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CONTENTS

4 Foreword by Inder M. Verma

7 Part I: Molecular markers
9 J. Michael Bishop
   Senescence and metastasis in mouse models of breast cancer
15 Joan Massagué
   Metastasis genes and functions
21 Zena Werb
   Transcriptional regulation of the metastatic program
25 Inder M. Verma
   BRCA1 maintains constitutive heterochromatin formation:
   a unifying hypothesis of its function
29 Tak Wah Mak
   The role of RhoC in development and metastasis

35 Part II: Motility and invasiveness
37 Robert Weinberg
   Mechanisms of malignant progression
43 Daniel Louvard
   Fascin, a novel target of β-catenin-Tcf signaling, is expressed
   at the invasive front of human colon cancer
49 Gerhard Christofori
   Distinct mechanisms of tumor cell invasion and metastasis
55 Douglas Hanahan
   Multiple parameters influence acquisition by solid tumors
   of a capability for invasive growth

59 Part III: Mechanisms of metastasis
61 Richard Hynes
   Cellular mechanisms contributing to metastasis
67 Ann Chambers
   Novel imaging approaches for studying tumor metastasis
73 Jeffrey Pollard
   Macrophages are a cellular toolbox that tumors sequester
   to promote their progression to malignancy
79 Wolf-Hervé Fridman
   T effector/memory cells, the ultimate control of metastasis in humans
85 Kari Alitalo
   Inhibition of lymphangiogenesis and metastasis
91 Shahin Rafii
   Contribution of CXCR4+VEGFR1+ pro-angiogenic hematopoietic
   cells to tumor oncogenesis

97 Part IV: Cancer stem cells
99 Paolo Comoglio
   Invasive growth: a MET-driven genetic program for cancer and stem cells
105 Hans Clevers
   Wnt and Notch cooperate to maintain proliferative compartments
   in crypts and intestinal neoplasia
111 Owen N. Witte
   Progression of prostate cancer from normal tissue stem cells

117 Abbreviations and glossary
121 Participants
124 Group picture
125 Fondation Ipsen
FOREWORD BY INDER M. VERMA
Under the Tuscan sun

Tuscany is beautiful, in large measure because nature was bountiful, but man has also contributed much to enhance its allure and charm. The site for the third cancer series on Invasion and Metastasis was right in the middle of Tuscany, a beautiful collection of marvelously decorated group of villas in Abbazia di Spinetto, from May 20-23, 2007. The sun and Tuscan food were both in splendor to accompany the exciting and cutting-edge science. As always, roughly half of the participants gave formal talks and the remainder participated in lively discussions.

In my introductory remarks I emphasized that, while we know a lot about the molecular mechanisms of carcinogenesis, we know little about metastasis, which is the ‘real killer’. Metastasis is often preceded by an intricate set of molecular changes, leading to the invasion of tissues by cancer cells. Understanding the molecular signatures of invasive processes is therefore essential to combating metastasis.

Mike Bishop and Joan Massagué described the multi-gene signatures that can identify tumors with a poor prognosis. Massagué also emphasized the importance of the role of the microenvironment in determining the fate of metastases. Zena Werb explained the role of GATA-3 as a marker of malignant progression in a breast cancer model. I described the role BRCA1 plays in maintaining heterochromatin integrity and thereby the genomic stability of a cell. Tak Mak’s presentation focused on RhoC’s role in breast cancer.

On the general theme of motility and invasiveness, Robert Weinberg presented his work on epithelial-mesenchymal transition (EMT), a key event in metastasis, and how there may be a selective pressure that determines which cells become metastatic. Daniel Louvard also talked about EMT and the role of fascin in migration, cell dissemination and metastasis. Gerhard Christofori discussed the three possibilities for metastatic tumor cell dissemination, as single or collective cells in the presence or absence of EMT, or by dissemination to local lymph nodes. Doug Hanahan also returned to the idea of selective pressure eliciting invasiveness and the idea that impaired angiogenesis is pro-invasive.

Richard Hynes focused on the later stages of metastasis and on host influences on metastasis. Ann Chambers talked about the roles of the size of the cancer cells, the size of the blood vessels and the rate of blood flow from the primary site to the secondary site — and the use of imaging to observe metastasis. Jeffrey Pollard also used imaging, but in his case to illuminate the role of macrophages in metastasis.

Wolf-Hervé Fridman also focused on the immune system and suggested that the local immune infiltrate may play an important role in controlling invasion and metastasis. Kari Alitalo discussed the role of the lymphatic vascular system in spreading tumor cells and the potential use of anti-VEGFR3 as a complement to anti-angiogenic cancer drugs. Shahin Rafii presented potential ways to block the growth and metastatic potential of hemangiogenesis-dependent tumors.

Throughout the talks, the speakers and discussants often referred to cancer stem cells. Paolo Comoglio suggested that targeting Met, which is expressed in both stem and cancer cells, can inhibit invasive growth and metastasis. Hans Clevers presented the first formal proof that GPR49 is a single marker for adult stem cells in the intestinal tract. And Owen Witte has developed in vitro prostate colony-forming and sphere-forming assays to measure stem cell activity, which can help identify cell surface markers that enrich for prostate stem cells.

As in the past, the discussions were lively, continued during lunch, dinners and even the excursions into the Tuscan countryside. We all owe a great deal of thanks to Jacqueline Mervaillie and Yves Christen for not only selecting a beautiful place for the meeting, but for their superb organization, without which this would have been just another meeting in Tuscany. Many thanks also to Apoorva Mandavilli, who once again has managed to find organization in the disorganized order of speakers, themes and areas. Finally, many thanks to you for attending the meeting. I hope you had as much fun and learned as much as I did.

Inder Verma
PART I: Molecular markers

Michael Bishop
Senescence and metastasis in mouse models of breast cancer

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BRCA1 maintains constitutive heterochromatin formation: A unifying hypothesis of its function

Tak Wah Mak
The role of RhoC in development and metastasis
The cause and molecular pathogenesis of breast cancer is poorly understood. Michael Bishop and his colleagues developed a technique that allows for easy expression of ectopic genes in precursor cells of mouse breast epithelium. This serves as an attractive model for exploring the genetic elements in the genesis and metastasis of breast cancer. Tumor progression must require active suppression of senescence. The Id1 gene, known to be overexpressed in a variety of tumors, appears to suppress senescence of human cells in vitro and developing breast cancer cells in vivo. Thus, inhibition of either Id1 itself or its effectors in the suppression of senescence could be a strategy for treatment of cancer, particularly those in which Id1 is over expressed. In tumors elicited by Middle T-Antigen, the macrophage stimulating protein expands cancer-initiating cells and augments both tumor growth and metastasis. Based on large datasets of tumors in relatively young women, the signaling complex also appears to be a strong independent predictor for poor outcome in human breast cancer.

Breast cancer is the second most common cause of death from cancer among women in developed nations. Each year in the United States alone, there are more than 210,000 new cases of breast cancer. The primary cause of death in most cases is metastasis, but the cause and molecular pathogenesis of breast cancer and of metastasis are poorly understood. The treatments now available to treat the disease can sometimes extend lifespan, but rarely cure metastatic disease.

Genes implicated in breast cancer suffer either gain of function or loss of function with some frequency. Among these, the normal allele of the Met gene is over expressed in up to 25% of human breast cancers. This subset of tumors is distinctive and does not express estrogen receptor, progesterone receptor or HER2. The tumors carry a high risk of metastasis and very poor prognosis. Some companies are developing Met-based drugs for tumors that otherwise are not amenable to effective therapy.

Met is a transmembrane protein tyrosine kinase receptor, and its ligand is known as scatter factor (SF) or hepatocyte growth factor (HGF). The kinase is extremely pleiotropic, and appears to have roles in cellular motility, invasion, cellular proliferation and even in normal tissue morphogenesis. Perhaps not surprisingly, it also has a role in some of those processes during tumorigenesis.

Human breast cancer appears to originate from a bipotential stem cell—and that tumor stem cell has ostensibly been identified and isolated. But no one has developed a mouse model for breast cancer that deliberately targets that cell.
In a new model system, normal breast epithelium is cultured and infected with the mouse stem cell vector bearing the gene of interest. GFP is typically included as a marker. When the transduced cells, which express GFP, are put back into a mouse fat pad, the cells reconstitute an entire mammary gland with all the elements expressing GFP, suggesting that the virus is transducing precursor cells.

When this method is used to transduce a normal allele of Met and over express it, distinctive ductal lesions develop. The lesions resemble common mouse breast cancer lesions, but they are too disorganized — either by luminal or myoepithelial markers — to be truly akin to human intraductal carcinoma in situ. They do not progress but remain in that state through the lifespan of the mouse.

Transducing Myc, in contrast, has an impact on the morphology of the ductal structure and gives rise to focal lesions that are a little better organized than the Met lesions. But these lesions also sit for many months, although they may eventually give rise to a neoplasm.

When Met and Myc are combined, however, there is very aggressive tumorigenesis. Large tumors arise within a month and are quickly lethal. The tumors are of mixed lineage, both luminal and myoepithelial, they are adenocarcinomas, and they carry the marker CK6, an apparent atavism of the progenitor cell.

This model facilitates the testing of diverse genes both alone and in combination and is quick in comparison to classical transgene models. It may also have greater authenticity than transgenic models, which tend to be uni-lineage in nature, and it appears to target a biopotential precursor cell, akin to that which gives rise to adenocarcinomas of the human breast.

The tumors from the combination of Myc and Met themselves do not metastasize, however, so the long range objective is to test these tumors further for metastasis genes.

### The question of Id

The Id genes are a group of HLH DNA-binding genes that act as control elements by virtue of being dominant negative inhibitors of other transcription factors. The Id genes figure in a variety of physiological phenomena: cellular proliferation, apoptosis, invasion, angiogenesis, differentiation and senescence.

Interestingly, Id1 is over expressed in a large variety of human tumors at a significant frequency. In breast cancer, its expression becomes more common as the tumor grade is more severe, and the expression itself seems to be a poor prognostic indicator — although that has not been studied in any rigorous prospective manner. Id1 is a member of Joan Massague’s [see page 15] 54-gene human metastasis signature and has been implicated in metastasis in functional assays. Some researchers believe that the gene is an angiogenic switch.

When tumorigenesis is initiated in mice by transduction of activated Ras into normal breast epithelium, the result is very small tumors that do not expand. When Id1 is added to the experiment, there is rapid tumor growth that leads to either spontaneous death or the need to sacrifice the mice. So Id1 increases the penetrance of tumors that arise from Ras. These tumors are also of mixed lineage and they are vigorously metastatic to the lung.

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**Figure 2**

Id1 is required for tumor maintenance.
With Ras under the control of doxycycline, as soon as it is shut down, these tumors, which would otherwise go on to kill the animal, simply dissolve away. If Id1 is shut off, the tumors stop growing and persist as palpable nodules, until the mice are eventually sacrificed.

**Switching to senescence**

Some years ago, several labs demonstrated *in vitro* that efforts to transform cells with certain oncogenes often led to senescence. The same has been demonstrated with tumors *in vivo*. The Ras tumors that are limited in their growth turn out to be senescent nodules. Massive senescence in the tumors is what limits their size, eventually making them cystic and necrotic.

The same thing is true of tumors that arise from a combination of Id1 and Ras. If Id1 is shut off, cystic elements develop and there is massive senescence in the lesions. In either instance, Ras alone leads to a senescent response, and withdrawing Id1 leads to a senescent response. So Id1 seems to facilitate tumor penetrance and tumor growth by suppressing the senescent effect of Ras.

This can be demonstrated *in vitro*. When well developed tumor cells from a combination of Ras and Id1 are put in culture, tumor cells grow rapidly. But if Id1 is withdrawn, the cells simply do not grow and go into senescence, both by morphology and by other markers.

In this system, Ras triggers senescence in mammary epithelium, limiting tumors to necrotic elements. Ras can elicit progressive tumors in animals that do not have the INK-ARF complex or ARF alone, p21, or p53 — even a heterozygous deficiency in p53. But in the absence of p16, it cannot elicit progressive tumors. This suggests that among the pathways that are thought to lead to senescence, the p53/p21 pathway, in this model at least, is essential to senescence, and the p16 pathway is not. This is in contrast to results from other labs, particularly *in vitro*.

In the absence of p16, there are not even minimal Ras tumors. But there is no change in the expression of various members of the p53/p21 signaling pathway and the p16 pathway in response to Id1, so Id1’s mechanism of action is as yet a mystery.

The two genes Id1 and HRasV12 cooperate to produce very aggressive metastatic mammary adenocarcinomas and they are both required for tumor maintenance after the tumors are well developed.

In the case of Ras, proliferation shuts down if you shut it off. In the case of Id1, shutting it off leads tumors to senesce. In a tumor that is over expressing Id1, if Id1 is countering the senescent response to oncogene stress or tumorigenic stress, then a therapeutic targeting of Id1 or some element in its signaling pathway could arrest the growth of tumors by eliciting senescence. The long range objective is to see whether this could lead to a therapeutic modality.
A role for macrophages

Metastasis is a particularly prominent phenomenon in breast cancer, with metastases to multiple organs, including lung, liver, bone and brain. In terms of the role of inflammation and macrophages in various aspects of tumorigenesis, the gene RON is particularly interesting.

