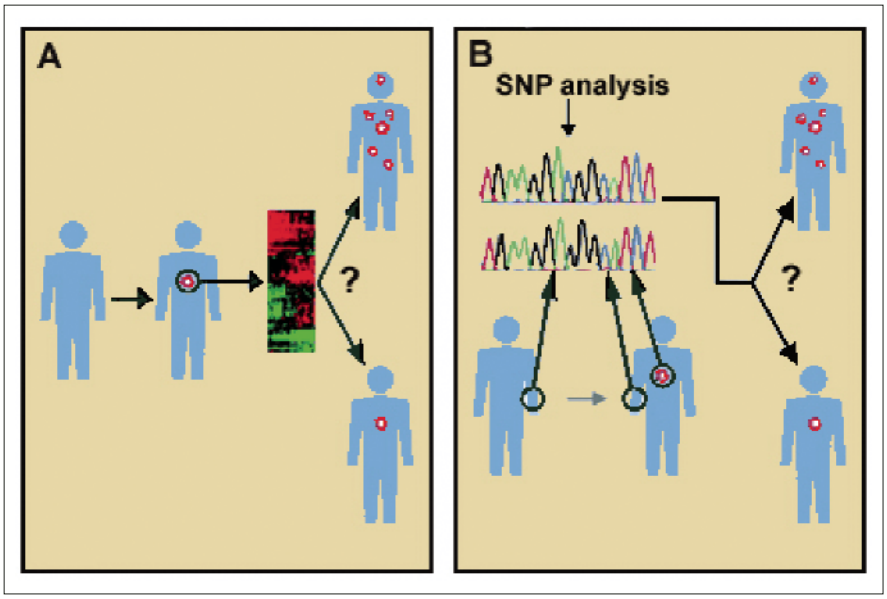

Figure 2.
Implication of germ line polymorphism versus somatic mutation for metastasis risk assessment. A: *for somatic mutation, tumor formation must have occurred to permit the isolation of tissue required for assay.* **B:** *by analysing germ line polymorphism, risk assessment can be made using any tissue, including blood, at the time of diagnosis, or even before diagnosis of primary tumor.*

dicate that human *anakin* is strongly associated with the presence of metastases (and not with tumor size) and that a single nucleotide polymorphism (SNP) in *anakin* is a predictor of survival. It is tempting to speculate that the products of *sipa1* and *anakin* genes function in the same pathway.

Are these germline polymorphisms responsible then for controlling the entire metastatic process? Probably not. Recent groundbreaking studies have demonstrated that gene expression signatures arise in subpopulations of metastatic cells that mediate metastases specifically to the lung [11] or to the bone.[12]

The ethics of genetic screening

If germline variation in the form of SNPs can be shown to modify metastatic efficiency in humans to a similar extent as they do in mice, then gene expression profiling might well be replaced as a prognostic tool by a panel of predictive SNPs. This approach would be preferable in that SNP-based assays have far less laboratory-to-laboratory variability and SNPs can be typed in any tissue, including blood (Fig. 2). As a consequence, it might be relatively easy to identify those at increased risk of both cancer and subsequent metastasis, and to enroll such individuals in chemoprevention regimens. In fact, the above mentioned treatment of PyMT mice with caffeine before and after the development of palpable mammary tumors demonstrated the feasibility of this approach.

This approach, however, also raises a serious ethical question. If there is no effective way to deal with metastasis in certain cancers, should patients be made aware at diagnosis that they are at high risk for disseminated disease? In many patients, this knowledge will probably add significantly to long-term stress and anxiety. Being at high risk is absolutely no guarantee that one will indeed develop life-threatening secondary lesions, but may open up the potential for genetic discrimination in the healthcare system.

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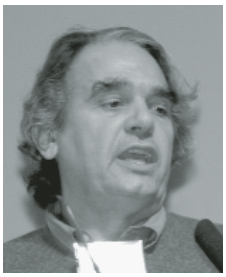
Watch out for tiny RNAs!

C.M. Groce, M.D., Ph.D., Ohio State University, Columbus, OH, USA



The name Carlo Croce is linked to many milestone discoveries. The demonstration that a chromosome translocation in Burkitt lymphoma cells join an immunoglobin gene on chromosome 14 to the *myc* oncogene on chromosome 8, was one of the first examples showing that cancer is caused by genetic damage. Croce's method of 'chromosome walking' enabled him and others to identify many new and wholly unrecognized cancer-related genes. In 1984 his research group identified a gene that causes follicular lymphoma, a gene they named *bcl*-2 and that soon appeared the founding member of a family of proteins crucial in the regulation of apoptotic cell death. He also led research that produced the first mouse model for chronic lymphocytic leukemia (CLL), the most common type of human leukemia.
Finally, Croce's laboratory became the first to link the mysterious tiny microRNAs to cancer development.

Solving a riddle

In human CLL, Croce's group detected in about 50 percent of cases DNA deletions at chromosome 13q14. It appeared that also in mantle-cell lymphoma, multiple myeloma, and prostate cancer this region most often contains deletions and that in CLL frequently it is the sole genetic abnormality. Many years were spent hunting for the possible tumor suppressor gene involved, but in vain. Then, in the fall of 2001, *Science* contained three articles describing tiny RNA molecules that were called 'microRNAs'.

Small interfering RNAs had been discovered in roundworms in the early nineties, but now it was reported that these RNAs were also abundant in fruit flies, plants and humans. MicroRNA genes (miR genes) appear to be large family of highly conserved noncoding genes involved in temporal and tissue specific gene regulation.

After reading these *Science* papers, Croce telephoned his postdoc Calin, asking him to see if he could find miR genes in the 13q14 region. Using positional cloning, Calin indeed identified two miR genes, miR15and miR16, located with a 30-kb region of loss in CLL, and deleted or downregulated in 70% of CLL cases.[1] Later it was found that the involving microRNAs target *bcl*-2 mRNA, the product of which is a strong inhibitor of apoptosis.[2]

Tiny RNAs set the tune

The initial product of a miR gene is a hairpin-like molecule that goes through several processing steps (involving the ribonuclease Drosha) before it is exported from the nucleus to the cytoplasm. Here it is processed into an active 21-22 nucleotide RNA by the ribonuclease Dicer. One strand of this double-stranded RNA is then incorporated into the 'RNA-induced silencing complex' (RISC). RISC can target mRNAs, either for inhibition, by blocking their translation into protein, or destruction (as in RNA interference). Base pairing between the microRNA and its complementary target mRNA gives the process its specificity (Fig. 1). The choice between translational inhibition and destruction is thought to be governed by the degree of mismatch

C.M. Croce

Carlo Croce attended medical school in his native Italy and turned his growing interest in oncogenic viruses into a fellowship for the USA, were he mapped his first viral integration site on a chromosome. He arrived at the Wistar Institute in Philadelphia in 1970, and by the late 70's he was using somatic cell hybrids to identify and map many important cancer genes like bcl2, myc, all1, and tcl1. It yielded dozens of high profiled publications and a nickname: 'gene hunter'. In the 80's he was both associate director of Wistar and Professor of Human Genetics at the University of Pennsylvania. In 1988 he became the director of the Fels Institute for Cancer Research at Temple University School of Medicine. He joined Thomas Jefferson University in 1991 as Director of the Kimmel Cancer Institute, a position he held until moving to Ohio State University in 2004 where he has been leading the human cancer genetics program. Croce is a member of both the Italian and American National Academies of Sciences and has over 700 publications to his name. He is also linked to the first mouse model for chronic lymphocytic leukemia, which is nearly identical to human CLL. More recently he defined the role that microRNAs play in cancer development.

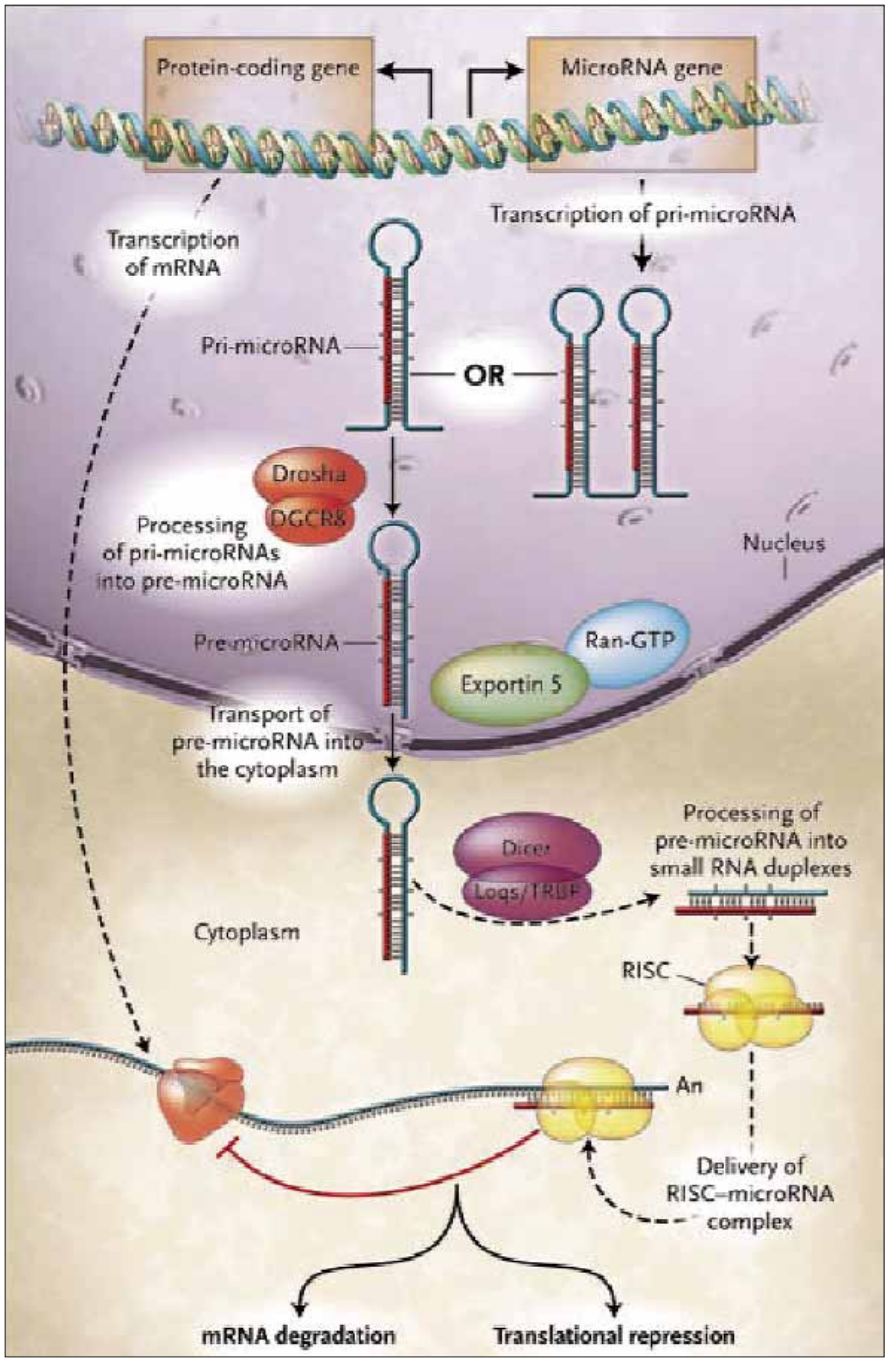


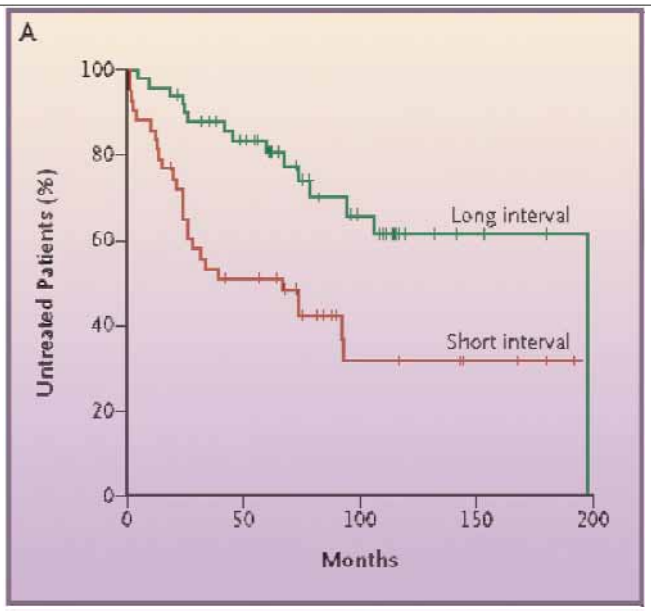
Figure 1. Biogenesis of microRNA and microRNA-mediated gene regulation in animal cells.

Mature functional microRNAs of approximately 22 nucleotides are generated from long primary microRNA (pri-microRNA) transcripts. First, the pri-microRNAs, which usually contain a few hundred to a few thousand base pairs, are processed in the nucleus into stem-loop precursors (pre-microRNA) of approximately 70 nucleotides by the RNase III endonuclease Drosha and its partner Pasha, a homologue of the human DiGeorge syndrome critical region gene 8 (DGCR8). The pre-microRNAs are then actively transported into the cytoplasm by exportin 5 and Ran-GTP and further processed into small RNA duplexes of approximately 22 nucleotides by Dicer RNase III enzyme and its partner Loqacious (Locs), a double-stranded RNA-binding-domain protein that is homologue of the human immunodeficiency virus transactivating response RNA-binding protein (TRBP). The functional strand of the microRNA duplex is then loaded into the RNA-induced silencing complex (RISC). Finally, the microRNA guides the RISC to the cognate messenger RNA (mRNA) targety for translational repression or degradation of RNA.

between the microRNA and its target mRNA, with degradation being the outcome for best-matched targets. Because microRNAs can inhibit the translation of imperfectly matched targets, it is possible that each microRNA may target multiple genes, and that several microRNAs may regulate a given target. The interplay between mRNA and microRNA is vital for the normal regulation of gene expression and the expression of microRNAs appears to be highly regulated according to the cell's developmental lineage and stage. Each type of cell is likely to have a specific microRNA milieu, a unique set of microRNAs dampening the translation of thousands of mRNAs. It has been estimated that no less that one third of our genes is regulated by microRNAs.

Superior tools

Given this global effect on gene expression, one would expect a general involvement in human cancers. Application of methods for high-throughput microRNA anaysis certainly identified



B

MicroRNA	Level of Expression of MicroRNA	
	Short interval	Long interval
miR-181a	High	Low
miR-155	High	Low
miR-146	High	Low
miR-24-2	High	Low
miR-23b	High	Low
miR-23a	High	Low
miR-222	High	Low
miR-221	High	Low
miR-29c	Low	High

Figure 2.
Relationship between the level of expression of microRNA and the time from diagnosis to initial therapy in patients with chronic lymphocytic leukemia.
*Kaplan-Meier curves in Panel **A** depict the proportion of untreated patients with CLL according to whether the interval from diagnosis to therapy was short or long. In Panel **B**, the patients are grouped according to the level of expression of nine microRNA genes (p<0.01).*

cancer-specific microRNA fingerprints in every type of analyzed cancer (Fig. 2).[3,4] MicroRNA profiles appeared surprisingly informative, reflecting developmental lineage and differentiation state of the tumors and a general downregulation of microRNAs in tumors compared with normal tissues. As might be expected from the role of some microRNAs in development, the microRNA profiles of tumors are in accord with the tumours' developmental history; tumors derived from tissues with a common embryonic precursor (such as gastric, colon and liver cancers, which are all derived from the embryonic endoderm) share similar microRNA expression patterns. Leukaemias are clearly separate from solid tumors and, strikingly, are subgrouped according to their underlying genetic abnormalities A large scale 'miRNome' analysis by Croce's lab on 540 samples including lung, breast, stomach, prostate, colon and pancreatic tumors identified many overexpressed microRNAs with targets supporting their function as either dominant or recessive cancer genes.[5]

In contrast to mRNA profiles, microRNA profiles are highly accurate when applied to the same samples. Thus, microRNA profiles are probably more effective in classifying human cancers and predicting their developmental

origins than mRNA microarrays that contain more than 16,000 protein coding genes.[6]

New therapeutics

Along with Stefan Costinean, Croce decided to isolate and study the miR155 gene in the above mentioned mouse model. Transgenic mice with overexpression of miR155 developed a lymphoproliferative disease resembling the human diseases, thus strongly suggesting that even one single miR155 can act as an effective oncogene.[7] Subsequently, overexpression of miR155 was observed in solid tumours such as breast, lung, and colon cancer. Particularly in lung cancers, overexpression of miR155 was an indicator of bad prognosis.[8]

Recently, Krutzfeldt and coworkers succeeded in engineering a novel class of oligonucleotides they termed 'antagomirs'.[9] These cholesterol-conjugated single-stranded RNA molecules are 21–23 nucleotides in length and complementary to mature target microRNAs. In mice, one intravenous injection of a specific antagomir silenced miR-122 in for more than a week, resulting in upregulated expression of hundreds of genes predicted to be repressed by miR122, because these genes had a miR-122 recognition motif in the 3' untranslated region. Antagomir treatment also revealed a significant number of downregulated genes that may be activated) by miR-122, e.g. by suppression of a transcriptional repressor.

Clearly, these antagomirs provide a powerful tool to further investigate the role of miR genes in cancer. Croce's study suggests that antagomirs might block miR155 expression and be an effective therapeutic strategy in patients with acute lymphoblastic leukemia or high-grade lymphoma, and possibly solid tumor types as well. Moreover, virtually all anticancer drugs used today are never totally specific. MicroRNAs fine tune gene expression in very critical pathways, so hopefully this research may lead to highly specific therapeutics.

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Micro's make a difference in testis cancer

L.H.J. Looijenga, Ph.D., Erasmus MC, Rotterdam, The Netherlands

Compared to tumors originating from somatic cells, germ-cell tumors (GCTs) tend to be highly sensitive to chemotherapy and/or irradiation. Recent research has been starting to provide clues about the unpredictable development of GCTs and their exquisite treatment sensitivity. Focusing on testicular germ cell tumors of adolescents and adults, research groups from The Netherlands Cancer Institute in Amsterdam and the Josephine Nefkens Institute in Rotterdam have been placing microRNAs in the spotlight.

Totally different view

GCTs can occur both in ovaries and testes and in different extragonadal sites along the midline of the body, including the midline of the brain. This curious anatomical distribution is likely related to the migration route of primordial germ cells (PGCs) during embryogenesis. Whereas GCTs traditionally are categorized in an organ-oriented way, recent clinical and experimental data reveal that, in fact, they constitute one single disease, with separate entities that can manifest themselves in different anatomical sites.[1]

An important phenomenon in germ-cell development is genomic imprinting: paternal and maternal sets of chromosomes have different functionality due to parental-specific epigenetic modification of the genome, e.g. by DNA methylation and histone acetylation. Somewhere between the stage of a PGC and a spermatozoa or an oocyte, the originally biparental pattern of genomic imprinting, present in the zygote, has to be erased, so that either a paternal or a maternal pattern is established. In GCTs, the status of imprinting has proven extremely useful in identifying the cells of origin and in explaining their developmental potential. Based upon maturation stage and imprinting status, Oosterhuis and Looijenga propose a classification in five GCT entities, an approach entirely different from the standard WHO-classification.

Within the testis, three separate entities of GCT are identified: the teratomas and yolk sac tumors of neonates and infants, the seminomas and nonseminomas (referred to as testicular germ cell tumors of adolescents and adults: TGCT), and the spermatocytic seminomas of elderly. These different types of testicular GCT are characterized by histology, clinical behavior, and chromosomal aberrations.

P53 poses a problem

TGCT constitute the most common malignancy in Caucasian males aged between 15 and 45 years and the incidence is still increasing. Certain families have an increased risk for TGCTs, but these are of small size, indicating complex genetics. TGCT are also highly curable, i.e., they are overall exceptional sensitive for irradiation and/or platinum-based chemotherapy.

Only a limited number of TGCT is resistant to these modalities. A crucial factor in sensitivity to irradiation and DNA-damaging anticancer drugs is the P53 protein, a transcription factor that becomes active as a response to a variety of cellular stress factors, including DNA breaks and mitogenic signals from oncogene products, such as

L.H.J. Looijenga

Leendert Looijenga studied biology at the University of Groningen and graduated cum laude with a specialization in medical cell biology in 1989. Until 1990, he worked as a visiting scientist at the Department of Anthropogenetics, under the supervision of Wolter Oosterhuis and Peter Devilee. In 1990, together with Oosterhuis, he started the Laboratory for Experimental Patho-Oncology at the Daniel den Hoed Cancer Center in Rotterdam. Four years later he defended his thesis, Pathobiology of germ cell tumors of the adult testis: views and news, at the Erasmus University Rotterdam, and became an initiating scientist and staff member. In September of 1998, the laboratory moved to the Josephine Nefkens Institute. The research is focused on the pathobiology of germ cell tumors, in particular those of the (adult) testis, and possible clinical applications. Looijenga was visiting professor at the La Jolla Cancer Foundation (USA) and the Umea University (Sweden) and in 2005 became Honorary Professor of Translational Patho-oncology at the Erasmus University.

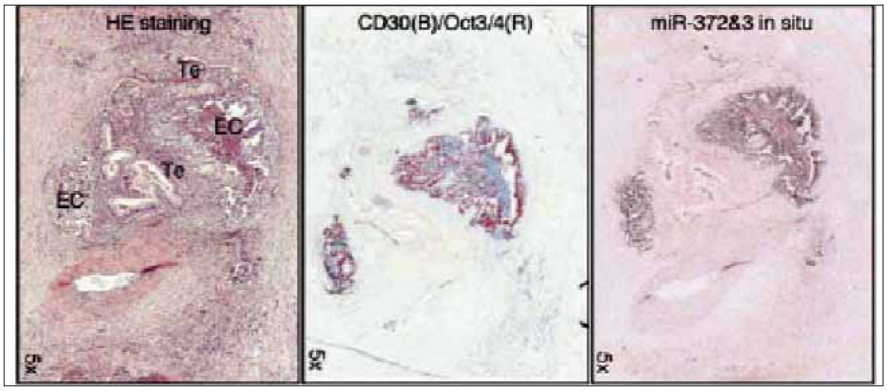

Figure 1.
In situ hybridization on a nonseminaoma of mixed histology to detect miR-372&3 expression. The embryonal carcinoma (EC) component of the tumor was morphologically determined using the haematoxylin/eosin (HE) staining and by immunohistochemistry with anti-CD30 and OCT3/4 antibodies of the next tissue sections. (Te: teratocarcinoma).

mutated RAS-protein. By inducing an arrest of the cell cycle, P53 allows the cell to repair the damage; if too much DNA damage has occurred, or in case of continuous RAS-signaling, it will induce apoptosis or an irreversible growth arrest (cellular senescence). Inactivation of P53 by gene mutation and loss of heterozygosity is most often not only involved in tumorigenesis, but, it is also a hallmark of resistance to irradiation and DNA-damaging compounds.