RON is frequently active, constitutively active or over expressed in breast cancer. It is the transmembrane receptor for the macrophage stimulating protein (MSP). Signaling from MSP is initiated by the cleavage of a precursor molecule by a recently identified protease. The active form of MSP then triggers RON, which is a pleiotropic effector, with effects on osteoclasts, epithelial cells and, of course, on macrophages.

The model used for these experiments is based on a pleiotropic oncogene, the polyoma middle T-antigen (PyMT). This is a classical transgene model which goes through a tumor progression that resembles human disease, but it fails to mimic typical human adenocarcinoma in that the tumors are of single cellular lineage rather than of mixed lineage and metastasize only to the lung.

In the experiments, either the tumor cells are transduced with MSP or the PyMT gene is transduced into normal breast epithelial cells. The main difference is that transduction with a mouse stem cell virus gives mixed lineage tumors with PyMT.

Transduction of PyMT tumors with MSP leads to more rapid tumor growth, although the tumor size tends to plateau at the same time as in the controls. MSP appears to kick up the frequency of cancer-initiating cells by ten-fold. More dramatically, however, there’s a profound effect on metastasis. Instead of metastasizing only to the lung, the PyMT tumors metastasize to every organ to which breast cancer normally metastasizes, except for the brain.

There’s a modest increase in metastasis to lung and liver, a big increase in metastasis to lymph nodes and spleen — and the tumors acquire the ability to metastasize to bone, which is among the most onerous of human breast cancer metastases. The ability of these cells to invade an artificial matrix also requires the presence of isolated macrophages along with MSP.

So MSP appears to have an effect directly on tumor cells: it augments tumor growth and increases the number of tumor-initiating cells. In vitro at least, it can elicit an epithelial-mesenchymal transition. It converts PyMT tumors to dual lineage dramatically.

Acting through macrophages, it also has an impact on the metastatic ability, eliciting osteolytic lesions. In a classic in vitro assay for osteolysis, tumor cells taken alone cannot erode either an artificial or a real osteogenic slice. But with MSP added to the mix, there is extensive osteolysis. There’s good evidence to believe that MSP is acting directly on osteoclasts to elicit this osteolysis.

Survival signatures

Analysis of a data set from the Netherlands Cancer Institute of 295 relatively young patients with stage I and II tumors from 1984 to the present has produced a 70-gene signature, called MammaPrint, to predict poor prognosis. The US Food and Drug Administration recently approved MammaPrint for use in the US.

In that same dataset, expression of MSP alone in the tumors has no predictive power. But when there is congruent expression of the MSP signaling complex at above average levels, there is a strong aggravating
effect on metastasis and on survival. Of all the parameters used to predict prognosis, this one has the strongest hazard ratio for both patient survival and metastasis.

The MSP complex bumps up MammaPrint’s prognostic accuracy, at least in retrospect, from 51% to 82%. Prognostic accuracy for both survival and metastasis in a certain period of time go up dramatically.

One other dataset from UNC/Utah also contains all three genes. With 162 primary tumors, this dataset has fewer tumors, but it is more representative in age and tumor stage than is the Netherlands set. The UNC/Utah set has been in observation relatively briefly, for about two years, but it’s the only other available set in which the MSP complex is present.

By array analysis, the MSP complex is expressed in 6.2% of tumors, about half the prevalence of the Netherlands set. There is a shorter time to relapse, and the MammaPrint accuracy improves from 28% to 56% in retrospect. The strength of these data are limited by sample size and relatively brief period of observation but the trend is in the right direction.

In summary, in this model system, MSP facilitates metastasis from an orthotopic site to sites that are clinically relevant and the signaling complex is a strong independent predictor for poor outcome in human breast cancer.

The MSP signature identifies patients who are at risk despite having a good prognosis by other parameters so it augments prognostic capability. It also appears to help identify patients who are good candidates for chemotherapy.

References

Metastasis allows cancer cells from a primary tumor to disseminate and invade distant organs. There are longstanding questions about the stage at which cancer cells acquire the ability to metastasize and the identity of genes that mediate process of metastasis. Different organ microenvironments may select for different species of metastasis. A patient with advanced multi-organ metastatic disease presumably harbors circulating cells that represent a cross-section of various organ-specific species of metastasis. Gene expression profiling of malignant pleural fluids of patients with advanced breast cancer identifies a lung metastasis gene signature that supports both lung metastasis and primary tumor growth. Other genes impart lung metastasis virulence without supporting primary tumor growth. Four LMS genes, encoding epiregulin, the cyclooxygenase COX-2, and the matrix metalloproteinases 1 and 2 are co-opted by breast cancer cells for a vascular remodeling program that promotes metastatic progression. Joan Massagué discussed the importance of these ‘metastasis progression’ genes and ‘metastasis virulence’ genes in metastatic colonization.

After the surgeon has removed a primary tumor, sometimes weeks, years or even decades later, metastases may emerge. There is a general understanding of the steps that tumor cells must undertake and the barriers they must surpass to accomplish metastasis. However, the mechanisms tumor cells employ to fulfill these requirements are still not well understood. One thing is clear, however: tumor cells co-opt whatever mechanism, providing a selective advantage under the enormous pressures facing these cells in the establishment of metastasis.

Evidence of such co-option can be found, unexpectedly, in studies on the TGF-β signaling pathway. The cytokine TGF-β normally acts as a potent growth inhibitor and a tumor suppressor. Through its membrane receptors, TGF-β activates Smad transcription factors. The activated Smads assemble transcriptional complexes that target specific genes for either activation or repression. Up to 150 genes respond immediately to TGF-β in this fashion, including genes that lead to a cytostatic response.

For tumor cells, this growth inhibitory pathway is a nuisance that they are eager to avoid. Gastrointestinal tumor cells, for example, accumulate inactivating mutations in the TGF-β receptors, which disable the entire TGF-β signaling program. But in breast cancer, glioblastoma and other types of tumors, the TGF-β receptors and the rest of the signaling pathway often remain intact. Instead, the tumor cells selectively lose only the cytostatic responses to TGF-β, allowing many other TGF-β gene responses to remain.

Malignant cells obtained from the pleural fluids of patients who are succumbing to metastatic breast cancer
have been shown to selectively avert the growth inhibitory action of TGF-b. In about half of the cases, the breast cancer cells accumulate LIP, an endogenous inhibitor of the transcription factor C/EBPb that Smads need for p15INK4b induction and for down-regulation of MYC. The impediment to C/EBPb function caused by LIP thus suppresses the TGF-b cytostatic response of breast cancer cells without affecting their other TGF-b gene responses.

The reason that some tumors chose this complicated route to escaping the tumor suppressor function of TGF-b is that, in doing so, they can use the crippled TGF-b pathway to their advantage in metastasis. For example, two normal TGF-b target genes, interleukin-11 (IL-11) and CTGF, have been separately identified as genes that breast cancer cells use for metastasis on reaching the bone marrow.

CTGF is thought to foster angiogenesis, whereas IL-11 induces the production of RANKL, that then stimulates osteoclasts to further the osteolytic process. Therefore, when breast cells avert the growth inhibitory action of TGF-b by over expressing LIP, they can use TGF-b to their advantage as a stimulus for their own production of IL-11 and CTGF.

These provocative findings provide a rationale for TGF-b’s dual action in cancer, and illustrate the concept that tumor cells may stop at nothing to extract a selective advantage to defeat any barriers.

**Organ-specific metastasis**

Primary tumor cells that manage to metastasize to the lungs, brain, bones or liver, have to surmount tremendous barriers in order to conquer these environments. The “seed and soil” hypothesis promulgated by Stephen Paget in the 19th century holds that certain tissues and organs present a favorable environment for each particular type of tumor cells.

But it seems improbable that organs that have evolved over hundreds of millions of years would simply welcome wandering tumor cells to invade and colonize them. Instead, tumor cells may develop a competence to evolve and adapt to life in hostile organ microenvironments.

Tissues such as the bone marrow, the pulmonary parenchyma or the brain present very different microenvironments. Their barriers against colonization by circulating tumor cell must be equally different. Consequently, the cells that succeed in colonizing each organ may represent different metastatic species. Indeed, this species heterogeneity can be exploited to identify organ-specific metastasis genes and functions.

In advanced disease, different metastatic lesions may release their well-adapted cells into the circulation, and from there to other body fluids such as the pleural fluid, where malignant cells coming from the bones, the lungs or the brain may commingling. Large build-up of pleural fluid is a common complication in advanced breast cancer and causes breathing difficulty in the patient. As such, pleural effusions must be drained, yielding a rich source of live metastasis cells for study.

According to this working hypothesis, these fluids would contain a demographic cross-section of various species of metastases that are present in a patient. Individual cells that are picked from a malignant pleural fluid cell population and grown out as clones show distinct gene expression profiles, reflecting their heterogeneous nature [see Chambers, page 67]. Interestingly, when tested in bone colonization or lung colonization assays, cell clones with distinct gene expression profiles have a distinct metastatic phenotype.

Some clones home to the bone and form aggressive osteolytic metastases, but fail to colonize the lungs. Others find their way to the bone but quickly disappear from this tissue, whereas they readily form lethal lung metastases. Yet others behave rather indolently and fail to colonize any organ.

Using mice for *in vivo* sorting of metastatic populations, distinct cell populations that form metastasis in
the bones, the lungs or the brain are derived from the same patient. When these lesions are excised and the tumor cells grown in culture, the resulting cell lines have clear organotropie preference for the organs from which they were extracted. After just two cycles of in vivo selection, the resulting cells may be so aggressive that it takes only 200 of them to give a mouse lethal lung metastasis.

If the lung metastatic cells are inoculated into the mammary glands of mice, and the growing tumors removed, metastases emerge later in the lungs from tumors that were formed with lung metastatic cells but not tumors formed by bone metastatic cells. Thus, these organ-specific metastatic phenotypes are evident even in metastasis from the primary site.

Mediating metastasis

By gene expression analysis, 54 genes in the case of the lung and 102 in the case of bone are associated with these organ-tropic phenotypes. These sets of genes are uniquely expressed in the lung and bone metastasis cell populations. The two lists are very different, with only about 10% overlap[^3].

The lung metastasis genes encode mostly factors that participate in interactions with the microenvironment: cytokines, chemokines, their receptors, proteases and extracellular matrix components. The list also includes cell autonomous functions such as, for example, the Id1 inhibitor of cell differentiation, and FSCN1 and NEDD9, both of which are implicated in tumor cell motility. Among the bone metastasis genes, CXCR4 has previously been implicated in bone metastasis. Others such as IL-11 and CTGF have since been shown to mediate metastasis.

This is not just in vitro phenomenology, but also has clinical relevance. The lung metastasis genes are also expressed in primary tumors in patterns resembling their expression in the experimental metastatic systems. In the first cohort analyzed, a subset of the 54 lung metastasis genes classify a tumor subtype that, when checked for clinical history, go on to develop lung metastases. Eventually these patients show metastases everywhere, but lung is the top noted clinical complication as a site of relapse. Bone and other sites are excluded.

This gene subset includes 18 lung metastasis–associated genes that together constitute the Lung Metastasis Signature (LMS). When this signature is bioinformatically transformed into a classifier trained in an independent tumor cohort, it provides a tool to separate those tumors that are negative for the LMS from those that are positive, with a clear difference in the risk of lung relapse. When this is expanded by analyzing with different tumor cohorts, which total more than 600 tumors, tumors classified as LMS+ once again differ significantly in terms of metastatic risk.

In conclusion, the LMS classifies tumors specifically for associated risk of lung metastasis. However, for the LMS to have an effect, the tumor has to grow to a certain size. The larger the tumors, the higher the propensity of metastasis. A molecular basis for this intriguing relationship between LMS+, tumor growth, and lung metastasis propensity, is eventually provided by studies on the function of specific gene components of the LMS.
Metastasis progression

The 18 LMS genes are a subset of the 54 genes whose expression endows tumor cells with the ability to colonize the lungs from the circulation. These 18 genes turn out to be substantially expressed in primary breast tumors in a manner that indicates a high risk of pulmonary relapse.

If these 18 genes are already expressed in primary tumors, it must be because they provide a certain advantage in the primary tumor and not just in the lung metastases. This advantage would not be a generic tumor growth property, because that would also be effective in bone metastasis, and therefore would have not emerged in differential screening assays.