TGCTs tend to have a high level of wild-type P53 protein and in unselected TGCTs, *p53* mutations have hardly been identified. However, Kersemaekers found no difference in chemosenstivity between TGCT-derived cell cultures with and without functional P53. Moreover, inactivation of P53 in a cisplatin-sensitive TGCT-derived cell line did not make these cells resistant. Also, the amount of P53 in TGCT tumor samples did not correlate with treatment results.[2] These remarkable findings have now been clarified by genetic screening for microRNAs.

Hunting for microRNAs

In order to identify novel functions of microRNAs, Voorhoeve and coworkers developed a genetic screening system, using the majority of cloned human microRNAs and based on the bar-code system described in chapter one. Being particularly interested in microRNAs that cooperate with the ras oncogene in cellular transformation, they used primary human fibroblasts transfected with the ras oncogene. The assay identified miR-372 and miR-373: these microRNAs permitted proliferation and tumourigenesis in cells that harbor both oncogenic RAS and active wild-type P53 protein, i.e., the cellular response to DNA damage remained completely intact, but somehow the

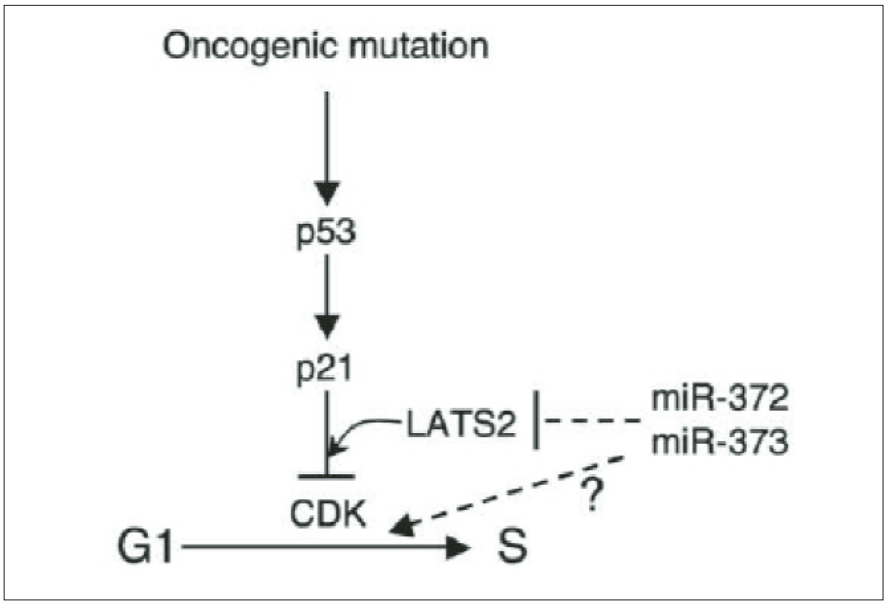

Figure 2.
A schematic model showing the mechanism through which miR-372&3 can suppress an oncogene-activated p53 pathway.

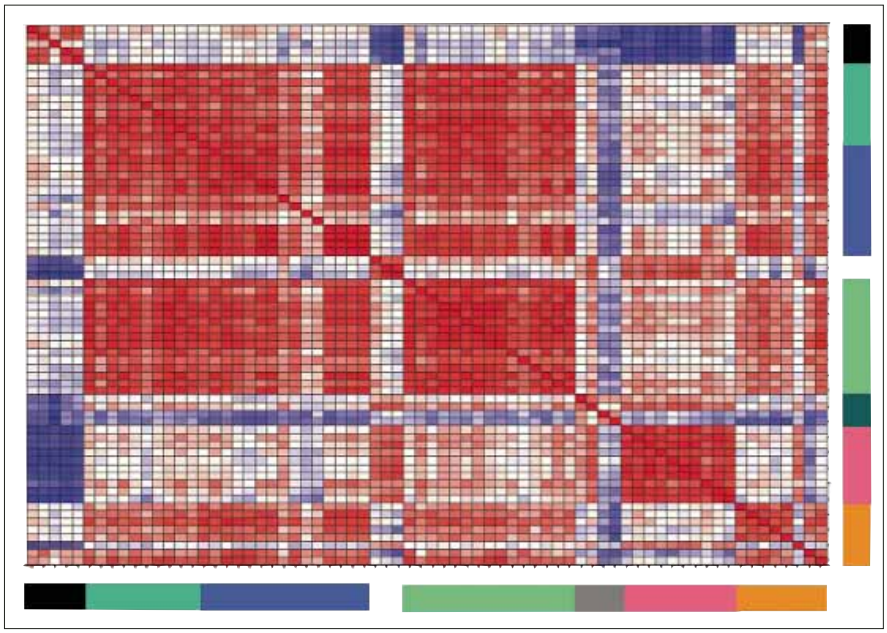

Figure 3.
Correlation plot (Peason's coefficent) of the various types of germ cell tumors and normal testis (indicated in different colours) based on the expression of 157 miRNAs. Note the specific clustering of the various entities.

senescence-inducing effect of P53 in the context of RAS-signaling was circumvented in these cells.[3] Would it be possible that miR-372 and miR-273 play a role in tumor types that retain wild-type P53 and, as a result, remain sensitive to DNA-damaging treatments?

Numbing the P53 pathway

Here TGCT enter the stage. Whereas in somatic tumors expression of miR-372 and miR-373 is seldom observed, most seminomas, and about two thirds of non-seminomas were found to (over) express miR-372 (Fig. 1) (whereas spermatocytic-seminomas and normal testis were negative). In non-seminomas and microRNA 372&373 expressing seminomas, no *p53* mutations were detected, but two out of four microRNA-372-negative seminomas had an inactivating mutation in the *p53* gene. Thus, expression of miR-372 and miR-373 suppresses the P53 pathway to an extent sufficient to allow oncogenic mutations to accumulate in TGCTs. Further experiments revealed that miR-372 and miR-373 regulate the expression of a protein (LATS2) that is part of the cell cycle machinery-inhibition pathway activated by P53: inhibition of LATS2 abrogates a brake on the cell cycle (Fig. 2). The function of miR-372 and miR-373 in embryonic stem cells probably is facilitation of rapid growth, but their deregulation predisposes cells for accumulation of carcinogenic events. Based on these findings, a high through-put expression analysis of miRNAs has been initiated, revealing histology-specific profiles (Fig. 3), which is currently under investigation.

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Lack of air makes tumors metastatic

P.M. Comoglio, M.D., Ph.D., IRCC, Torino, Italy

What started as a gut feeling is becoming a consistent concept; invasive growth and metastasis are not mistakes of Mother Nature, but a physiological programme, used in organ formation during embryonal development, and, in the adult, used for wound healing and regeneration. There is increasing evidence that this program is executed by (cancer) stem cells and progenitor cells. The proto-oncogene *met* has found to be a key regulator: this tyrosine-kinase receptor senses adverse microenviromental conditions (such as hypoxia) and drives invasion and metastasis by the activation of genes that control blood coagulation. Is Met (the product of the protooncoogene *met*)[1] an ideal target for the development of clinically relevant metastasis inhibitors?

Invasive growth in normal development

During embryonic development, the transformation of the flat, two-layer germinal disk into a three-dimensional organism depends on the transient conversion of some cells from an epithelial to a mesenchymal phenotype, i.e., cells with a spindle-shaped morphology and motile behaviour. This epithelial-mesenchymal transition (EMT) is followed by ordered cell migration and the morphogenesis of new structures (Fig. 1). The same process can be reactivated in post-natal life during tissue repair and organ regeneration.

Two sets of molecules rather selectively control this transition: scatter factors and semaphorins (the latter being closely related to the structure of scatter factor receptors.)

Scatter factor 'hepatocyte growth factor' (HGF) was independently identified as a platelet-derived mitogen for hepatocytes and as a fibroblast-derived factor capable of inducing epithelial cell dissociation and motility ('scattering').

During the early phases of development, HGF and its receptor Met are co-expressed in stem and progenitor cells, which generates self-stimulating circuits in cells of the endoderm and the mesoderm. HGF-Met signaling has a role in the migration and positioning of mesoderm cells through the primitive streak. During organ genesis, HGF is produced by cells of mesenchymal origin, whereas the receptor is expressed by the adjacent cells of endodermal origin, or myoblasts. As has been observed in knock-out mice lacking HGF or Met, HGF-Met signalling is an absolute requirement for development of the placenta and for the migration of muscle-forming myoblasts from the somites to the limbs. Furthermore, HGF stimulates tubulogenesis in the liver and kidneys during organ regeneration and promotes differentiation of the ductal tree in the mammary glands during early pregnancy.

Met-switch starts the program

Cancer cells hijack the strategies by which the embryo grows and develops (Fig. 1). Morphogenesis and metastasis seem to arise from the same genetic program that instructs cells to detach from a primary colony, cross tissue boundaries, adhere to and migrate through extracellular matrices, and, most importantly, to escape death

P. M. Comoglio

Paolo Comoglio graduated in Medicine at the University of Torino, Italy, in 1969, and studied Immunology with Herman Eisen at the Washington University in Saint Louis. In 1973 he investigated the biological properties of cancer cells with Leonard Warren at the University of Pennsylvania in Philadelphia. Returning to Italy in 1974, he became Associate Professor and then, in 1980, Professor of Histology at the University of Trieste Medical School, Italy. In 1983, he moved to the University of Torino Medical School. He has been Director of the Division of Molecular Oncology at the Institute for Cancer Research and Treatment in Torino since 1996. He was then appointed Scientific Director of the same Institute in January 2000.

Comoglio reached a significant milestone of his scientific activity when he developed new technologies for the study of oncogenes encoding tyrosine kinases (anti-phosphotyrosine antibodies and related technologies). By the end of 80's, through these tools, he identified the Scatter Factor Receptors encoded by the oncogenes met and ron. More recently, his group discovered a family of genes encoding Plexins, the transmembrane receptor for Semaphorins, and demonstrated that these molecules cooperate with Scatter Factor Receptors in the control of cancer progression and metastasis.

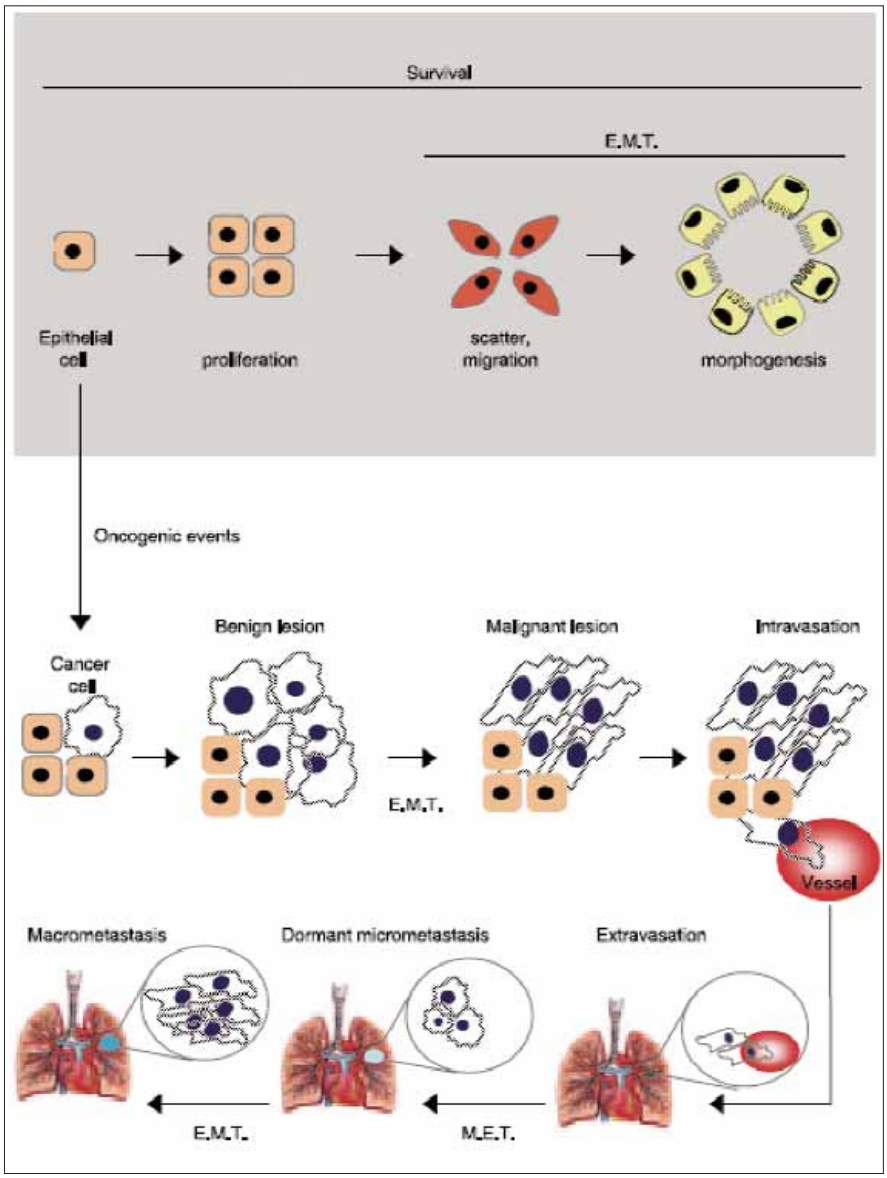

Figure 1.
Invasive growth is a physiological program aberrantly activated during cancer progression. *Invasive growth is a physiological program that results from the integration of different biological activities, including cell proliferation, survival, cell-cell dissociation ('scattering'), migration, invasion, and morphogenesis. Normally occurring in development and adulthood for the generation and maintenance of organ complexity and architecture, it is aberrantly recruited by cancer cells during tumor progression. By acquisition of genetic alterations, a normal epithelial cell is transformed into a cancer cell. Through a mechanism known as epithelial-mesenchymal transition (E.M.T.), cancer cells acquire a metastatic phenotype. Metastatic cells reach a secondary site via blood or lymphatic vessels. Strikingly, in the established metastases, the EMT process could be reverted through a mesenchymal-epithelial transition (M.E.T.). After this reversion, the metastasis - in a loop fashion - can undergo another EMT, giving rise to a lesion, which is often significantly different from the primary tumor.*

caused by an unfamiliar tissue context *(anoikis)*. Invasive growth involves distinct, sequentially activated events: how do these emerge from HGF-Met signaling? Where is the specificity?

Extensive studies by Comoglio's research group revealed that, apart from the possibility that specificity occurs from the strength of a signal or its duration, it likely results from cooperation with other specific transmembrane receptors. These different receptors tend to cluster, forming 'receptosomes' that at the intracellular domain attract a variety of proteins. For example, the tyrosine kinase-activity of Met activates Ras-protein, that transiently stimulates a cascade of kinase activities that initiate the cellular proliferation machinery. But after binding of Met to Plexin B1 and integrin$\alpha 6\beta 4$ receptors, an entirely different kinase cascade is being activated, providing sustained signals for invasion and morphogenesis. In this context, the integrin$\alpha 6\beta 4$ receptor provides a docking platform form for multiple intracellular subunits with different structures and functions.[2] Thus, no special receptors are required here, but a combinatorial assembly of multiple public signaling pathways in a way that a special combination of these pathways delivers a private meaningful message. Subsequently Comoglio's lab provided data that this message evokes transcription of a large variety of genes

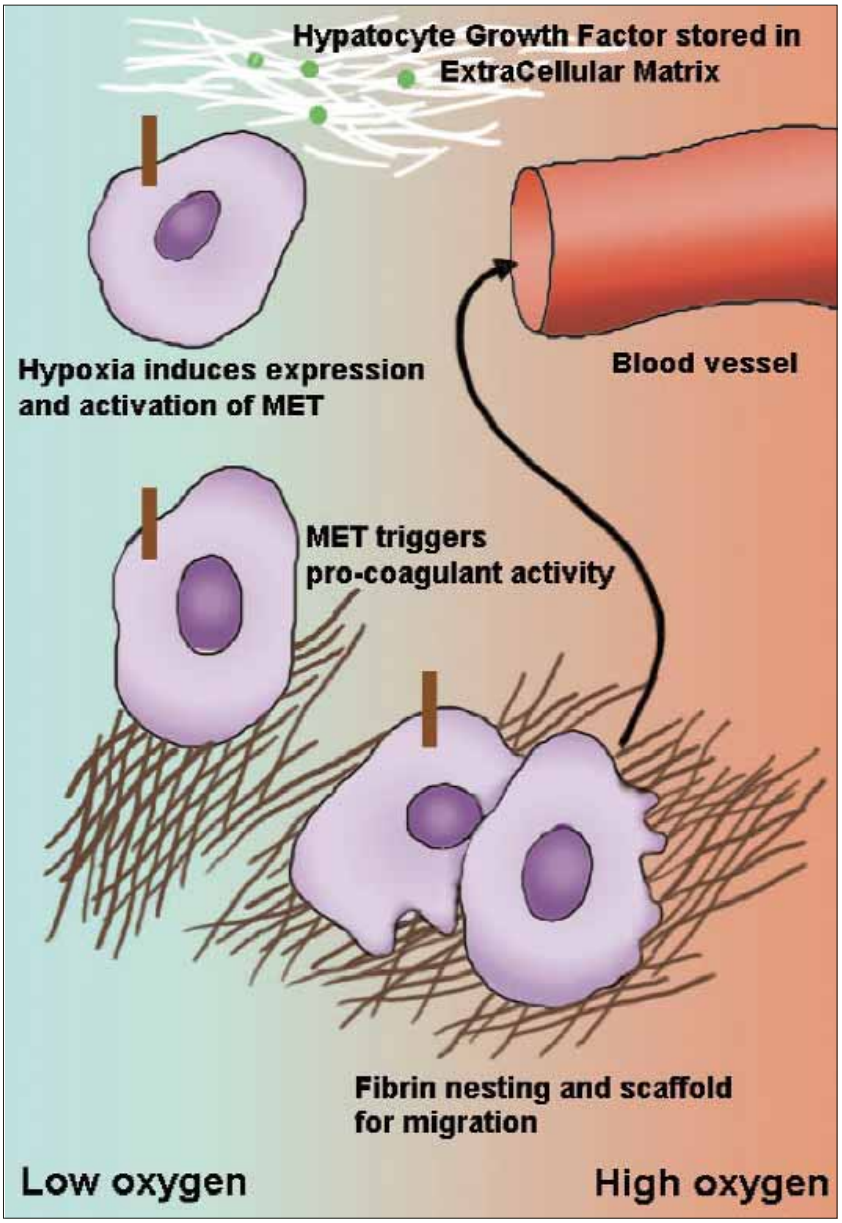

Figure 2.
MET drives the journey from hypoxia to normoxia (passing through a fibrin trail). *Hypoxia promotes MET expression and synergizes with HGF in inducing the invasive-growth program. Following MET-triggered upregulation of haemostasis genes, cells foster pro-coagulant activity ending in fibrin deposition (nesting). Fibrin provides a provisional matrix that attracts new vessels and provides anchorage for cell growth and migration.*

(the '*met*-transcriptome'). In a clonal cell line of hepatocyte progenitor cells in which HGF induces a branching morphogenesis, microarray analysis revealed changes in the expression levels of no less that 250 genes. Responses could be immediate (genes that are being translated into transcription factors that target other genes), delayed, late (comprising virtually all genes relevant for invasive growth: matrix metalloproteinases, collagens, cytoskeletal proteins, contractile proteins, etc), biphasic (genes being turned on and off in a biphasic way), and permanent.

Cells grasping for air make Met

Considering its crucial role in invasion, it may not be a surprise that Met is overexpressed in many cancer types, including hepatocarcinomas, gastrinomas, and carcinomas of the pancreas, stomach, prostate, ovary and breast. Although extremely rare, *met*-activating germline mutations are found in patients with hereditary kidney cancer. Somatic *met* mutations are associated with increased aggressiveness in various carcinomas, but the detection of these mutations is a rare event: *met* gene amplifications or tyrosine kinase activating point mutations have been observed in only 66 out of 1078 case studies. Interestingly, tumors harbouring met mutations tend to be extremely aggressive. Unpublished

data show that from 25 patients with disseminated cancer, but with a primary cancer too small for detection, some indeed harbored a *met* mutation.

Autocrine and paracrine mechanisms of Met activation are seen in tumors that are derived from mesenchymal cells which physiologically produce HGF. But Met is most frequently overexpressed in the absence of autocrine HGF production (in fact HGF is normally ubiquitously present in the extracellular matrix and therefore no limiting factor.

What then regulates *met* expression? How is *met* switched on in cancers?
As a rule, met is turned on by a hypoxia-driven mechanism.[3] Oxygen sensing is based on the function of hypoxia inducible transcription factor HIF and the promoter of the *met* oncogene contains no less than five HIF-responsive elements. In tumor sections, antibodies that specifically stain activated HIF target the same cells that are strongly stained by antibodies to Met. So, in most tumors, Met expression is almost exclusively restricted to hypoxic areas. Extracellular HGF tends to be present in sufficient amounts to induce an EMT; the HGF-Met interaction mobilizes epithelial cells to look for fresh air!

In vivo, tumor adaptation to hypoxia is a complex phenomenon. It includes the induction of genes that affect the microenvironment, such as genes coding for angiogenic factors. Treatment modalities aimed at deprivation of blood supply therefore may have a grim side; besides the desirable tumor necrosis, anti-angiogenesis treatment may induce a dangerous tumour invasive switch![4]

Blood-curdling discovery

The idea was to construct an unconventional mouse tumor model for liver cancer in which *met* is overexpressed exclusively in slowly-proliferating stem cells. For that purpose, *met* was delivered by a lentivirus that targets non-dividing cells. Unexpectedly, in addition to causing liver cancer, the presence of *met* in the liver also caused thrombosis: intravascular coagulation was even observed prior to the clonal expansion of the *met*-transformed cell clones. It was noted that this phenomenon closely resembles a syndrome, described 150 years ago by the French physician Trousseau, namely, the increased propensity of blood to clot as a sign of occult malignancy.[5]

Expression analysis of 20.000 genes subsequently provided evidence for a strong upregulation of the plasminogen activator inhibitor-1 enzyme and Cyclo-oxygenase2 - both being key players in blood clotting cascade. Pharmacological inhibition of these proteins prevents both coagulation disorders and cancer progression. The induction of coagulation is therefore likely to be an important effector mechanism of the invasive growth programme; pro-coagulant activity ends in fibrin deposition (nesting), which forms a provisional matrix that forms a scaffold for protection against *anoikis*, attracts new vessels, and provides anchorage for cell growth and migration. Possibly cells then move towards the oxygen gradient, reach blood vessels and become metastatic (Fig 1).

Wounds that never heal

It has been repeatedly demonstrated that HGF and Met are deeply involved in processes in the very early embryo. That justifies the hypothesis that in adults they are involved in processes of tissue repair and regeneration. Chronic tissue injury causes persistent activation of stem cells and the process of tissue repair, leading to an increased risk for tumour development, which can be viewed as wounds that never heal, but keep regeneration mechanisms constitutively activated.