Based on this reasoning, the 18 lung metastasis genes provide "metastasis progression" functions, which enable tumor progression as well as metastasis to a particular organ. The remaining 36 lung metastasis genes, which are not substantially expressed in primary tumor, would constitute "metastasis virulence" genes, or genes that specifically foster tumor growth in the context of the lung parenchyma but not in the mammary gland. These predictions are confirmed by reducing the expression of LMS genes by means of RNAi in lung metastatic cells. The knockdown of LMS genes inhibits the ability of these cells to form tumors in the mammary gland and to form lung metastasis. But, in most cases, knocking down these genes individually has a very small effect. The metastatic functions provided by these genes must act synergistically to be effective, so multiple LMS genes must be knocked down simultaneously in various combinations in order to functionally assess their role in metastasis.

Fortuitously, a recently reported list of vascular endothelial growth factor (VEGF) target genes in endothelial cells includes four of the LMS genes: the prostaglandin-synthesizing enzyme gene COX2, the EGFR ligand gene epiregulin (EREG), and the matrix metalloproteases MMP1 and MMP2. Based on these observations, tumor cells may co-opt for their own advantage part of the gene expression program that is normally activated by VEGF in endothelial cells.

The expression of these four genes in the lung metastatic cells is an intrinsic capacity and not the result of stimulation by autocrine VEGF. Knocking down these genes in combination, but not singly, causes a strong inhibitory effect on the growth of the tumor cells as mammary tumors or as lung metastases. The tumors that do form have elevated incidence of apoptotic cells, suggestive of a general stress on the tumor population. When three of the four genes are put back in by transfection into the knockdown cells, the highly tumorigenic and metastatic phenotype is rescued.
Sequential steps

In tumors formed by quadruple knockdown cells, a staining for endothelial cells reveals a failure of these cells to assemble into capillaries. Vascular endothelial and smooth muscle cells are present in the tumors, but they fail to assemble into functional vessels. In a permeability assay using red dextran, vessels can be seen to be highly permeable in tumors formed by the control cells, but tumors formed by quadruple knockdown cells are devoid of permeable capillaries. So these genes in combination allow the assembly of a vascular network in these tumors that is typically fenestrated and permeable.

The single knockdowns do not decrease the incidence and growth rate of lung metastasis, as tested by an intravenous inoculation assay. However, the double knockdown of EREG and COX2, and more so the triple and quadruple gene knockdowns, have marked inhibitory effects on lung colonization from the circulation and lung metastasis from mammary tumors.

Interestingly, this inhibitory effect occurs early in the lung colonization process, suggesting that these metastasis genes are required for an early step in the seeding of the lung parenchyma. Indeed, confocal microscopy reveals a decreased rate of tumor cell extravasation from the lung capillaries into the parenchyma. This correlates with a defect in the ability of these cells to migrate across an endothelial cell layer in vitro.

There are pharmacological inhibitors of these genes products in clinical use, including the Cox-2 blocker celecoxib, and the EGFR inhibitor cetuximab. Experimental drugs against MMPs are also available. As in the case of genetic inhibition by means of RNAi-mediated knockdown, effective inhibition of tumor growth and metastasis can be achieved when the drugs are administered in combination, but not singly.

In combination, celecoxib and cetuximab are effective at decreasing the rate of primary tumor growth and the ability of the tumors to seed the lungs. There is also a decrease in intravasation from these tumors into the circulation. The drug combinations are effective at impairing the ability of the tumors to assemble vasculature, to disseminate, and to extravasate into the pulmonary parenchyma.

Similar results are seen with malignant cells isolated from pleural fluids from patients treated at MSKCC. These cells are kept in culture for the minimum time needed to transduce them with luciferase, and then injected into immunodeficient mice to select for lung metastatic variants. The cells selected through one single passage in vivo express increased levels of COX2, EREG and MMP1 compared with the unselected parental cell population. And when these cells are tested for their ability to lodge into the lung, the ones selected in vivo are more aggressive, but their metastatic advantage is blunted by a combined treatment of the mice with cetuximab and celecoxib.

In conclusion, these analyses identify sets of 18 or more genes, constituting a lung metastasis signature that is associated with risk of metastasis to the lung, but not the bone, in patients. Four of these genes act in concert to promote vascular assembly and intravasation in primary tumors, and tumor cell extravasation through the capillary network in the lungs. The combined use of pharmacological inhibitors against these gene products has clinical implications, which may be tested in clinical trials of patients with metastatic disease.

References

**Transcriptional regulation of the metastatic program**

A report on a lecture by

**Zena Werb**

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*Tumor cells must successfully accomplish a number of cellular processes to form metastases. Pathologic analyses of epithelial cancers have established a strong negative correlation between tumor grade and metastasis: well differentiated breast tumors such as fibroadenomas tend to have a low capacity for metastasis formation, whereas poorly differentiated breast tumors such as invasive ductal carcinoma have a high capacity for metastasis, suggesting that loss of differentiation may be a factor in metastasis formation. Zena Werb presented data showing that when gene expression in these two types of tumors is compared, the transcription factor GATA-3 emerges as one of the strongest predictors of tumor grade and prognosis, with its low expression predicting a poor prognosis. GATA-3 specifies and maintains luminal cell differentiation in the mammary gland and is down regulated in carcinomas. Its loss marks the loss of tumor differentiation and the onset of tumor dissemination, and adding back GATA-3 restores tumor differentiation and represses tumor dissemination to distant sites, suggesting that GATA-3 is a bona fide marker of malignant progression in this model.

The program of tumor metastasis has many steps. During this process, tumors lose their differentiation, although this is a step that hasn’t been much studied.

In a mouse mammary tumor model, normal mammary ducts consist of a bilayered epithelium surrounded by adipogenic stroma. As cells progress to the pre-malignant stage, the lumens fill in and there is a gain in proliferation. At the carcinoma stage, the events that facilitate tumor dissemination include a gain of invasive and migratory behavior, and a loss of differentiation. There may be a transcriptional program that is related to this change in differentiation.

The transgenic mouse models of breast cancer have multiple initiating sites in each of ten mammary glands. That makes it difficult to figure out the stepwise processes in this putative program. The MMTV LTR driven polyoma MT model facilitates the study of development of tumors. At three weeks of age, the mammary glands have normal looking ducts and hyperplasias.

Each of these hyperplasias is a clone. When these animals are crossed into animals that express GFP, each one of the hyperplasias can be visualized, excised and transplanted into a recipient mammary fat pad, generating a whole series of tumors, each originating from the single clone. The tumors develop with kinetics similar to the normal multiple tumor system. At various times between 5-18 weeks, the mice are sacrificed or undergo a mastectomy to remove the mammary gland into which the tumor is transplanted.

By three weeks, the tumor starts to grow a little bit, developing into adenomas by five weeks. By eight weeks, they turn into early carcinomas, which then progress to late carcinomas. The time to palpable tumor is 63 days. Between seven and eight weeks after the initial transplantation, cells begin to appear in distant sites.
Cells make it to the lung, which is not surprising for a breast tumor. But tumor cells also appear in many other sites, including spleen, kidney, brain and liver, although metastases form only in the lung.

More and more cells are seeded into the lung over time. In most cases, only one or two foci grow to a big metastasis. On average, between 1 in 100,000 and 1 in a million cells has the ability to make a metastasis.

This model can help study which features — such as tumor size, proliferation and apoptosis, inflammation, angiogenesis, necrosis and differentiation — distinguish those cells that disseminate from those that don’t.

**Malignant conversion**

Compared with tumors that have low or no metastasis, those that show high seeding have large areas of necrosis or hypoxia. They all have lost substantial differentiation, have angiogenesis and huge numbers of inflammatory cells. Of the different characteristics, two events are properties of the tumor cells themselves and two are properties of the microenvironment.

As the tumors progress, fibrillar collagen accumulates in the extracellular matrix as seen by Picro-sirius red and crossed polaroids. Collagen is thought to be the material to encapsulate the tumor but it may in fact be the super highway for tumor cells [see Pollard, page 73]. Collagen genes are usually among the top five up regulated genes in human cancer.

In terms of vasculature, inside the tumor, no cell is more than 50 µm from the nearest capillary. In carcinomas, large numbers of macrophages are present, mainly at the edges of the tumor and some in the tumor itself, but mostly along the blood vessels in the tumor. As seen by *in vivo* imaging, macrophages and other myeloid cells at the margin of the tumor are quite active. They move at about five microns per minute. The ones in the tumor don’t move.

At the sites of the tumor, there is a lot of necrosis and debris. If the debris is taken from a tumor and put into a normal mammary gland, cells see the debris, recognize it and attract other cells, perhaps by sending out a secondary signal.

In the inflammatory response to a tumor, the response is always mediated by recruited cells. The cells that are already present don’t respond to the stimulus. If, instead of debris, bacteria labeled with dTomato are used, there is the same sort of secondary piling up of cells.

**Parallel pathways**

There are many problems that are solved in both development and cancer — for example, proliferation, differentiation, migration, invasion and matrix remodeling. One way to solve the differentiation problem in cancer may be to understand it first in development.

In a microarray profiling of the invasive ends, the terminal end bud and the duct, many interesting genes come up. The most abundant transcription factor in the mammary gland turns out to be GATA-3. There are six members in the GATA family of transcription factors. They play fundamental roles in cell fate specification as a group, and operate in hierarchical networks of transcription factors to activate target genes of the differentiated cell. They bind to a GATA DNA sequence via a DNA-binding domain containing one or two zinc-finger domains. GATA factors are also very important in hematopoiesis.
GATA-3 is present in all luminal epithelial cells. At the terminal end bud there are GATA-3 negative progenitor cells. Mice that have a floxed GATA allele, crossed into the constitutive Cre, the MMTV-Cre, show deletion during puberty and development. With constitutive Cre, the mammary glands look normal at two to three weeks of age, which is pre-puberty, but at puberty, they don’t develop. With an inducible Cre on the whey acidic protein promoter (WAP-rtTA-Cre), doxycycline can be added at any time to delete the GATA gene.

With inducible Cre, within five days of giving mice doxycycline in their drinking water, the luminal epithelial cells show an increase in proliferation followed by the death of most of the cells, very disorganized epithelia and infiltration of inflammatory cells. Eventually there is no epithelium left that has deleted the Cre. But there is some outgrowth because the WAP isn’t expressed in every cell.

Surprisingly, a differentiated adult epithelium, which normally does not proliferate, could begin to do so and then undergo apoptosis in this case. Most important, the loss of GATA triggers a loss in differentiation, suggesting that the GATA factor is required to initiate and maintain the differentiation state.

Among the GATA-3 target genes is FoxA1, which regulates the estrogen receptor. These are all co-regulated in the mouse mammary gland but they may also be co-regulated in human breast cancer.

Interestingly, there are progenitor cells that are GATA negative. Under the influence of GATA-3, they undertake a luminal fate. They don’t default to a myoepithelial fate, and it is unclear what’s involved in this differentiation.

**Crucial loss**

Based on an analysis of all published microarray studies, it’s clear that well differentiated tumors that are estrogen receptor positive have high GATA and a good prognosis with low metastasis. In contrast, poorly differentiated tumors, the invasive ductal carcinomas, are estrogen receptor negative, have low levels of GATA and poor prognosis. Among human breast cancer cell lines, the ones that are poorly metastatic or not metastatic when introduced into mice have GATA expression whereas others, such as MDA231, have very low GATA levels.

In the progression from hyperplasias and adenomas to carcinomas, there is a loss of differentiation genes and of GATA. At the adenoma stage, virtually every cell is GATA positive. As the cells progress to early carcinoma, there are areas still that haven’t become carcinoma that are all full of GATA. By late carcinoma, there’s no GATA expression.

In the early carcinoma, cells begin to break through the basement membrane and enter into the bloodstream. It’s at that point of converting to an early carcinoma that GATA is lost. Cells that disseminate as early as seven to eight weeks express keratin 18 but are all GATA negative. This loss of GATA-3 may be necessary for tumor dissemination in breast cancer, but the loss of GATA doesn’t explain why tumor cells won’t grow in the distant sites.

If primary tumors are taken from a late carcinoma, infected with a retrovirus that expresses GFP, IRES and GATA-3, or an empty vector and transplanted to the mammary gland, an empty vector generates undifferentiated tumors and there’s no GATA expression.
But if GATA is over expressed, the tumor cells are GATA positive and have a very differentiated appearance with ducts and lumens, recreating the signs of tumor differentiation\(^5,6\). These tumors express the redifferentiation genes that are characteristic of the mammary gland, such as caseins and amphiregulin, an estrogen receptor regulated gene.

Dissemination is rare and even when it happens, it is only in a few cells, all GATA negative, suggesting that they have turned off the transfected GATA gene. It’s very clear that differentiation controls a program that determines this dissemination.

The loss of GATA marks the loss of differentiation in breast cancer both in the mouse and in human, and restoring it is sufficient to induce tumor differentiation and prevent metastasis.