There is circumstantial evidence that *met* expression is important for stem cells. For example, it is a functional marker for erythroid precursor cells

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and in the villi of the gut, met expression is restricted to the bottom of the crypt, where the presence of stem cells is suspected. This concept has recently been substantiated by recent experiments in nude mice injected with tumor cells containing a *met* gene with a tetracycline-inducible promoter region. When *met* is switched off, after 45 days the lungs are clean. Switch it on and 100 percent of the mice develop metastasis foci. But switch it off at a time when cell clones are already growing in the lung, the outgrowth of these lung tumors is radically prevented. Could it be that Met is prerequisite for clonogenic self renewal?

Cross talks

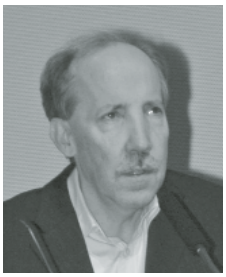
Interestingly, the *met* signalling pathway has been shown to cross talk with another pathway involving the Notch receptor.[6] Activation of the latter results in transcriptional down-regulation of *met*, suppression of HGF-dependent Ras signalling, and impairment of HGF-dependent cellular responses. But on the other hand, Met activation leads to transcriptional induction of Notch ligand Delta. Autocrine activation of the Notch receptor then culminates in activation of HES1, a strong inducer of self renewal genes. So, Met is able to self-limit its own protein levels and simultaneously starts a program that leads to sustenance of cellular stemness. It is tempting to speculate that this inhibition of met signals occurs when cellular oxygen levels are restored: the migration of the disseminated tumour cell stops and clonogenic activity blossoms at a new site.

In literature, it has been repeatedly stated that other oncogenes or growth factors, such as K-*ras* and TGFβ, are involved in the EMT as well. But although Comoglio admits he is biased, he is convinced that scatter factors and semaforins are the only *bona fide* regulators of this process.

Clearly, provided that a crucial link between cancer stem cells and Met will be definitely proven, Met could be a very attractive target for treatment. Preliminary experiments in mice with soluble decoys of Met receptors or HGF antagonists have offered proof of principle that Met-inhibitors can indeed be effective anti-metastatic agents.[7-9]

Targeting tumor lymphatics

K. Alitalo, M.D., Ph.D., University of Helsinki, Finland



The induction of angiogenesis and neovascularization is an important mechanism by which tumors promote their continued growth and subsequent metastasis. The lymphatic vascular system offers another particularly apt conduit for the spread of tumor cells, as this network of vessels drains interstitial fluid from tissues to return it to the blood, and functions as an exit route for immune cells from tissues. Lymph vessels already were described in the 17th century, but only ten years ago the research group of Kari Alitalo discovered growth factors and molecular markers specific to these vessels. Development of blocking antibodies or small molecules that interfere with lymphangiogesis could have therapeutic potential as anti-metastatic agents.

A drainage system

The lymphatic vasculature complements the blood vascular network, but rather than forming a circulatory system, the lymphatic network comprises a tree-like hierarchy of vessels that transports extravasated fluid and macromolecules unidirectionally from tissues back to the blood circulation. Unlike blood vessels, lymphatic vessels have a discontinuous or fenestrated basement membrane, lack tight inter-endothelial junctions, and are therefore permeable to interstitial fluid and cells. Through specialized anchoring filaments (e.g., fine strands of elastic fibers connecting lymphatic endothelial cells with their surrounding matrix), the lymphatic vessels stay open when the tissue pressure rises. Compared to the blood vessels, lymphatics are a low flow, low pressure system and much less coagulable due to lack of platelets and erythrocytes. They also send out fewer sprouts and are organized in a less complex network (Fig. 1A).[1,2]

The VEGF-C/D-VEGFR-3 pathway

Blood vessels in the adult are normally quiescent and require signals to growth ('angiogenic switch'). Members of the vascular endothelial growth factor-(VEGF) family and their receptors play critical roles in this switch; especially activation of VEGF receptor-2 (VEGF-R2) provides crucial signals. Because during development lymphatic vessels arise from blood vessels,[3] it may not be surprising that some angiogenic mechanisms are being conserved in lymphangiogenesis. Atali's lab discovered that VEGF-C and VEGF-D interact with VEGFR-3 in lymphatic endothelial cells, that VEGF-C and its receptor VEGF-R3 are usually co-expressed at sites were lymphatic vessels sprout, and that overexpression of VEGF-C in transgenic mice causes lymphatic vessel hyperplasia.[4]

Lymphangiogensis often accompanies angiogeneis: nascent blood vessels are leaky and lack of accompanying lymphatic growth would result in increasing tissue oedema. Nascent VEGF-C and -D bind to VEGF-R3, but this binding is enhanced by proteolytic processing of these factors. In addition, the fully processed VEGF proteins can also activate the angiogeneic VEGF-R2. Thus, both angiogeneic and lymphangiogenic signals can be co-ordinately generated from a single molecule, depending on the degree of processing and the relative expression of receptors.

K. Alitalo

Kari Alitalo obtained a MD from the University of Helsinki in Finland and after receiving his PhD in 1982, carried out post-doctoral research with Michael Bishop and Harold Varmus in San Francisco. He discovered several novel receptor tyrosine kinases and showed that these receptors and their ligands are important in tumorangiogenesis. Among his most original findings are the cloning and characterization of FGFR-4, Src, the first endothelial specific receptor Tie, as well as VEGFR-3 and VEGF-B (and identification of its receptors VEGFR-1 and NP-1). A further significant achievement was the isolation, cloning and characterization of the first lymphangiogenic growth factor VEGF-C and the isolation of lymphatic endothelial cells, opening up the lymphatic vascular system to molecular analysis after over a hundred years of descriptive pathology. Alitalo has also devised molecular therapies for lymphoedema that are now entering clinical trials. At the University of Helsinki, Alitalo leads the Molecular/ Cancer Biology Program and Centre of Excellence in the Biomedicum Helsinki Research Institute. He is (co)author of over 300 research papers, member of the Finnish Academy of Sciences, and the editorial board of the EMBO Journal. He has received several distinguished scientific awards, including the 'Europe Medicine Senior Prize'.

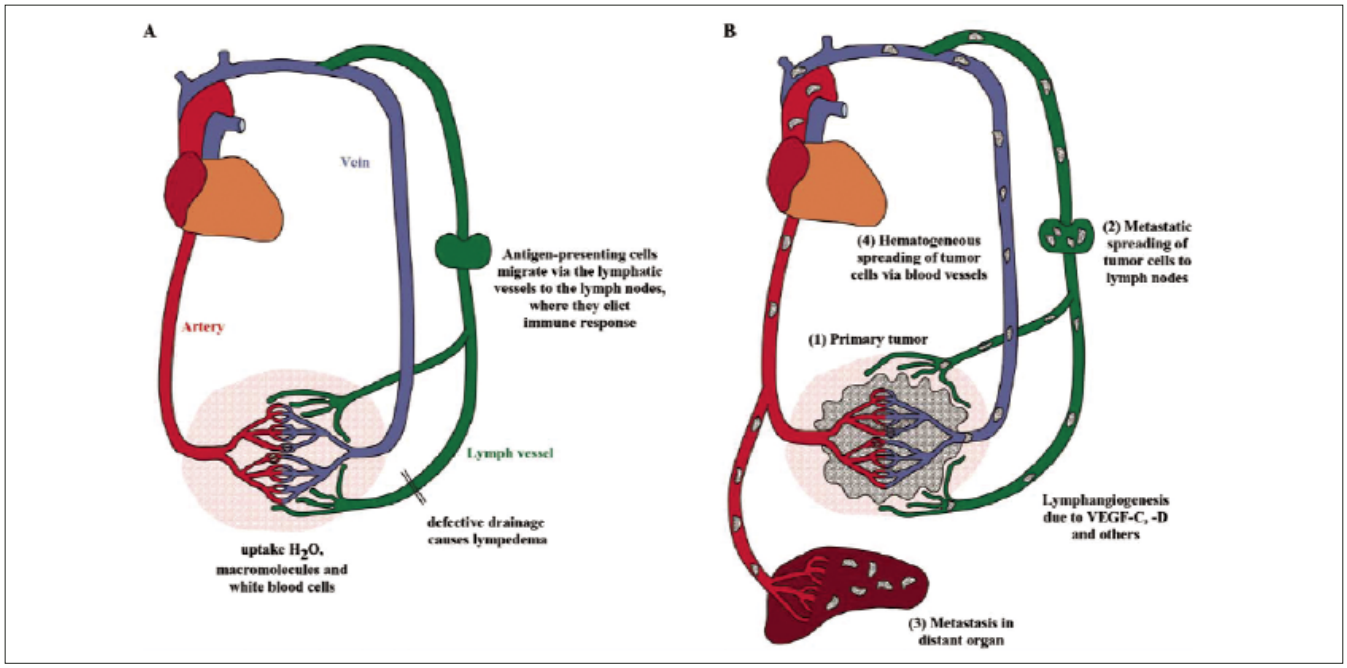


Figure 1.
Scheme illustrating the relationship between blood vascular and lymphatic system in normal health and in cancer
A: *In a healthy individual, lymphatics drain extravasated fluid, proteins, and cells to the lymph nodes and, via the thoracic duct, to the venous circulation. Immune cells such as antigen-presenting cells, patrolling through the body for foreign antigens, traffic to the lymph nodes, where they elicit an immune response. Lymphedema develops when lymphatic drainage is insufficient as a result of primary hypoplasia, surgical resection, radiation, infection (such as filariasis), etc.*
B: *In cancer, as a result of lymphangiogenesis around and possibly also within (controversial) the primary tumor (1), dislodged tumor cells drain to the lymph nodes, which represents a poor prognosis (2). Tumor cells then traffic to the vascular circulation, through which they metastasize to distant organs and kill the patient (3). Since tumors are highly angiogenic, tumor cells may also metastasize via an hematogeneous route to distant organs (4), which may explain why inhibition of lymphangiogenesis blocks lymph node but not lung metastasis in an experimental tumor model.*

A matter of balance

Tumor vascular vessels are highly disorganized, tortuous, and dilated with uneven diameter, excessive branching, shunts, and incomplete or absent muscular coverage. Their walls have numerous 'openings', widened intercellular junctions, and a discontinuous or absent basement membrane. Consequently, tumor vessels are leaky and blood flow is chaotic and variable.

When there is imbalance in the growth of both vascular systems, as is most often observed in tumors, the interstitial pressure will rise. This increased intratumoral pressure may cause complete collapse of lymph vessels; probably only lymph vessels at the tumor margin are important for spreading tumor cells. Here endothelial cells send long filopodia towards the VEGF-C producing tumor cells and then form tumor-directed vessel sprouts, where vessel lumen opens up and allows facilitated access of tumour cells to the lumen. Also the collecting lymphatic vessels, draining fluid from the tumor area, are stimulated by intraluminal VEGF-C to dilate through the process of endothelial proliferation in the vessel wall. Clumps of metastatic tumour cells could then undergo an easier transit in lymph, flowing in the dilated hyperplastic vessels (Fig. 1B).[5]

In case of lymphoedema, e.g. caused by surgery or radiotherapy for breast cancer, or in non-healing wounds, treatment with VEGF-C is likely to restore the lymphatics and preliminary clinical data are most encouraging in that respect.

Treatment opportunities

VEGF-C and VEGF-D expression correlate with vascular invasion, lymphatic vessel and lymph node involvement, distant metastasis, and, in some instances, poor clinical outcomes. Studies in mouse models have shown that VEGF-C and -D overexpression can enhance lymphatic metastases. Furthermore, a soluble VEGFR-3 fusion protein (VEGF-C/D Trap) catches VEGF-C and -D and so reduces lymphatic metastases in several models by inhibiting sprouting and vessel dilation and restoring the integrity of the vessel walls (Fig. 2).[6]

Alitalo's group tested a variety of human tumours growing as xenografts in nude mice with monoclonal antibodies that block signaling via VEGFR-3; seven out of twelve tumours showed a statistically significant inhibition of tumor growth. Moreover, recent data provided evidence that these antibodies work synergistically with chemotherapy.

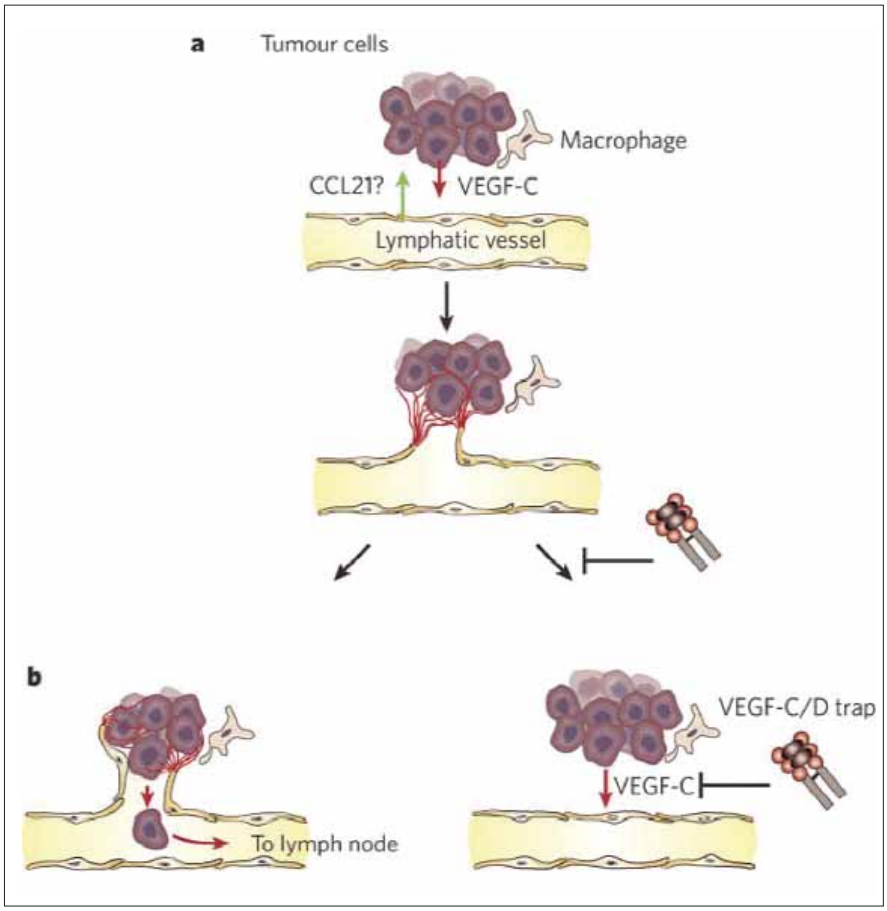


Figure 2. Role of VEGF-C/D in lymphatic metastasis in cancer

A. *Tumor cells and tumor-associated macrophages secrete lymphangiogenic growth factor VEGF-C or VEGF-D, which induces sprouting of nearby lymphatic vessels, facilitating the access of tumor cells into the vessel lumen.*

B. *Aggregates of tumor cells are transported to the regional lymph node, from which they can spread to distant organs through either blood or lymphatic vessels. Blockage of VEGFR-3 signaling with a soluble VEGFR-3 fusion protein (VEGF-C/D Trap) inhibits metastasis in most mouse tumor xenograft models by stabilizing lymphatic vessels.*

Although in adult tissues, VEGFR-3 expression is restricted to lymphatics, various types of human cancer also express this receptor in their blood vessels (probably a relict from the expression of VEGFR-3 in embryonic vasculature), suggesting that at least in some tumours, VEGFR-3 directed treatment may address both lymph and blood vessels. Is there a danger then that, as a result of increased hypoxia and concomitant Met expression (see chapter five), tumor cells will acquire a more invasive phenotype? Alitalo is not concerned about this, as the antibodies preferably block sprouting and dilating of lymph vessels.

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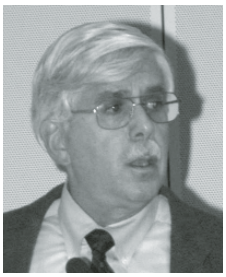
Mammary (cancer) stem cells: searching for a niche

J.M. Rosen, Ph.D., Baylor College of Medicine, Houston, TX, USA



For many years, Jeffrey Rosen has been studying critical periods in the development of the mouse mammary gland: ductal proliferation and branching that occur during sexual maturity, lobulo-alveolar proliferation that occurs during pregnancy, terminal differentiation and lactation, and involution characterized by increased apoptosis and extensive tissue remodelling. His laboratory is focussed on the role of systemic hormones and local growth factors on these processes. Studies from the laboratory of Ken DeOme performed more than fifty years ago demonstrated that transplantation of random fragments of mammary tissue into the cleared mammary fat pad of recipient mice leads to outgrowth into all cell types of a functional mammary gland. Over the past several decades, direct evidence for the existence of mammary gland stem cells has accumulated. One of the Rosen Laboratory objectives is to localize the stem cell niche(s) within the normal mammary gland and, within mammary tumors, to determine the alterations in regulatory mechanisms leading to tumor initiation.

Nagasaki

Mutations that initiate breast cancer (BC) appear to accumulate slowly in cells that persist throughout a woman's lifetime, since there is an exponential increase in BC incidence with age, and since girls exposed to excess radiation in adolescence have an increased risk of BC 20-30 years after the exposure. In fact, the best epidemiological data are from women who were as a teenager exposed to the atomic bomb at Hiroshima and Nagasaki: many of these women developed BC 30-40 years later. It is also well known that 40% of the recurrences after BC surgery occur after 10 years and that these recurrences usually are genetically identical to the primary tumor.

Another striking feature is that BC is a very genetically and clinical heterogeneous disease. Already in the ductal carcinoma *in situ*-stage (DCIS) you may see a large amount of the same genomic instability as can be observed in infiltrating BC-metastases. As there actually are a relatively small number of genetic changes going on in this stage, it seems likely that in many cases epigenetic changes are involved in the early stages of BC progression.
You can classify BC in various subtypes and these can be already present very early on. Therefore, any model of BC has to explain the appearance of these subtypes very early in progression.

Explaining breast cancer pathology

Rosen suggests a model (Fig. 1) not so different from that of suggested by Bernards (see page 10): a stem cell can go from a multipotent progenitor to a more bipotential progenitor that is committed to give rise to either myoepithelial cells or luminal cells. There are luminal progenitors that are either estrogen receptor-(ER)-negative or ER-positive and give rise to respectively ER-negative or ER-positive ductal alveolar cells. So, depending on the tumor initiating cells and the genetic events that occur, different subtypes of cancer develop. The primitive multipotent progenitor tends to give rise to ER-

JM. Rosen

Jeffrey Rosen studied chemistry and received a BA degree in 1966. His PhD research at the Roswell Park Cancer Institute, Buffalo, New York, was on the mechanism of action of glucocorticoids in lymphoid tissue, where he helped elucidate the mechanisms for glucocorticoid resistance in lymphomas. Postdoctoral studies at Vanderbilt University School of Medicine, Nashville, Tenessee, were concerned with the mechanism of action of estrogen on growth and differentiation in the chick oviduct. These involved the isolation of ovalbumin mRNA and the first demonstration of steroid hormone induction of a specific mRNA. In 1973, Rosen joined the faculty of Baylor College of Medicine and was a founder member of the first Department of Cell Biology in the USA. In a sabbatical leave during 1987-88, he participated in early studies to elucidate the mechanisms of interferon action that helped lead to the discovery of the Jak/Stat pathway. Rosen, who is the recipient of a prestigious MERIT award from the National Institutes of Health, is currently a Distinguished Service Professor and the C.C. Bell Professor of Molecular & Cellular Biology and Medicine at Baylor College of Medicine. His laboratory has authored over 195 publications and book chapters dealing with hormonal regulation of gene expression, signal transduction, normal mammary gland development, breast cancer, transgenic animal models of breast and prostate cancer, and mammary gland stem and progenitor cells.

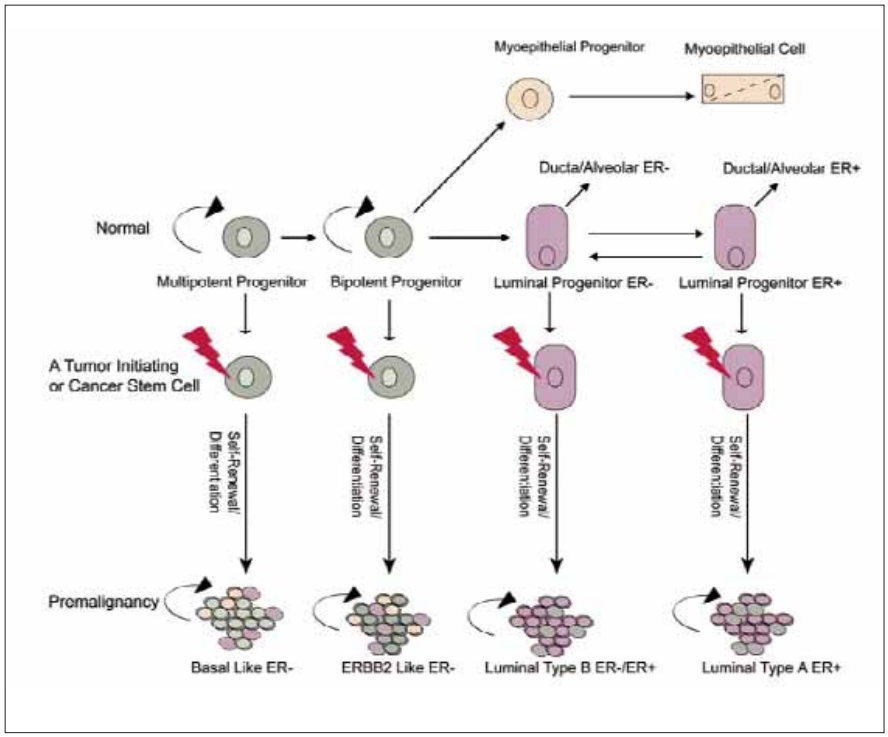


Figure 1.
A hypothetical model for breast cancer development.

negative, very aggressive cells, with the poorest outcome, while a ER-positive progenitor may give rise to the luminal subtype that responds very well to antiestrogen therapy. On contrast, a luminal subtype from an ER-negative progenitor is far less responsive.

This model does not require dedifferentiation of more mature cells (as was suggested by Bernards), but rather acquirement of a self-renewal pathway in a progenitor that normally would be committed to a specific function. Thus, the pathology of BC reflects not only the different oncogene and tumor suppressor pathways, but also the various types of progenitors in which the genetic changes occur.

Searching breast cancer stem cells

In stem cell research, two classic assays are used to provide a proof of principle. Adapting a technique pioneered in studies of leukemias, solid tumors are dissociated to single cells and then stained for specific (cell surface) markers and sorted with a FACS-machine. Small subfractions of the original cell population are then transplanted into the cleared mammary fat pad of mice. About one in 2000 cells will generate a normal ductal outgrowth and, as you get the animal pregnant, this outgrowth will form lobular structures. This is easy to do in syngeneic animals, but extremely difficult to do with clinical material.