References

Breast cancer susceptibility gene 1 (BRCA1) was first identified as a hereditary breast and ovarian cancer susceptibility gene in highly penetrant families. BRCA1 has been implicated in disparate cellular functions, including DNA damage repair, cell cycle checkpoint activation and transcriptional regulation, but how these functions relate to BRCA1 and its role in tumor suppression is unknown. In a brain specific knockout of BRCA1, there is increased apoptosis and defective proliferation in the developing brain, leading to numerous defects in brain structure and organization. Cells devoid of BRCA1 display a defect in constitutive heterochromatin formation and maintenance. BRCA1 binds to satellite repeats within heterochromatin in the mouse. Purified recombinant BRCA1 possesses a RING ubiquitin ligase activity, and preferentially mono-ubiquitinates Histone H2A in vitro. Cells devoid of BRCA1 may be impaired in heterochromatin formation by a failure to ubiquitinate histone H2A. Inder Verma presented a unifying hypothesis that might explain many of the functions that BRCA1 has been attributed with in the past dozen years.

About ten percent of breast cancers are probably due to the two familial predisposition genes, BRCA1 and BRCA2, although there are some other genes that are known to be risk factors.

Of the many different types of breast cancer that have been identified, including those attributed to BRCA1, the most common forms are infiltrating ductal carcinoma, which is seen 63.6% of the time and infiltrating lobular carcinoma, which occurs 5.9% of the time. In the case of BRCA1 and BRCA2, the histopathology is much more aggressive, much more extensive and most of these cancers belong to the infiltrating ductal and lobular categories.

One impressive aspect of BRCA1 is its high preponderance and penetrance. Nearly 90% of those who develop cancer carry this mutation. Younger women with family history, who have two or three cases of different cancers will, by age 30, show between 30 and 40% penetrance. By age 70, there is nearly 90% penetrance.

BRCA1 was identified nearly ten years ago by a group in Utah. The gene codes for a big nuclear protein with unremarkable features. The carboxy terminus is a transcriptional activation domain. If a mutation from a woman with breast cancer is added to the carboxy terminus, it no longer causes transcriptional activation, suggesting that transcription is one of the functions of this gene.

There are some BRCA domains that appear to be involved in DNA damage. The gene has a nuclear localization signal and there are sequences with which RAD51 and MRE11 associate. More important, there is a ubiquitin ligase domain, contributed by the RING domain, which appears to be important.
Over the years, there has been a plethora of research on BRCA1. Dozens of proteins have been associated with it in two-hybrid screens. But it’s a very sticky protein so those results may not carry much significance. The protein is involved in DNA damage repair, cell-cycle control, transcriptional regulation, ubiquitination, cell proliferation, apoptosis and defective X-inactivation. Until recently, BRCA1 was also linked with transcriptional coupled DNA repair, although that has not been reproducible. But among all these activities, which is BRCA’s primary function and which are the work of proteins associated with it?

**BRCA in the brain**

BRCA1 is highly expressed in embryonic neuroectoderm, which is rather unusual. The BRCA1 knockout mouse can be used to ask why the gene is expressed in those tissues.

BRCA1 has a large number of introns and exons in its sequence. One particularly large exon, exon 11, constitutes nearly 70% of the gene. When BRCA1 is deleted, animals die by embryonic days five to seven, and definitely by day nine. If these animals are crossed with p53-negative mice, they do a bit better. In these experiments, almost the entire allele is deleted and the mice have Nestin Cre, which is selectively expressed in the neural progenitors.

Unlike the other knockouts, these animals are born, but they are small. Remarkably, however, they are highly ataxic, very agitated in the absence of the mother, lack coordination and have many of the phenotypes of ataxic mice. Eventually, all of them die, usually by day 12 or 14 but at most by day 20.

The cortex in the mouse is small, but is still of interest because it has an important role in the brain. The size of the cortex in the knockout mice is only about a third the size of the cortex in control animals. The midbrain is not that different, however. Because BRCA1 is a tumor suppressor, the knockout might have been expected to have greater proliferation but in this case, the knockouts eventually die, likely due to defective proliferation.

In brain sections from the control animal, there are six perfectly organized cortical layers, and the dentate gyrus is normal, as is the cerebellum. In the knockout, in contrast, the cortical layers are present, but are pretty disorganized. The cerebellum’s foliation is fully gone, and the dentate gyrus is abnormal. Interestingly, different regions are present, but just not fully differentiated, which is often a hallmark of cancer. More important, by microarray analysis, there are hundreds of unexpected genes that are misexpressed.

For example, ganglionic eminences are the source of interneurons, which connect motor neurons to sensory neurons, and are a major component of the brain. The ganglionic eminences in the BRCA1 knockout mouse are almost gone compared with the control, indicating a clear loss of certain neural cell types due to increased apoptosis and lack of cell growth.

This is even more apparent with BrdU staining. There is a decrease in BrdU staining in the knockout and far fewer cells as compared with the controls. All these results indicate that the loss of BRCA1 leads to the loss of cells, leading to a smaller animal with a smaller brain. BRCA1 is also known to play a role in apoptosis. There’s a substantial increase in apoptosis in the cortex and in the ganglionic eminences as compared with control.

**Chaos in transcription**

Under the microscope, the first surprise is that the deletion of BRCA1 reduces the number of heterochromatic centers per cell in the mouse brain from five in the controls to one in the knockout mouse. In fact, in mouse embryo fibroblasts or other tissues, it is really difficult to spot heterochromatic centers. They are easier to spot in neurons whose heterochromatin is more prominent.
Euchromatin is involved in transcription and heterochromatin is largely responsible for the control of gene expression. There are two types of heterochromatin: facultative and constitutive. Constitutive heterochromatin is responsible for controlling the expression of the major and minor satellite DNA at the centromeres and sub-telomeres.

Normal heterochromatin functioning requires methylation of the histone H3. Suv39h, a major repressing protein, methylates H3 at K9. Once H3 is methylated, it binds the chromodomain of the major heterochromatic protein HP1 and suppresses satellite DNA transcription.

In the knockout mice, there’s a 15- to 20-fold increase in induction of satellite DNA transcripts above the levels seen in the control. This enormous amount of satellite DNA presumably leads to chaos in transcription. Hundreds of genes that should either not be expressed or should be suppressed get turned on. For example IGF2 and H19, which are known to be imprinting genes, are expressed at high levels. So the lack of BRCA1 leads to the high expression of satellite transcripts, which are normally shut off during cell growth and differentiation.

The HCC1937 cell line, a human breast cancer cell line, has a truncated form of BRCA1 and is therefore very useful in studying BRCA1 function. If HCC1937 cells are irradiated, the cells die. If BRCA1 is added back to the cells, they survive. This is a six-week assay, but is more efficient than having to breed mice to study mouse brains.

Because HCC1937 cells don’t have BRCA1, they show a high expression of satellite DNA transcripts. Adding back BRCA1 by infection with a retroviral vector suppresses the expression of the satellite DNA. Adding back BRCA1 from patients whose BRCA1 has mutations in RING domains and in ubiquitin ligase domains gives the opposite result as the wildtype.

Even in HeLa cells, knocking down BRCA1 with siRNA induces satellite DNA. These are easy, convenient assays that can be used with various BRCA1 mutations to ask which ones are responsible for which functions.

**Satellite repression**

ChIP analysis shows that BRCA1 physically associates with both major satellite DNA and minor satellite DNA. At least formally, the ability of BRCA1 to shut off these satellite DNA bands is by virtue of its ability to bind these sequences.

More interestingly, the three forms of HP1, a, b and g, also bind to major and minor satellite DNA and prevent transcription. The sites of BRCA1 binding are the same as of HP1 a and g.

HP1 is involved in repression and performs many of the functions ascribed to BRCA1. For example, HP1 is involved in gene repression by formation of heterochromatin, and gene repression by being part of euchromatin. HP1 can cause transcriptional arrest by directly associating with transcriptional factors. It’s also involved in heterochromatin integrity, binds to telomeres and is a transcriptional activator.

There is a reduction of HP1 in the foci of BRCA1 deficient brains as compared with the controls. In neurons of knockout mice, for example, there are larger, but far fewer, HP1 foci. More important, the cerebellum has less HP1 in the knockouts than in wildtype. But because this is a Nestin Cre mouse which only affects the brain, there is no change in HP1 levels in the thymus of knockout animals.

**Figure 3**  
Deletion of BRCA1 reduces the number of heterochromatic centers/per cell in the mouse brain.

**Figure 4**  
BRCA1-deficient brains show a decrease in heterochromatin protein 1 (HP1) foci.
The deficiency is at the level of the HP1 protein. There’s no effect on the RNA level, so the change could be because of HP1 protein stability of HP1 and not necessarily at the level of HP1 transcription.

Once again, if BRCA in HeLa cells is knocked down with siRNA, there is a reduction of HP1 proteins, particularly HP1-a. RNA is again not affected. BRCA1 only affects major and minor satellite DNA and some imprinted loci transcription in the mouse. It does not affect the LINE elements.

**RINGing the functions**

Histones are modified in various ways. They are acetylated, methylated and, more interestingly, there is ubiquitination of yeast Ub-H2B upon DNA damage, and silencing of Polycomb complex requires Ub-H2A. In the last case, H2A, a histone protein, is ubiquitinated and this has been shown to be important.

BRCA1 has a ubiquitin ligase activity, so there may be a connection with the ubiquitinated histone proteins. In BRCA1 knockouts, for example, the number of ubiquitinated H2A heterochromatic centers is substantially lower as compared with controls. These elements may all be brought together to make a formal model about how heterochromatin might be suppressed.

BRCA1 has a RING domain, which has ubiquitin ligase activity. Another protein called BARD1, which associates with BRCA1, synergizes with the BRCA1 ubiquitin ligase activity. Although BARD1 also has a ring domain, the domain has no ligase activity by itself. BRCA1 and BARD1 can form heterodimers, however. In the absence of BRCA1, BARD1 is a very unstable protein and knockouts of BARD1 are dead by embryonic day 7.5-8.5. H2A can only be ubiquitinated by a combination of BRCA1 and BARD1.

Mutants in the RING domains are the same as in women who have pathological disease. For example, mutant cysteine 24, which was in a patient, does not reconstitute BRCA1 activity in a knockout.

Ubiquitin-H2A binds to satellite DNA in the wildtype but the binding is greatly reduced in the knockout. There is almost no association because BRCA1 is missing, and it’s unable to catalyze the ubiquitination of Histone H2A, and presumably repress transcription of these satellite DNAs.

In conclusion, the loss of HP1 and the loss of ubiquitination of H2A allow for the lack of suppression of the satellite DNA transcription, which then leads to non-specific derepression, and may thereby be responsible for many of the observed functions of BRCA1. All these functions — genomic instability, transcriptional silencing, DNA damage checkpoints, DNA replication and centromere formation — can be explained by the turning on of the heterochromatin.

In the animal model, knocking out BRCA1 leads to a small cortex and ataxia. There are also examples of this in two twin sisters. One sister got epilepsy at age eight and took anti-epileptic drugs, some of which are anti-estrogenic. At age 24, the second sister came down with BRCA1-linked cancer, although both of them are heterozygous for BRCA1. In a brain scan, there’s enormous heterotopia on one side of her cortex as compared to the other. She also has that same sort of ataxic phenotype. The woman is still alive.

The final question is why BRCA1 loss causes breast cancer. BRCA1 is lost in most other tissues but doesn’t cause tumors in other cells. One possibility is that there are other genes that substitute for BRCA1. Perhaps these other genes are present in all other tissues and are missing in breasts.

If HCC1937 BRCA-deficient cells are grown in culture, and infected with each one of a lentiviral library of 17,000 full length cDNAs, the assay identifies five interesting genes, including protein kinase C binding protein (PRKCBP1). But the sequences are not full length, they are only 3’ untranslated regions, suggesting the involvement of non-coding RNA.

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**References**

The progression of a tumor from benign and localized to invasive and metastatic growth is the primary cause of poor clinical outcome in cancer patients. Over expression of RhoC has been shown to enhance the ability of melanoma cells to exit the blood and colonize the lungs. RhoC could lead to increased motility and vascularization of a tumor and possibly increased entry of tumor cells into the bloodstream. To understand how RhoC’s functions are altered in cancer development and particularly in metastasis, mice carrying RhoC mutations have been created. Mice expressing a Polyoma middle T transgene under the MMTV promoter rapidly develop multifocal mammary tumors without evidence of a hyperplasic precursor lesion. Studying the effect of RhoC mutation on the kinetics of mammary tumour occurrence in PyV MT transgenic background supports published data that 100% of PyV MT females develop metastatic lesions in the lung. Tak Mak presented data investigating the mechanism of RhoC mutation on metastasis in this transgenic model.

To what extent is metastasis determined by the ability of the primary tumor cells to survive in a secondary site? RhoC’s involvement in metastasis is one approach to addressing that question.

The absence of RhoC may impair motility and vascularization of a tumor. To assess RhoC’s role in tumor formation and metastasis, knockouts of RhoC are created. The knockout mice are viable, with no difference in T cell and B cell function. Using a MMTV driven Polyoma virus-mT, there is no change in the knockouts in the primary breast site number, the size or structure of tumors, proliferation as measured by Ki67, angiogenesis as measured by CD31 and factor VIII, or the amount of cell death as measured by Tunel.