The other assay works with mammospheres: you culture mammary cells in suspension on a nonadherent substratum in a serum-free medium enriched with growth factors and subsequently count the generated cell colonies that have a minimal size (spheres). It's a tricky measure of self renewal, as you must be aware of spontaneous aggregation of the suspended cells.

Until recently, the prospective identification of the real tumor-initiating cells, i.e. cells giving rise to *all* components of a heterogeneous tumour, had remained elusive. But Clarke and coworkers have now used cell-surface markers to isolate from the bulk of human breast tumor cells a subpopulation that, compared to the other cells, have a hundred times increased ability to form tumors when injected into the cleared mammary fat pad of immunedeficient mice. These cells are positive

for marker CD44 and negative for CD24 (in mice, CD44 being replaced by CD29).[1] Still, in the hands of Rosen these cells are not completely quiescent: more than 95% of these cells are in cell cycle. So, the really quiescent stem cell has not yet been identified!

Keeping stem cells quiescent

Based upon a model first proposed by Phil Beachy and colleagues, in most adult tissues *bona fide* stem cells are normally kept quiescent. They simply do not divide, unless there is some kind of inflammatory response or tissue injury or a specific activation program; only then these stem cells exit their niche and start to proliferate. In a regeneration response, this trigger eventually fades away, but things are different in case of chronic stress: persistent activation of these pathways can lead to a population of activated cancer stem cells and subsequent genetic alteration in the latter may give rise to malignancy.

What keeps these primitive stem cells quiescent is their (not yet very well defined) niche environment. A crucial factor is adhesion between stem cells and surrounding cells or extracellular matrix molecules. Studies on this subject in *Drosophila* revealed an important role for the presence of adhesion molecules, such as cadherins and α- and β-integrins. Decreased adhesion of one of the daughter cells after asymmetric division of a stem cell can result from up-regulation of the activity of transcription factor c-Myc, as suggested by Andreas Trumpp and colleagues in the hematopoietic system. Myc has been estimated to regulate about 15% of the genes in the human genome and also down-regulates expression of 'glue-molecule' E-cadherin. Myc in turn is regulated by many different intracellular signalling pathways, such as the Notch pathway and the Wnt/β-catenin-pathway. Activation of these pathways can result from epigenic events, e.g. hypermethylation of the promoter of the soluble Frizzled-1 (loss of this protein, which happens to be an extracellular inhibitor tor for Wnt-sigaling, is very often seen in BC; it leads to an autocrine stimulated Wnt-pathway, resulting in increased Myc-activity.) Inhibition of β-catenin signalling in mammary alveolar progenitor cells leads to the inhibition of mammary gland development and pregnancy-induced proliferation, implicating β-catenin as an important stem cell survival factor in the mammary gland.[2]

LIP-LAP

Another mechanism involves the C-EBP-β, a member of the basic leucine zipper family of transcriptional regulators, playing important roles in cell proliferation, differentiation and oncogene-induced senescence through both positive and negative effects on gene expression.[3] The *c-ebpβ* gene has no introns, but can be expressed as differently sized isoforms (named respectively LAP and LIP), as a result of translational regulation. The smaller isoform LIP lacks activating domains and in fact functions as an inhibitor of LAP. Rosen's group showed that the LIP/LAP ratio is increased in many in BCs, especially in the more aggressive phenotypes.

Studies over the last ten years have shown that LIP/LAP-ratio can control cell fate: the choice between proliferation, commitment and terminal differentiation. Cynthia Zahnow, a former postdoctoral fellow from the Rosen Laboratory, has demonstrated that alterations in the epidermal growth factor (EGF)-signalling pathways lead to alterations in the LIP/LAP ratio. Furthermore, a very recent publication from the lab of Joan Massagué has provided evidence that this ratio is at the core of the cytostatic effects of transforming growth factor β. TGFβ is the most potent and widespread growth-inhibitory cytokine known in mammals, and its effects are based in part on elevating expression of cycle cycle-dependent kinase (CDK) inhibitors p15 and p21 and concurrent repression of Myc and anti-differentiation factors. In fact, resistance to TGF-β action is a hallmark of cancer. It was found that C/EBPβ is essential for TGFβ's induction

of p15 and repression of Myc. These responses were absent in cells from half of the metastatic BCs tested and correlated with an excess of LIP.[4]

Based on these data, Rosen proposes an hypothetical model: stem cells are normally quiescent as a result of low Myc levels, but you can have signals (e.g. though the EGF receptor or epigenetic regulation through silencing of soluble Frizzled) leading to up-regulation of Myc. In the case of the TGFβ-mediated cytostatic response, you can overcome this inhibition by changing to a higher LIP/LAP ratio, now allowing these cells to become transient amplifying cells.

Resistance

About 45 million Americans are uninsured, which is an indictment of the US health care system, but also provides the unique opportunity; in the Ben Taub hospital in Texas, especially African Americans and Hispanic arrive with very large tumours and in fact they often need treatment with size-reducing chemotherapy before the tumors can be surgically removed. Taking biopsies before and after this neo-adjuvant chemotherapy allowed Jenny Chang, a clinical oncologist and colleage of Rosen, to evaluate the nature of treatment-resistant cells. Genetic profiling studies revealed elevation of CD44, β-catenin, Wnt etc. FACS sorting followed by growing mammospheres, comparing material from treated and non-treated patients showed a significant increase in $CD44^{+}CD24^{-}$ cells. Interestingly, there is evidence that the treatment resistance of these cells is in part due to an increased activity of cellular pumps that rapidly remove certain chemotherapeutics from the cellular interior.

The identification of mammary stem cells also highlights the need for a dramatic shift in the way radiation therapy is designed. This is clinically given in small daily doses to reduce normal tissue toxicity, yet still achieving adequate tumor cell kill. Studies in the past have shown that tumors can undergo accelerated repopulation between daily fractions of radiation dose and it is tempting to hypothesize that this accelerated growth derives from tumor stem cell clonogens responding to radiation-induced cellular stress. But there is also increasing evidence that stem cells are simply more apt to repair the radiation-induced DNA damage and that this corresponds to increased levels of β-catenin and the Wnt/β-catenin-target survivin, a mitosis inhibitor that allows cells to repair their DNA damage in stead of undergoing a mitotic catastrophe. In a mouse model, radiation selectively enriched for progenitors with activated Wnt/β-catenin and surviving expression, suggesting that the Wnt/β-catenin signaling pathway may be an attractive target for directed anti-stem cell therapeutics.[5,6]

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The Wnt signalling pathway and cancer stemness

R. Fodde, Ph.D., Erasmus MC, Rotterdam, The Netherlands

What makes a stem cell a cancer stem cell (CSC) is the loss of homeostatic mechanisms that normally regulate proper cell numbers within a niche. In the adult stem cell niche hierarchy, the *bona fide* stem cell is the only one with total self renewal capacity. This cell gives rise to multipotent progenitors, which on their turn develop into more committed progenitors, eventually giving rise to the terminally differentiated cell types. Probably only few signal transduction pathways, e.g. those normally stimulated respectively by extracellular Wnt, Notch and Sonic Hedgehog ligands, are capable of controlling stem and progenitor cell numbers within adult stem cell niches; uncontrolled activation of these pathways determine the expansion of these stem/progenitor cell populations (the 'CSC niche'), thus resulting in tumors which are well- or poorly differentiated, depending the stage of the hierarchy at which oncogenic events occur. At the Erasmus Medical Center in Rotterdam, Riccardo Fodde's research group has been unravelling the role of Wnt-signalling in the onset and malignant behaviour of cancer stem cells.[1,2]

The Wnt/β-catenin pathway

In intestinal stem cells not exposed to the soluble Wnt, ligand β catenin is degraded by a specialized 'protein-shredder', the so-called destruction complex, and is thus unavailable for functioning as a transcriptional activator of Wnt target genes. This destruction complex is composed by several proteins that bind and earmark β-catenin by phosphorylating it at specific sites. Members of the destruction complex include kinases like GSK3β (glycogen synthase kinase-3β) and CK-1 (casein kinase 1), but also other proteins with a scaffolding function, such as tumour suppressors APC (adenomatous polyposis coli protein), Axin and Conductin. In the presence of the Wnt-ligand, formation of the destruction complex is inhibited, thus leading to intracellular β-catenin accumulation and its consequent translocation to the nucleus, where it binds to members of the family of transcription factors TCF and LEF, thus modulating the expression of a broad range of target genes (Fig. 1).

In the vast majority of 'sporadic' colorectal cancers, a mutation has occurred in APC, preventing formation of the β-catenin destruction complex. In the remaining 15% of the cases, β-catenin itself has acquired oncogenic mutations that make it resistant to proteolytic degradation. The outcome is essentially the same: notwithstanding the presence or absence of the Wnt-lignad, β-catenin is now able to accumulate in the cell and eventually translocates to the nucleus (Fig 2).[3]

At the bottom of the crypt

How does this work in stem cell niches of the gut? At the bottom of the crypt there are probably very few stem cells which divide very infrequently and in an asymmetric way. The upper daughter cell migrates upwards, enters a mitogenic phase (the transient amplifying cells) and eventually differentiates in one of four cell types of the adult intestine: the absorptive cell, the Goblet cell, the endocrine cell and the Paneth cell (the latter being the only

R. Fodde

Ricardo Fodde studied biology and molecular genetics at the University of Pavia, Italy. His Ph.D. work on hemo- and haptoglobins was carried out at the Department of Human Genetics at the University of Leiden, the Netherlands, and has led to the characterization of the spectrum of mutations leading to haemoglobinopathies in the Netherlands. In 1990, he started post-doctoral work on the molecular genetic basis of colorectal cancer and, as a fellow of the Royal Dutch Academy of Science (KNAW) he visited the laboratory of Raju Kucherlapati at the Einstein College of Medicine in New York, where he developed the first targeted mouse model for intestinal tumorigenesis. In 2001, he became Professor of Cancer Genetics at the LUMC in Leiden. His group has contributed to the elucidation of molecular basis of hereditary colorectal cancer in man, developed a large number of mouse models for solid tumors, and characterized novel functional aspects of the APC tumor suppressor. Most recently, has been focussing his research on the role of APC and β-catenin in stem cell renewal and differentiation in tumour initiation, progression and metastasis. In 2003, he was awarded the VICI-NOW grant, and became Professor of Experimental Pathology at the Josephine Nefkens Institute of the Erasmus MC in Rotterdam.

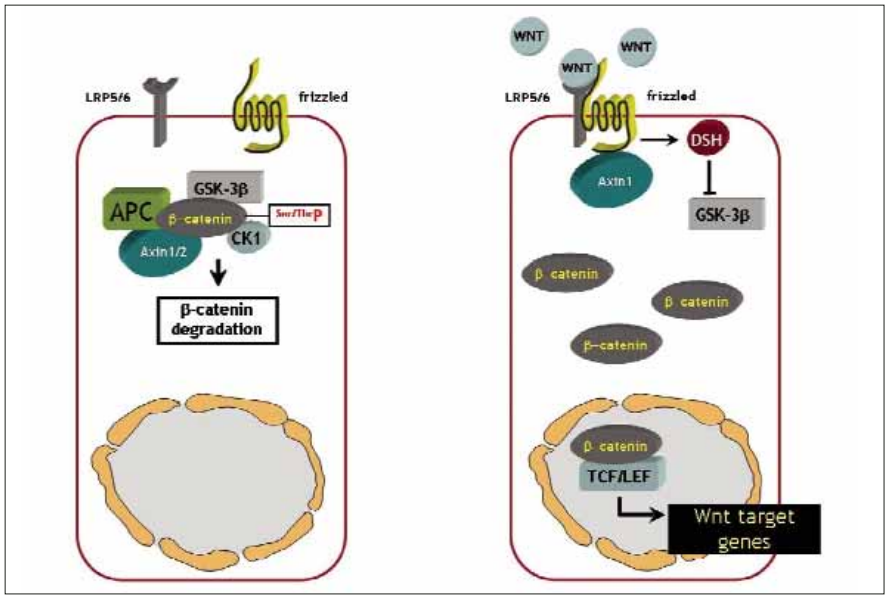

Figure 1.
The Wnt/β-catenin pathway

one that moves downward and accumulates at the crypt bottom). Two to three days after stem cell division the differentiated cells eventually undergo apoptosis and are exfoliated in the intestinal lumen. It should by now be evident that such a highly dynamic tissue requires a finely tuned equilibrium between asymmetric cell division, proliferation, cell migration, differentiation, and apoptosis.