But RhoC does appear to be essential for metastasis. There is a dramatic decrease in the number of lung metastatic colonies in the heterozygous situation versus the knockouts. More important, the size of the tumors is one log lower, suggesting that there is a substantial decrease in the amount of tumor burden going to the lung.

Other Rho molecules are known to be involved in motility and cell-cell adhesion so it is not completely surprising that there is also a deficiency in motility, as measured by the transwell assays. In the absence of RhoC, tumor cells can still be found in the lung in large numbers. Compared with the heterozygous situation, however, there are many caspase-3 cleaved (apoptotic) breast tumor cells in the lungs of the RhoC-deficient mice, suggesting that some of the tumor cells can travel to the lung but don’t survive.

When mammary tumor cells are established from either wildtype Py-mT or the RhoC Py-mT or RhoC Pymt, maintained in culture for five days and injected into the tail vein of mice, it’s again clear that metastasis to the lung is self autonomous. In other words, it isn’t anything in the environment of the RhoC-deficient lung that’s responsible.
Identifying how RhoC controls metastasis and how that process is regulated may help provide targets for therapeutic intervention.

Caveolin caveats

Caveolin-1 and caveolin-2 are over expressed in inflammatory breast cancer. By immunohistochemistry staining of caveolin-1 and caveolin-2, there is a significant correlation between caveolin-1 or caveolin-2 protein expression and RhoC expression.

Caveolin also interacts with the chaperone complex TCP-1 and modulates its protein folding activity. TCP-1 is a hetero-oligomeric molecular complex composed of eight highly conserved polypeptides. TCP-1 is abundant in the eukaryotic cytosol and is essential for cell survival.

TCP-1 could chaperone caveolin-1 to the cytoskeleton where caveolin-1 binds to filamin, providing evidence for the organization of caveolin-1 associated membrane domains by the actin cytoskeleton. PTP-PEST also docks on filamin. PTP-PEST in turn interacts with a Crk-associated substrate called p130 CAS, which when phosphorylated, induces the cell to move.

Rho GTPases bind caveolin-1, possibly stabilizing it, and triggering a series of events. Knocking down RhoC may affect the phosphorylation of p130 CAS, which is essential for a signal that allows the cell to survive.

In B16 murine melanoma cells, there is a correlation between invasiveness and p130 CAS phosphorylation. There is also a correlation between the level of expression of caveolin-1 and phosphorylation of p130 CAS. Knocking down RhoC reduces the phosphorylation of p130 CAS, but does not down regulate caveolin-1.

There is an 87% overall homology between the Rho family members, RhoA, RhoB and RhoC. All of the members contain a putative caveolin binding domain and in principle, can bind caveolin-1. A construct expressing the caveolin binding sequence can disrupt the putative interaction between RhoC and caveolin, with a resulting effect on p130 CAS phosphorylation, similar to what is seen with in the knock down of RhoC.

Through its effect on p130 CAS, the caveolin binding sequence construct prevents cell migration and invasion. The disruption of the RhoC/caveolin interaction, by over expressing the caveolin binding sequence, has a more severe effect on migration than the RhoC silencing. This effect can be seen in wound healing assays, a migration assay in collagen-coated transwells and in invasion assays through Matrigel. Both over expression of the binding sequence and RhoC silencing have the same inhibitory effect on invasion.

The same experiment can be repeated with MDA231 cell lines using synthetic peptides representing the caveolin binding domain, one with the wildtype sequence and the other with alanine substitutions. The wildtype peptide decreases the phosphorylation of p130 CAS at Y165 and Y410 in a concentration dependent manner. The mutated synthetic peptide doesn’t have the same effect.

This is a preliminary result, but suggests that the mechanism for metastasis is a general mechanism in murine melanoma cells as well as in human adenocarcinoma cells.
The disruption of the Rho/caveolin interaction as well as the silencing of RhoC may affect the activation of Rac1. Use of the caveolin binding sequence or silencing of RhoC reduces Rac1 activation. This is almost certainly the cause of impaired movement in these cells. The projected mechanism is a failure to activate Wave and therefore a failure to initiate actin remodeling to form lamellipodia or invadopodia, necessary for cell migration and invasion.

**PTEN pathways**

It’s not clear what the many therapeutic drugs on the market, such as EGFR or Her2/neu inhibitors, target when they hit tyrosine receptor kinases. It could be the more growth related Ras pathway, which is involved in the cell cycle. On the other hand, the effect on the PI3-kinase component, affecting more the survival pathway, may be the most important target.

The PI3 kinase pathway and the Ras pathway show crosstalk, so Ras is not categorically involved in growth and cell cycle and PI3 kinase in survival, protein synthesis nutrient sensing and hypoxia. But there may be a dominant role for these two pathways in the two categories of cellular function.

Most tumors are hypoxic and the most malignant tumors have been found to prosper under conditions of low oxygen tension or hypoxia. Tumor progression requires an increased adaptation to hypoxia. Hypoxia could be involved in nurturing an environment in which the tumor cells become more aggressive and metastatic. It also increases angiogenesis and drug resistance and may affect glucose metabolism, whether aerobic, anaerobic or glycolysis.

From the viewpoint of the PI3 kinase pathway, some kinds of tumors, such as glioblastomas and endometrial carcinomas, have a high frequency of PTEN mutations. But despite some earlier evidence, many other kinds of cancers don’t have many PTEN mutations. For example, only about 5% of breast cancers have PTEN mutations, and non-small cell lung cancers and cancers of the pancreas, colon and hematopoietic tissues have almost no PTEN mutations.

In the dataset from the Dutch group (see Bishop, page 9), the basal-like subtype, which is generally negative for all three receptors, estrogen receptor, progesterone receptor and HER2, is the most aggressive breast cancer. The luminal A subtype, which has a high level of ER and PR, has an almost normal level of PTEN expression.

Beyond PTEN, the PI3 kinase pathway can integrate multiple inputs during tumorigenesis. There are many different ways to affect the downstream effects of PI3 kinase pathway — through mutations in PTEN or PI3 kinase that confer loss or gain of function, phosphorylation, methylation, crosstalk with p53 or crosstalk with Ras/Raf/ERK.

But even when all this is added up, there still might be a big player that is completely missing. To find this, PTEN is over expressed in the eyes of *Drosophila*, which gives a startlingly dramatic effect. Screening 1,600 mutants reveals DJ-1. Over expression of the fly DJ-1 can rescue the PTEN phenotype, suggesting that DJ-1 opposes PTEN function. DJ-1 is a highly conserved protein, with a nearly 50% homology between *E. coli* and humans.
Parkinson’s connection

DJ-1 has been identified as PARK7, an early onset Parkinson’s disease gene, and it is autosomal recessive. It was first found in Dutch and Italian early-onset Parkinson’s disease families but has since been found all over the world. Although the protein is evolutionarily related’s, it has no known function. It has an important cysteine 106, using which the protein forms a homodimer through a disulfide bond.

But it’s unclear how DJ-1 is involved. If DJ-1 is over expressed, even in the absence of serum, there are high levels of phospho-AKT, phospho-GSK-b, cyclin D1 — almost as if it has the properties of an oncogene. Indeed, one of the earliest studies showed that DJ-1 collaborates with Ras as an oncogene when evaluated by transfection in NIH-3T3 cells.

Because it emerged from a PTEN screen, it’s not inconceivable that DJ-1 inhibits the function of PTEN directly, and not upstream or indirectly. By knocking down DJ-1, phospho-Akt can be completely wiped out, but only when PTEN is present. In the absence of PTEN, that doesn’t work.

Several labs have shown an interesting correlation of DJ-1 with breast, lung and colon cancers. The level of expression of DJ-1 and phospho-Akt in breast tumors has a correlation of 0.007, suggesting that when DJ-1 is over expressed, there is deregulation of the PI3 kinase pathway.

The majority of lung cancers, when matched to their normal counterparts, have high levels of DJ-1 expression. More important, the level of expression of DJ-1 in stage II and stage III lung cancers correlates with survival. This value is approximately 0.027, which is marginally significant.

With stage IV tumors and those with Ras mutations, the correlation disappears, suggesting that it is an early event that puts the brake on the whole system. But with stage I tumors, the correlation to patient survival becomes highly significant, with a p value of 0.0069. In other words, DJ-1 raises the risk of early relapse and death two- to three-fold. As stage I lung cancer is mainly treated by resection, so DJ-1 may play a role in metastasis.

Although DJ-1 is an early onset Parkinson’s disease gene, the knockout does not have a phenotype. If neuronal cells are treated with hydrogen peroxide or the oxidative stress agent MPTP, those with lower levels of DJ-1 are more likely to die. There is no difference in the response to other apoptotic stimuli, such as camptothecin, radiation and UV. The only difference is in response to reactive oxidative species.

When dopaminergic neurons are treated with MPTP in the absence of DJ-1, the mice come down with more severe Parkinson’s disease-like symptoms. In individuals without DJ-1, nothing presumably happens until they’re 30 or 40 years old, and then perhaps in response to some stimuli, the number of dopaminergic neurons become scarce and Parkinson’s disease sets in.

Low oxygen response

DJ-1 also has a role in hypoxia, which might provide an explanation for its involvement in metastasis. If DJ-1 is knocked down from human lung cell lines and the cells are then exposed to hypoxia, there is a dramatic increase in cell death. DJ-1 knockouts are more sensitive to hypoxia-induced apoptosis.
When DJ-1 wildtype cells are shifted to hypoxia, vascular endothelial growth factor (VEGF) is induced. In the absence of DJ-1, there is less VEGF, even when there is no change in DJ-1 levels in hypoxia. For some reason, higher levels of DJ-1 induce higher expression of VEGF, presumably leading to the creation of more blood vessels and more metastasis, or less cell death. In the absence of p53, there is more DJ-1 than in the presence of p53. The proteins pulled out in yeast two-hybrid experiments show these experiments include 14-3-3-theta, which is known to be highly expressed in lung cancer, glyceraldehyde 3-phosphate dehydrogenase (Gapdh) and cytochrome c oxidase CoxII.

These all suggest a link with metabolism. DJ-1 appears to play a role in hypoxia, and perhaps in shifting metabolic pathways.

Cancer cells wire themselves completely differently in terms of metabolism. Warburg said in 1920s that tumor cells mainly rely on glycolysis, which is inefficient and generates only 2 ATPs per molecule, compared with 36 ATPs generated during normal glucose metabolism, if it is metabolized in the Krebs cycle. What’s more, some tumor cells lines are estimated to burn up to 400 times more ATP than normal cells — although these estimates are based on *in vitro* observations.

So what do cancer cells burn, if it’s not glucose?

Withdrawing glucose from cells up regulates p53, while the deprivation of oxygen from cells activates HIF-1a. If cancer cells switch from low glucose to low oxygen and they can still survive with a high rate of metabolism, they must rely on an alternative energy source. In order to do this, they must activate genes that can steer the cancer cells towards a different metabolic pathway.

Using a temperature-sensitive p53, genes that are activated by this tumor suppressor are identified. These genes are assessed to see whether they are over expressed with stress stimuli such as staurosporin or 5FU, and whether they are over expressed in a hypoxic situation. Knockdown of these genes using RNAi shows that the cells are sensitive to hypoxia and glucose deprivation, implying that they code for alternate metabolic pathways used by tumor cells in low oxygen and glucose conditions.

All of these observations lead to the suggestion that it may be time to reconsider metabolic targets in cancer.
PART II: MOTILITY AND INVASIVENESS

Robert Weinberg
Mechanisms of malignant progression

Daniel Louvard
Fascin, a novel target of b-catenin-Tcf signaling, is expressed at the invasive front of human colon cancer

Gerhard Christofori
Distinct mechanisms of tumor cell invasion and metastasis

Douglas Hanahan
Multiple parameters influence acquisition by solid tumors of a capability for invasive growth
The progression of primary tumors to disseminated metastases may be a result of selection — in which rare variants from the primary tumor are selected to metastasize — or of adaptation of the primary tumor cells to the stroma in which they are located. The epithelial-mesenchymal transition (EMT) is a process that many carcinoma cells undergo in order to acquire traits such as motility, invasiveness and increased resistance to apoptosis. Many of the transcription factors responsible for programming EMTs during embryonic development are used by carcinoma cells to acquire the traits associated with high-grade malignancy. EMT is induced in carcinoma cells in response to heterotypic signals released by mesenchymal cells within the stroma. Once carcinoma cells land at distant sites, they must adapt to the local microenvironment, which is unlikely to be hospitable to the cells. Robert Weinberg discussed how selective pressure may favor the outgrowth of micrometastatic cells that can assemble traits that enable their survival and proliferation, making selection crucial at the last step of the invasion-metastasis cascade.

Just five changes are enough to transform a normal human cell into a tumor cell. These five changes can take human cells through to a primary carcinoma, the penultimate product in tumor progression. But the resulting tumors rarely, if ever, invade and metastasize, indicating that there are likely to be other determinants for those last steps.

A new medium for propagating human mammary epithelial cells, derived from organoids from reduction mammoplasties, has helped create BPLER cells. These are in contrast to HME cells, which are propagated in the standard medium. These two cell types have distinct gene expression array patterns, and the cells grown in one medium cannot be switched to the other.

The two cell types remain polyclonal throughout the course of their derivation, suggesting that no other mutational events defined their genomes; the two cell types are genetically identical, but epigenetically programmed differently.

The BPLER cells create invasive ductal carcinomas of the breast, similar to those seen routinely in the clinic and have a desmoplastic rich stroma as seen with a smooth muscle actin stain. In contrast, in the squamous cell carcinoma made by HME cells, smooth muscle actin is present only in the pericytes surrounding the vessels.

Another crucial difference between the cell types is that BPLER cells are metastatic and HMLER cells are not, suggesting that the differentiation program of a normal cell-of-origin may determine the behavior of the...
tumor long before tumor progression has proceeded to the penultimate step. Understanding metastasis will therefore require knowledge of this differentiation program.

In ductal carcinomas created from BPLER cells, tumor-initiating cells make up between a tenth and a hundredth of the total cell population, compared with traditional tumor cell lines, in which a million cells are needed to seed a tumor. These cells may therefore have self-renewing cancer stem cells present at a high frequency.

The much higher metastatic capability of the BPLER cells may in part be because they have a higher percentage of these cancer stem cells, compared with the squamous cell carcinomas. Thus, self-renewing cells that metastasize have the possibility of growing into macroscopic metastases, while disseminating cells that lack self-renewal capability will be unable to spawn macroscopic growths.

**Complex cascade**

For the last step in the invasion metastasis cascade, during which a micrometastasis grows into a macrometastasis, at least two things are required: the disseminating cell must either have self-renewing capability or the ability to acquire self-renewing capability and, second, it must adapt to the local tissue microenvironment in which it happens to land (See Massague, page 15).

The complexity of the invasion metastasis cascade rivals that of the steps leading to the formation of the primary tumor in the so-called Vogelgram. But, based on mouse breast cancers and expression array analysis, the number of genetic alterations required for the metastasis cascade may be simpler than for tumor progression.

The second most over expressed gene in a line of highly metastatic mouse breast cancer cells is Twist. Twist was originally discovered in the context of *Drosophila* developmental biology, during which cells undergo epithelial mesenchymal transition (EMT). One way by which cancer cells may acquire traits of high-grade malignancy is to resurrect the genetic programs that are used in early embryogenesis and perhaps transiently in wound healing.

If BPLER cells are planted in a mouse stroma, the human cells shut down expression of cytokeratin and instead express vimentin, indicating that these cells have undergone EMT — triggered not by an additional mutation, but rather by the heterotypic signals from the stroma. Identifying these signals is crucial for understanding cancer progression.

At least a dozen papers have shown over the past years that mesenchymal stem cells (MSCs) are recruited in large numbers to the tumor-associated stroma. Admixing the MSCs to MDA-231 breast cancer cells has a minimal effect on the ability of those cells to form a primary tumor, but increases the number of metastases five- or six-fold.

If the metastatic cells resulting from the MSC admixture are taken out, grown *in vitro* and reimplanted back on their own, they no longer have a high metastatic competence, indicating that the effect of the MSCs is an adaptation rather than a selection.
Signaling metastasis

When MSCs and cancer cells are mixed together and analyzed for cytokines or chemokines expressed at higher levels than in either of the parental populations, one cytokine, called RANTES or CCL5, shows a 60-fold increase in its levels.

If CCL5 production in the MSCs is shut down, CCL5 is no longer produced in the mixed culture. But using shRNAs in the cancer cells has virtually no effect on CCL5 production, indicating that the CCL5 production is elicited in the MSCs in response to their contact with breast cancer cells.

This enhanced metastasis can be mimicked by ectopically expressing CCL5 in the breast cancer cells, or by taking normally inactive fibroblasts, forcing them to produce CCL5 with an expression vector and commingling them with breast cancer cells. CCL5 is both necessary and sufficient for this reversible induction of metastatic ability. Thus, once cancer cells leave the primary tumor and reach the lungs, they forget their previously acquired ability to metastasize.

But these results don’t explain how cancer cells acquire the multiple phenotypes associated with an EMT. The Twist transcription factor, when it is ectopically expressed, can induce the scattering associated with an EMT. Twist is a pleiotropically acting transcription factor, which can cause the down regulation of several epithelial markers and induce many mesenchymal markers.

Shutting down Twist expression in the otherwise highly metastatic mouse breast cancer cells has no result on the rate of growth of the cells implanted subcutaneously. Loss of Twist also has no effect on their growth rate in vitro.

But the absence of Twist leads to an 85% reduction in lung metastasis. Importantly, all of the metastases that survive the shRNA treatment continue to show high levels of Twist expression, indicating that they derive from cancer cells in the primary tumor in which Twist expression was never successfully shut down.

Twist confers motility, invasiveness and resistance to apoptosis. It affects the invasion-metastasis cascade until its penultimate step – the formation of a micrometastasis, but not the last step of macrometastasis formation. That last step appears to be solved locally, when disseminated cells attempt to adapt to the foreign microenvironment in which they have landed.

All this suggests that rather than a series of mutations, metastasis may require the activation of an embryonic transcription factor or factors by contextual signals received from the microenvironment.

Multiple players

There is a series of other transcription factors associated with cancer pathogenesis. One of these, Goosecoid, is known to be active in the Spemann organizer at the blastopore lip of a Xenopus embryo. When ectopically expressed in breast cancer cells, Goosecoid greatly enhances metastatic capability. Goosecoid is also able to program the various steps of the EMT, but may not be sufficient on its own to push cells to metastasize.
A third transcription factor, Slug, was discovered during the study of melanocyte transformation. Slug is expressed upon transformation of these cells at levels as much as 1000-fold higher than in human breast cancer cells. Slug is the same transcription factor that the ancestors of melanocytes, when they migrate from the neural crest, use in order to disperse throughout the skin.

Shutting down Slug with siRNA results in a 93% decrease in the metastatic competence of experimentally transformed melanocytes. As is the case with the other EMT-inducing transcription factors Slug may collaborate with other transcription factors to induce invasion and metastasis.

A fourth transcription factor is called FoxC2, known to be important for specifying mesodermal fate and discovered by gene expression array analysis. FoxC2 is only highly expressed in the nuclei of the most metastatic of breast cancer cells. Also known as mesenchyme forkhead1, it induces a 30-fold increase in the motility of cells and an equally large increase in their invasiveness. It also induces the release of matrix metalloproteases 2 and 9 in large amounts.

FoxC2 has a strong association with basaloid cancers of the breast, which are triple receptor negative, lacking the estrogen receptor (ER), progesterone receptor and HER2/Neu. Interestingly, these basaloid cancers stain for both cytokeratin and vimentin, suggesting that EMT is not a binary step where cells are either epithelial or mesenchymal; many cancer cells may be only part way down the road from an epithelial to a mesenchymal state.

**Working in tandem**

Basaloid cancers constitute 15% of the total breast cancer load and have a poor prognosis. About 44% of these cancers express FoxC2, but only 1.5% of the far more common luminal cancers, which constitute about 70% of breast cancer burden in the clinic, express FoxC2 at high levels.

There’s a similar association of FoxC2 with ER-negative tumors, which have a worse prognosis than the ER-positive tumors, suggesting that FOXC2 may be responsible for some of the malignant properties of these cells. Unlike other transcription factors, FoxC2 is good at inducing mesenchymal markers, but rather poor at shutting down preexisting epithelial markers.

There are various other transcription factors, including Twist, Snail and Goosecoid, that can induce FoxC2 expression. Given FOXC2’s potency in inducing mesenchymal traits, it may assume the job of programming the mesenchymal portion of EMT, and the other transcription factors may take on the job of shutting down pre-existing epithelial genes. None of these transcription factors appears to act on its own, instead acting collaboratively.

FoxC2 induction causes cancer cells to release TGF-b1. At the same time, if TGF-b1 is added to epithelial cells or to transformed breast cancer cells, FoxC2 expression is induced, creating a self-reinforcing positive loop. These feed forward loops may in part be responsible for the sluggish induction of many of the biochemical traits associated with the EMT.

TGF-b1 also induces the expression of various transcription factors, each of which can program EMT. FoxC2 can be induced by these other transcription factors even in the absence of TGF-b1 signaling.
In different kinds of human tumors, multiple transcription factors can induce EMT, probably acting in combination with each other. In this model ‘interactome’, each transcription factor can turn on several of the others in a complicated circuit.

The nature of the contextual signals that impinge on this from the outside of the cell are unknown. TGF-β1 is undoubtedly one of these signals, but in a living tumor, it has not been proven to be enough to induce EMT.

A subset of human cancer cells have permanently lost E-cadherin, shutting it down either through promoter methylation, or through nonsense mutations in the reading frame, or through gene deletion. This has implications for the biology of EMT.

Among the cytoplasmic proteins that link E-cadherin to the actin cytoskeleton are α-catenin, β-catenin and p120 catenin (See Clevers, page 105). When E-cadherin is removed from the cell, in principle, β-catenin can move to the nucleus, where it collaborates with the TCF/Lef transcription factors to activate transcription.

If E-cadherin expression is shut down, either using shRNA or by expressing dominant negative E-Cadherin — the former removes almost all E-Cadherin from the cell surface and the latter removes only the protein’s ecto-domain — islands of epithelial cells disperse.

Both of these perturbations have little effect on the overall tumor size. But if the cells are injected in an orthotopic site, there’s a profound difference in metastases. What’s more, use of the shRNA triggers an EMT, inducing vimentin and N-cadherin and reducing levels of α-catenin and γ-catenin. This EMT is accompanied by the acquisition of a strong degree of motility. In contrast, the dominant negative E-cadherin, which lacks the ectodomain but has the cytoplasmic domain, does not evoke this EMT response.

**Double jeopardy**

If, in addition to shutting down E-cadherin, β-catenin is also shut down, then EMT is not induced, suggesting that the β-catenin liberated into the cytosol when E-Cadherin is removed is responsible for evoking these traits by activating transcription.

In the absence of β-catenin, the cells don’t acquire highly metastatic traits — and this adds another component to the interactome. It’s known that Twist can shut down E-cadherin and E-cadherin can shut down β-catenin. But it now appears that β-catenin can turn on Twist.

This self-sustaining positive feedback loop may also be responsible for maintaining the mesenchymal state and has implications for the notion of selection versus adaptation. If the E-cadherin gene is lost through mutation or promoter methylation, for instance, that trait is permanently in the descendant cancer cells and therefore on its own creates a mesenchymal state that may be a selectively advantageous trait.

If BPLER cells, which form aggressively growing tumors, are grown on one side of the mouse, slowly growing HMLER cells placed on the other side of the mouse don’t do anything after 8 weeks.

But if the HMLER cells are grown contralateral to the BPLER cells, they start growing after about 40 days. If the BPLER cells are grown on their own for 30 days and then the HMLER cells are put in, the HMLER cells start taking off after just 8 or 9 days, suggesting that there’s some kind of contralateral influence.

Even in a mouse that has a relatively small tumor burden, the bone marrow is already perturbed and has cells that, when mobilized into the circulation, can be recruited by slowly growing tumor cells. This effect can be recapitulated by extracting bone marrow cells from the tumor-bearing mice and admixing them directly with the slowly growing tumor cells.
In conclusion, among the rate-limiting determinants of metastasis is the importance of the cell of origin; the self-renewing ability of the cell leaving the primary tumor; the genotype of the tumor cells, which may affect their ability to respond to heterotypic signals from the stroma; the nature of these signals; the ability of the primary tumor to mobilize cells from the bone marrow; and the ability of disseminated cancer cells to recruit the activated bone marrow precursors from the circulation.
Colorectal carcinomas carry mutations in a variety of oncogenes and tumor suppressor genes that contribute to tumorigenesis. However, not much is known about mutations that contribute to the formation of metastases. Fascin1 is an actin bundling protein that is essential for the formation of sensory organelles known as filopodia, which are thought to be a critical hallmark of the invasive phenotype. By participating in filopodia formation, fascin1 may promote cell migration. Fascin1 is absent from normal epithelial cells, but high levels have been reported in many types of cancer cells. Daniel Louvard presented evidence that fascin1 is a potential target of $\beta$-catenin-TCF signaling in colon cancer cells. Fascin1 is exclusively localized at the invasive front of tumors also displaying nuclear $\beta$-catenin. Forced expression of fascin1 in colon cancer cells increases their migration and invasion in cell lines and causes cell dissemination and metastasis in vivo. Genes involved in cell migration and invasion, such as fascin1, could serve as novel targets for anti-metastatic therapy.

Many of the details of the initial events in colorectal cancer are known. The loss of control correlates well with histology and the invasiveness of the tumor arising in the gut. At the point of real metastasis, cells start to move from the primary site to distant metastases. For gut and colonic cancer, the primary organ of metastasis is the liver. More information on the biology of this process would help develop therapies and diagnostic tools.