Wnt appears to be a crucial factor in the maintenance of this equilibrium: when the Wnt signaling is off,a genetic program of migration, terminal differentiation and apoptosis is triggered, whereas, at the other end of the spectrum, full blown Wnt signaling activates a distinct genetic programs, possibly regulating both 'stemness' and self renewal. Research in hair follicles revealed that the dosage of Wnt determines the nature of the different genetic programs that are activated in these pluripotent stem cells.[4]
Mutations in the APC gene are likely to affect this finely tuned equilibrium, thus triggering loss of tissue architecture and the formation of a benign tumor.

The β-catenin paradox

Whether β-catenin destruction is prevented by one way or the other, one should expect nuclear accumulation of the protein in virtually all tumour cells within a tumor triggered by either APC or β-catenin mutations. Surprisingly, this is not the case: the presence of nuclear β-catenin within these tumors is restricted to cells at the tumour invasion front, the site where tumor cells are in direct contact with the surrounding stromal mesenchyme (Fig. 3). This remarkable finding led to the hypothesis that only these 'cells on the edge' are CSCs. Additional data from the scientific literature have that these cells not only accumulate at the invasive front, but, when they detach and move into the stromal layer, acquire mesenchymal markers like fibronectin, and loose epithelial markers like E-cadherin. In other words: they undergo epithelial to mesenchymal transition (EMT), a well-known transdifferentiation process.[5]
EMT makes these migrating cells even more dangerous, as they can adapt to the context where they reside, thus efficiently disseminating to distant organs where they form metastases.

Thus, the model arising from these observations is that intestinal cancers arise from adult stem cells niches as a result of mutations in members of the Wnt signalling pathway that disturb the finely tuned equilibrium of cell turn over in the crypt. The latter alters the tissue architecture and triggers the

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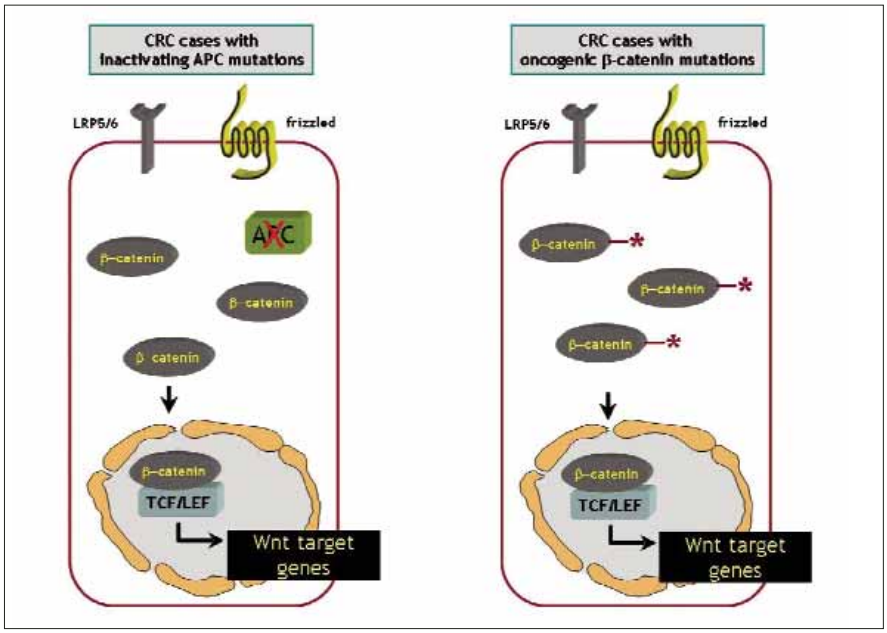

Figure 2.
Mutations that lead to colorectal cancer

formation of tumors, characterized by a pronounced cell heterogeneity, showing all cellular differentiation types, but also a relatively small CSC population. This is reflected by the heterogeneity of the tumor cells that detach from the primary mass and are disseminated throughout the body: among these, only CSCs are able to undergo transient and the reverse process MET (mesenchymal to epithelial transition), thus efficiently forming micro-metastases at distant sites. At these sites, CSCs recapitulate tumor formation, in a very similar fashion as at the primary site in the GI tract. Therefore, the histology and expression profile of (macro)metastases tend to resemble that of primary tumours (behave very similar as they do in the gut), and therefore the histologies of (macro)metastases tend to resemble that of primary tumours.[6,7]

One APC, different outcomes

After having generated a novel animal model heterozygous for s specific mutation in the mouse Apc gene, Fodde encountered yet another surprise: these mice do not get any intestinal cancer (as all other Apc-mutant mice do), but instead develop very aggressive multifocal mammary cancers. As for the intestinal tumors, these mammary neoplastic lesions are very heterogeneous, showing both luminal epithelial and myoepithelial cells, as well as a more hair follicle-like cell type, likely resulting from aberrant differentiation. Interestingly, whereas macrometastases recapitulate the heterogeneity observed at the primary site, tiny micrometastases do not (yet) show significant features of differentiation; in the latter, the majority of cells showed intracellular accumulation of ,-catenin, possibly indicating that these cells may represent cancer stem cells.

A second APC mouse model exclusively develops intestinal tumors, preferably in the upper GI tract; they start as benign adenomas and then degenerate into adenocarcinomas with invasion of parenchymal cells into the underlying stromal cells. Also in this model, intracellular β-catenin is only observed in a subpopulation of tumour cells.

Recently, Shackleton and coworkers reported the isolation of normal mammary stem cells by the cell membrane markers $CD29^{high}CD24^{+}$. Upon transplantation into the cleared mammary fat pad of syngeneic mice (see also page 10), even only one $CD29^{high}CD24^{+}$ stem cell is capable of reconstituting a complete and functional mammary gland. In support of a potential role for these markers to identify CSCs in breast cancer, the $CD29^{high}CD24^{+}$ sub-

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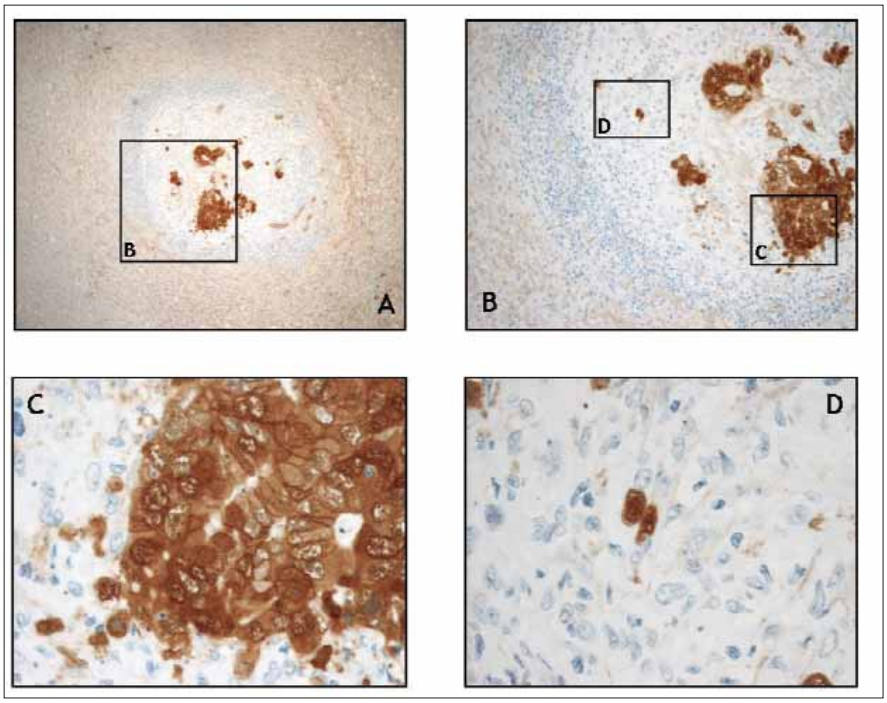

Figure 3.
Immunostaining of β-catenin (brown) in metastasis from colorectal cancer.

population was significantly expanded in pre-malignant mammary tissue from another Wnt mouse model for breast cancer when compared with a different, non-Wnt, mouse model for mammary tumourigenesis.[8]

In Fodde's lab, tumor cells isolated from both mammary and intestinal tumors from the above APC-mutant mice were sorted for the presence of CD24 and CD29 markers; only a small fraction (~1.5%) of the total tumour cells were $CD29^{high}CD24^{+}$. Accordingly, both these cell fractions show a marked enrichment of intracellular β-catenin accumulation.

These interesting results raise many questions: how can different constitutive APC mutations in mice result in such entirely different phenotypes (i.e., either intestinal cancers or mammary cancers)? According to Fodde, the basic idea is that every other stem cell niche has its own specific threshold of Wnt-levels: cells have to stay in a certain window of Wnt-concentrations to maintain homeostasis in their particular niche. A high level of Wnt-signalling activation somehow always first hits the gut, but the mammary gland might be sensitive to much lower Wnt levels.[9]

Silencing APC is not enough

Needless to say, mutation in APC gene represents the rate limiting initiating event, but other factors are likely to play equally important roles in progression towards malignancy, either with an additive or synergistic mode of action. One of the first genes to be mutated during intestinal tumour progression is the oncogene KRAS. Patients with tumors carrying both APC and KRAS mutations are characterized by poor prognosis. Breeding APC-mutant mice, which develop a relatively mild intestinal cancer phenotype, with mice bearing a transgenic KRAS mutation, results in compound APC/KRAS-mutant animals that develop ten times more intestinal cancers than APC-only mice and show an accelerated adenoma-carcinoma sequence (they even develop liver metastases).[10] Hence, in this model, oncogenic KRAS influences both tumor initiation and tumor progression. Whereas APC-mutant mice seem to form a preclinical model of the colorectal cancer patients with a relatively good prognosis, the APC/KRAS compound animals mimic the clinical course of the poor prognosis patient with frequent morbidity and mortality. By applying expression profiling analysis of tumors from both groups, the researchers in Fodde's laboratory were not able to distinguish poor from bad prognosis tumor types, thereby casting severe doubts upon the capacity of expression profiling to accurately predict prognosis in cancer patients.

Could it be that these negative results arise as a consequence of the CSC mode? And that the only relevant difference between good and poor prognosis groups consists in the relative abundance, within each tumor, of a cell with intracellular β-catenin accumulation? By counting the absolute number these alleged CGCs, Fodde's colleagues confirmed this hypothesis. The exact mechanisms of the synergism between mutations in APC and KRAS is now being elucidated and is likely to reside in the capacity of oncogenic KRAS to activate downstream tyrosine kinases, thus triggering Tyrosine-phosphorylation of β-catenin and prompting its detachment from E-cadherin at the cell membrane. Hence, it may well be KRAS-mutations significantly enlarge the pool of β-catenin molecules in the cytoplasm!

Calendar

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