Over the past decade, working on the cell biology of the intestinal cell led to the discovery of a gene that has helped to study morphogenesis in epithelial cells and to control the expression of virus transgenes. The villin gene is highly specific to intestinal epithelial cells and is expressed in all lineages of differentiated cells. Animal models have shown that it is also expressed in intestinal stem cells.

To develop a better model of intestinal tumors than those that exist, the villin promoter is used to target oncogenes, tumor suppressor genes and other genes of interest and to develop various animal models. Based on available mouse models, mice that have a single transgene, either APC or K-Ras, gives rise to very few tumors, and don’t kill the mice very quickly. But the combination of Ras and APC, the double transgene, generates a large number of tumors. These double transgenic mice have a limited life expectancy.

Tumors in these mice display the characteristics of invasive adenocarcinoma, but there has not been any evidence of liver metastasis. The mice die before the metastasis stage as a result of numerous tumors. None of the existing models generate liver metastases in a reproducible manner.

What’s needed for the initial steps of metastasis is the degradation of the basement membrane, an epithelial-mesenchymal transition (EMT) and migration of the cells. There are a number of gene candidates that can accomplish these complex actions. Candidates of interest include genes involved in cell motility and the actin cytoskeleton.
It is well accepted that differentiated intestinal cells emerge from the crypt, move rapidly toward the tip of the villus and begin to die by anoikis (See Clevers, page 105).

But the question is, how do these cells move? Normal epithelial cells do not move but form tight cell-cell adhesions. The mechanism for the movement of these cells, whether they move actively, or whether they’re being moved by the underlying mesenchymal tissue, has not been resolved in the gut and in many other tissues. What’s more, how do malignant cells move from the primary tumor site to distant metastatic location?

**Dynamic control**

A scan of the literature for candidates involved in cell motility comes up with a little-known molecule called fascin. This molecule controls actin dynamics and the formation of a specialized structure at the surface of a eukaryotic cell that triggers cell motility.\(^2\)

Fascin is expressed in specialized cells such as neurons and mature dendritic cells — but not immature dendritic cells — and in various tumor cells. It is a globular monomer with two actin binding sites, which makes the protein a very potent cross-linker for actin filaments.

The protein is localized in specialized structures called filopodia, which are in the lamellipodia, which in migrating fibroblasts or migrating cells is the locomotion apparatus in which actin dynamics takes place.

The filopodia have a tight bundle of actin filaments and are not static. Filopodia, structures that are numerous in neuronal cells, behave like sensory organelles, playing a role not in the locomotion *per se*, but in orientation and navigation of the cells.

Fascin is a major structural protein in filopodia, and an actin-binding protein, different from other such proteins in the lamellipodia. If the protein is deleted using siRNA, the cells no longer produce filopodia.\(^3\) The number of filopodia is reduced and those that remain have aberrant structures.

Phosphorylation of the fascin protein demonstrates the link between its structural aspect and cell signaling. If an important serine residue is mutated to alanine, the protein is constitutively active and makes very big filopodia. The phosphorylated form of fascin protein is inactive and the protein must be dephosphorylated to be activated. Protein kinase C is responsible for phosphorylating fascin, but the phosphatase that activates fascin is unknown.\(^3\)
Filopodia are built by using the existing network of filaments and by recruiting fascin, when it is expressed or when it is activated, forming a highly specialized structure.

Normal intestinal cells do not produce fascin, but malignant intestinal cells do. Fascin’s expression correlates with the grade of the tumor as defined by pathologists. When the tumor is more advanced, as defined by the international classification of colorectal cancer stages 1 through 4, 4 being the metastatic stage, fascin is expressed.

Intriguingly, fascin is expressed in a very peculiar position in the tumor. In the normal gut, epithelial cells do not express fascin, but endothelial cells express it at high levels. Fascin is not expressed at the center of the tumor, but at the invasive front, where the tumor progresses, its expression is significant.

**Inducing invasion**

If SW480 cells derived from human adenocarcinoma are used to try to mimic the contrast of migratory cells with cells that have cell-cell contact, in a sparse culture, the level of expression is high. If the culture is denser, the level of fascin is down. Interestingly, in the dense culture, β-catenin is seen at points of cell-cell contact but in sparse culture, β-catenin is often found accumulated in the nucleus.

In contrast with SW480 cells, HT29 cells, also derived from a human adenocarcinoma, do not express fascin. If HT29 cells are induced to express fascin with GFP, the cells that express the transgene for fascin can easily be visualized. From a structural point of view, fascin accumulates in the dorsal region, where many microvilli exist. When it is expressed, fascin can interact with the actin binding protein in microvilli, and also induce filopodia at the ventral surface of the cells.

In a migration or invasion assay in transwell filters coated with collagen or matrigel, cells that express fascin have an increased migration efficacy as well as increased invasion activity as compared with either GFP alone or the control, HT29 wildtype.

In a second assay, HT29 cells are injected into SCID mice by tail vein injection, and analyzed a few weeks later for the presence of lung metastases. Mice that express fascin-GFP have an enhanced capacity to produce large number of lung metastases. The foci of these cells express fascin and villin, which is an intestinal marker, not expressed in the lung.

Even among the few metastases from wildtype HT29 cells that express villin, there are a few cells expressing endogenous fascin, not derived from the fascin that is artificially introduced into the system.

Epithelial cells undergo EMT during migration to their secondary sites. Epithelial markers and mesenchymal markers can be used to define EMT. By definition, epithelial markers should be down and mesenchymal markers should be up during the transition.
When human metastases in the liver or at the site of invasion in the primary tumor are analyzed, however, the cells do not lose cytokeratin in the liver and do not express vimentin, a mesenchymal marker. Similarly, the epithelial marker E-cadherin is not lost. These observations suggest that when colorectal tumors undergo metastasis, they do not undergo classical EMT.

During metastasis, colorectal cancers may preferentially move as a cluster. If they do undergo EMT and lose E-cadherin, it must be too transient to be detected. Further study and in vivo imaging can help answer whether a combination of strategies or only one strategy is used in this situation.

In an invasive primary tumor, the level of expression of fascin correlates with the degree of invasion as defined by pathologists. There is also a correlation with lymph node status: the more lymph node invasion there is, the more fascin is found expressed in the primary tumor. Finally, if at the time of biopsy, the patient already has distant metastases, the level of fascin in the primary tumor is higher.

The vast majority of liver metastases from human specimens are made up of differentiated cells. Epithelial cells of the malignant tissue are fascin-negative; endothelial cells are fascin-positive as are the few undifferentiated cells in the metastasis.

**Tight regulation**

Bearing in mind the importance of β-catenin and the Wnt signaling pathway in colorectal tumor progression, one possibility is that the fascin promoter is tightly regulated during the metastatic process by the Wnt signaling pathway or by nuclear β-catenin.

It’s well known that in the adenoma, there is activation of c-Myc and cyclin D, for instance, through β-catenin signaling. And in the next phase, other genes such as L1, MMP and MT1 are activated. Fascin may be another gene that is activated at this time of tumor progression, although how fascin expression is spatially and temporally regulated is still not known.

The promoter for human and mouse fascin genes has at least five putative TCF binding sites, which is consistent with its regulation by β-catenin, which acts on the TCF binding site [See Clevers, page 105].

When a construct of the fascin promoter with luciferase is used in cell culture, adding β-catenin enhances the activity of the luciferase. A non-functional TCF co-transfected with an active β-catenin can abrogate this response.

If the fascin promoter is hooked to GFP, the promoter drives the expression of GFP in SW480 cells. In contrast, in HT29 cells, which don’t have an activated β-catenin signaling pathway, GFP is not produced. Adding an activated β-catenin — by deletion of the 89 first amino acids — drives expression of GFP in these cells.

Finally, using chromatin immunoprecipitation and various probes for RT-PCR, an antibody against TCF4 pulls down at least three of the putative TCF binding sites on the fascin promoter, suggesting that fascin is a direct target of the β-catenin-TCF complex.

The role of fascin in colon cancer metastasis is complex. The protein is tightly regulated in space and time. During tumor progression, there is an accumulation of β-catenin in the nucleus and when it reaches a certain threshold, perhaps also as a result of other accessory proteins, that triggers the expression of fascin and the cells start to migrate away from the primary site.
The cells journey across endothelial tissue, extravasate and reach a secondary site where fascin expression is turned off. The cells then reestablish differentiation, b-catenin leaves the nucleus, cell-cell contact is resumed and the cells establish a metastasis that can no longer move.

This animal model is useful because the fascin gene drives further the process of tumor progression and metastasis before killing the animal. By developing a triple transgene that combines APC, K-Ras and the fascin gene expressed under the control of the Villin promoter, crossed with a promoter for Villin-Cre under the control of tamoxifen, fascin can be expressed at any time in these mice. For instance, the triple transgenic animal can be allowed to develop tumors and then fascin activated when the tumor has already developed.

There are tools already available to study how these tumors move. By immuno-localization of fascin in epithelial cells, fascin has no effect on cell proliferation and organization and finds its place in the brush border.

Using two-photon microscopy, it is possible to visualize the movement of cells from the bottom of crypt to the villus. Once the transgenic mice develop tumors, fascin can be activated and its effect on the movement of tumor cells assessed.
Tumor cell invasion into the surrounding tissue can occur by either single cell or collective cell migration. Single cell migration is usually accompanied by an epithelial-mesenchymal transition (EMT), which involves several genetic and epigenetic alterations. The loss of E-cadherin function appears to play a central role in triggering EMT in a number of epithelial cell types. Up regulated expression of neuronal cell adhesion molecule (NCAM) is an early event during EMT induced by the loss of E-cadherin. Deletion of E-cadherin is accompanied by higher NCAM expression at the invasive tumor front. NCAM localizes to lipid rafts and switches its signaling complex to activate β1-integrin-mediated cell adhesion, an increased number of focal adhesion contacts and cell migration. Gerhard Christofori proposed the existence of at least three distinct mechanisms of metastatic tumor cell dissemination: single cell invasion involving EMT, collective cell migration in the absence of EMT, and lymphogenic dissemination to local lymph nodes facilitated by increased tumor lymphangiogenesis.

Tumor progression often involves the transition from a benign tumor — which in the case of epithelial cells is called an adenoma — to a carcinoma, at which stage the tumor cells profoundly change their behavior and identity. The cells permeate the basal lamina and invade into surrounding tissue to disseminate either by the lymphogenic pathway to reach lymph nodes, or by the hematogenic pathway to distant organs, where they seed metastases.

This transition involves several molecular genetic and epigenetic changes. One model of invasion is for the tumor as a collective tissue to invade into surrounding tissue. But in the classical model of metastasis, single cells invade into surrounding tissue and dramatically remodel it, along with the release of growth factors and the involvement of various cells of the immune system. Both these pathways of invasion exist. The classical model of metastasis involves the loss of cell-cell adhesion. The dissociation of tight junctions and adhesion junctions occurs via an epithelial-mesenchymal transition (EMT), a multi-stage process during which sessile epithelial cells, which hold on to each other and are highly differentiated, are transformed into migratory mesenchymal cells.

This transition goes hand in hand with changes in gene expression. There is a loss of E-cadherin and of tight junction proteins such as claudins, occludins, desmosomal proteins and cytokeratins, and a simultaneous gain of mesenchymal markers such as fibronectin, vitronectin and vimentin. EMT is a reversible process.
Many metastases, for example in colorectal cancer, express E-cadherin. There is a transition period during which the cells on the invasive front, which have disseminated as single cells, have lost E-cadherin. But once they have seeded into the target organ, they most likely re-differentiate and re-express E-cadherin to form a tumor or a metastasis that is very similar to the primary tumor.

EMT also underlies many processes in development, for example in gastrulation or in the neural crest. The reverse of EMT, or MET, is found in the development of the kidney.

E-cadherin, the central player in EMT, is a cell adhesion molecule. It is a prototype family member of the cadherin family and essentially makes up the epithelium. One molecule of E-cadherin recognizes and binds a molecule on a different cell, resulting in the adhesion of the two cells.

When the cells lose E-cadherin, they become migratory. In a tumor in which only half the cells have lost E-cadherin, the nuclei of the cells that still express E-cadherin are homogeneous in size and roundness, whereas the cells that have lost E-cadherin have nuclei that are big or elongated, indicating that the cells have started to migrate and pull away. There are many ways to get rid of E-cadherin, including proteolytic degradation of the extracellular domain and transcriptionally repressing E-cadherin via signaling pathways.

**A family of Snails**

In a hepatocellular carcinoma model, the hepatocyte growth factor (HGF), also called scatter factor, can induce scattering or migration of cells by repressing E-cadherin. HGF binds to its cognate receptor c-MET and the classical tyrosine kinase signal transduction leads to the activation of the MAP kinase pathway, which then activates c-Fos. One of the genes that is involved early on in this pathway is EGR-1, the early growth response gene, which is up regulated either by MAP kinase directly or by c-Fos.

EGR-1 is a transcriptional activator of Snail, which binds to the E-cadherin promoter. This represses the expression of E-cadherin and of Claudin-3. Snail also represses its own expression and the expression of EGR-1. Within the first few hours of HGF expression, there is a peak of EGR-1 and it is then down regulated. Snail, which is a very unstable protein, comes up later on. Snail is phosphorylated by GSK3-β in the nucleus. The first phosphorylation allows the protein to be exported. The second phosphorylation earmarks it for degradation.

The E-cadherin promoter is hypermethylated in many cancer types. Initial repression by Snail may lead to long-term repression by hypermethylation and, later on, even by epigenetic down-modulation of the E-cadherin locus. That hasn’t yet been proven, however.

E-cadherin can also be regulated by any of the other members of the Snail family of proteins, which includes Slug, Twist (see Weinberg, page 37), ZEB1 and 2 and a few other transcriptional repressors. These proteins are activated by TGF-b signaling. Also, Wnt signaling, certain growth factors, Notch and hypoxia can all up regulate Snail genes. EMT is thus securely controlled because Snail proteins are so tightly regulated, have a short half life and are kept in check at all times — apart from in cancer.
After cells lose their adhesion ability, they need to gain the aggressiveness needed to migrate and invade tissues. To study the signals downstream of the loss of E-cadherin, the Rip1Tag2 mouse is a useful model (see Hanahan, page 55). In this model, the rat insulin promoter is used to drive expression of the SV40 T antigen in the insulin-producing beta cells of the islets of Langerhans.

These mice recapitulate the multistage tumor development seen in most human cancers, including early hyperplasia, the angiogenic switch, formation of a benign tumor and carcinomas. The mice do not usually metastasize because they succumb to hypoglycemia from an excess of insulin, but they can be modulated at certain times to produce metastasis.

**Cause or consequence?**

In an adenoma that is still encapsulated, the tumor cells are still benign with defined nuclei and express E-cadherin. In the case of an invasive carcinoma, the cells have invaded into the exocrine pancreas, are heterogeneous with less cytoplasm and have almost completely lost E-cadherin expression.

To address whether the loss of E-cadherin the cause or consequence of tumor progression, transgenic mice have been generated that use the insulin promoter to express wildtype E-cadherin. Such b cell-specific expression of E-cadherin makes it impossible for the invading cells to lose E-cadherin.

When these mice are crossed to get double transgenics, tumor progression is arrested at the benign stage, generating mostly adenomas and no carcinomas. Conversely, expressing a dominant negative E-cadherin causes accelerated invasion with carcinomas and, for the first time, metastasis. This indicates that the loss of E-cadherin makes tumor cells invasive.

A newer method for testing this is to use E-cadherin floxed mice, which generate early hyperplastic lesions. Even early in hyperplasia in these mice, the cells are already invasive. These mice later develop lymph node metastasis and lung metastasis, which in Rip1Tag2 mice has only rarely been discovered. So triggering the loss of E-cadherin creates a highly invasive model.

Using MCF7 breast cancer cells to study the signals downstream of E-cadherin also reveals that stable knockdowns of E-cadherin show an increase in migration and invasion. In E-cadherin floxed cell lines, created by crossing floxed mice with MMTV-NEU mouse models, the loss of E-cadherin triggers EMT. The third model is the well established NMuMG system with normal murine mammary gland epithelial cells, which undergo EMT upon stimulation with TGF-β.

By microarray analysis, more than 400 genes are up regulated when E-cadherin is lost. Based on the kinetics of gene expression, these genes are clustered into early and late genes, which may serve as markers for early and late EMT, and help diagnose a tumor as early or late in the invasion process.

**Signaling shift**

Of the 400 genes, neural cell adhesion molecule (NCAM) is one of the earliest, and is expressed right after E-cadherin is knocked out. NCAM is an Ig-domain cell adhesion molecule that comes in three differently spliced isoforms, a GPI-anchored isoform and two transmembrane isoforms.

In a model of lobular breast cancer, there is no NCAM in the tumors. But when E-cadherin is knocked out, NCAM is highly up regulated on the invasive front of the tumor. NCAM is not heavily transcribed in human...
patient samples, but a similar molecule called L1, which is also upregulated during EMT, is more frequently expressed. Both NCAM and L1 seem to confer the same type of activity to tumor cells.

In a TGF-β induced NMuMG system, if TGF-β is knocked out, cells revert to an epithelial phenotype, they lose expression of NCAM and N-cadherin, which is a mesenchymal marker, and regain expression of E-cadherin. So NCAM seems to be required for maintaining mesenchymal properties.

If cells expressing E-cadherin are transfected with NCAM, the cells profoundly change their phenotype. They delaminate from the layer and start to put out neurite-like extensions and migrate away. So NCAM is sufficient to induce cell migration and invasion in matrigel invasion assays.

When NCAM is knocked down, the cells not only lose the neurite-like extensions but also lose cell spreading, which is usually an indication of cell matrix adhesion. Upon loss of E-cadherin, the mesenchymal cells gain focal adhesions. NCAM induces the formation of these focal adhesions. High levels of NCAM localize to cell-cell adhesion borders of mesenchymal cells. This cell-cell adhesion does not have the regulated and demarcated border seen with E-cadherin cell-cell adhesion, however.

When the loss of E-cadherin induces EMT, a subset of NCAM shifts to lipid rafts. Normally, low levels of NCAM may stimulate signaling via PLC-γ and MAP kinase. When EMT is induced, high levels of NCAM are shifted to the lipid rafts, where it associates with the Src family kinase p59-Fyn. This then phosphorylates focal adhesion kinase, thereby inducing β1 integrin-mediated focal adhesions and cell migration.

Three routes

One marker frequently used to stain lymphatic endothelial cells is Podoplanin. Podoplanin is a highly glycosylated mucin-type surface protein with a short 9-amino acid cytoplasmic tail, and is seen in some cancer cell types.

In squamous cell carcinomas, Podoplanin is expressed in the outer layer of the invasive front of a tumor. These cells, despite their migratory behavior, are still connected by E-cadherin to a neighboring cell. Podoplanin somehow plays a role in this collective invasion, mainly seen in squamous cell carcinoma.

If Podoplanin is expressed under the insulin promoter in Rip1Tag2 mice, the single transgenic mice don’t have an obvious phenotype. When the mice are crossed and the tumors are allowed to mature, the invading tumor cells still express E-cadherin, in contrast to normal mice where invading tumors have lost E-cadherin. So Podoplanin induces invasion in the absence of a loss of E-cadherin, or the absence of EMT.

When Podoplanin is transfected into MCF7 cells, podocytes or filopodia form. The cells that form filopodia still adhere to their neighbors. The cytoskeleton is heavily remodeled and the actin stress fibers are essentially dissolved, indicating that Podoplanin is sufficient to induce migration and invasion of these cells.

It’s unclear how Podoplanin has this effect but Podoplanin expression directly regulates the three famous Rho family GTPases, RhoA, Cdc42 and Rac1, and thereby the actin cytoskeleton.
Apart from the single cell and collective invasion types, there’s a third type of metastasis that is dependent on lymphangiogenesis, induced by the members of the vascular endothelial growth factor (VEGF) family, VEGF-C and VEGF-D, which bind to VEGFR3 in lymphatic endothelial cells\textsuperscript{7,8}.

If VEGF-C is expressed in the beta cells of the islets of Langerhans, it induces lymphangiogenesis and the islets are surrounded by lymphatics. When these mice are crossed with the tumor mice, all the mice develop intra-lymphatic clusters of tumor cells, some of which are E-cadherin positive. A third of the mice develop lymph node metastasis.

These observations indicate that just increasing the lymphatic vessel density in a tumor is enough to induce lymph node metastasis, in contrast with active metastasis, which requires cells to change their behavior, invade into the surrounding tissue, intravasate, disseminate, extravasate and seed metastasis.

In conclusion, at least three distinct mechanisms of metastatic tumor cell invasion and dissemination may exist: single cell invasion involving EMT, collective cell migration in the absence of EMT, and lymphogenic dissemination to local lymph nodes.
The RIP-Tag2 transgenic mouse model of pancreatic islet carcinogenesis represents the stepwise transition from pre-malignant stages to invasion and metastasis. In the tumor microenvironment, several cysteine cathepsin proteases facilitate invasion, at least in part by degrading the extracellular adhesion domain of E-Cadherin. The enzyme heparanase, which degrades the extracellular matrix, is exclusively supplied by inflammatory immune cells, and also facilitates invasion. Selective pressure from the microenvironment can also elicit increased invasiveness. For example, pharmacological inhibition of VEGF receptor signaling of tumor angiogenesis produces more highly invasive tumors that may partially circumvent the impaired angiogenesis by invasive co-option of normal tissue vasculature to sustain tumor growth. Finally, analysis of microRNA expression profiles is revealing candidate genes and multi-gene signatures of the invasive and metastatic capabilities. Douglas Hanahan discussed these complexities of invasion and metastasis and the new notion that impaired tumor angiogenesis is pro-invasive.

In the prototypical model of multistage tumorigenesis in pancreatic islets, normal islets are progressively transformed into islet carcinomas, going through the various stages of progenitor lesions, hyper-proliferative lesions and angiogenic switching. In this model, angiogenic switching happens early on, in the pre-malignant phase and prior to the emergence of solid tumors.

Eventually, there is an adenoma-like tumor, which is highly vascularized and progresses with the down regulation of E-cadherin into invasive carcinoma. But metastasis is relatively rare in this model. Overall, the model is representative of some human cancers, such as colon cancer, that go through an adenoma stage and on to invasive carcinomas, providing a means to study the acquisition of the invasive growth phenotype [see Christofori, page 49].

Histologically, hyperplastic and dysplastic islets are full of hyper-proliferative and apoptotic cells. An angiogenic islet turns on neo-vascularization in a so-called angiogenic switch, which is a discrete event. These then progress to islet tumors, which have a fibrous capsule and are fairly well defined, and on to two grades of invasive carcinoma, type 1 and type 2. The type 1 tumors are more micro-invasive and the type 2 are more widely invasive.

A number of factors influence the progression to invasive capability as well as to other aspects of the pathway. Signaling from insulin growth factor 2 (IGF-2) is activated both to attenuate apoptosis and to facilitate invasive growth. This happens in part through the IGF-1 Receptor, which when up regulated accelerates...
progression to invasive growth\(^1\). The fetal form of the insulin receptor may also be involved in IGF signaling for invasion and attenuating apoptosis.

Down regulation of E-cadherin is deterministic for the invasive growth phenotype. There are also contributions from the larger microenvironment, particularly extracellular matrix (ECM) degrading enzymes supplied by immune inflammatory cells. Interestingly, impaired tumor angiogenesis appears to facilitate an invasive growth phenotype. Polymorphic genetic modifiers also influence the propensity of these tumors to become invasive.

**Angiogenic switching**

Two effectors of invasive growth are provided largely by bone marrow-derived infiltrating immune cells. These are cysteine cathepsin proteases and heparanase.

All cells have cysteine cathepsins in lysosomes and in certain other intracellular compartments. But in a gene expression profiling assay, up regulation of several cysteine cathepsin proteases is observed in tumors, and confirmed in biochemical assays for protease activity.

Six cysteine cathepsin genes are up regulated, but not all are necessarily functionally important. To ask whether these proteases are collectively of importance, JPM, a pharmacological inhibitor of cathepsin activity, is used to interfere with their potential functions. JPM is a covalent probe that binds to the active site of the enzyme, irrevocably inactivating it. In trials, the inhibitor impairs tumorigenesis in this model, establishing their functional importance.

In an early “prevention trial” of about 400 islets in a mouse that are undergoing neoplastic progression, around 40 or 50 mice islets in control mice switch on angiogenesis. In the presence of the cathepsin inhibitor, in contrast, there is reduced angiogenic switching and impaired tumor growth at both early and late stages of the tumor, suggesting that the cathepsins functionally contribute to this process.

Interestingly, moreover, the cathepsin inhibitor produces a more benign tumor type. In the controls, an invasive phenotype appears in about half of the tumors, but in the cathepsin inhibitor treated tumor, the tumors are more adenoma-like with well defined margins, suggesting that the cathepsin inhibitor blocks invasive growth.

What’s more, control tumors switch off E-cadherin, but in mice treated with the cathepsin inhibitor, there’s largely a retention of E-cadherin. In biochemical assays, these proteases can cleave E-cadherin, suggesting that part of the mechanism of down regulating E-cadherin may be a proteolysis by the cysteine cathepsins\(^2,3\).

In a separate set of genetic experiments, four of the six cathepsins implicated — cathepsin B, C, L and S — are knocked out in the tumor-prone mice. These genetic crosses are feasible because mice carrying these gene knockouts are viable and fertile, with minimal abnormalities. When the cathepsin knockout mice are crossed with the RIP-Tag2 model and tumor burden is assessed at a defined endpoint, cathepsins B, L and S appear to impair tumor growth compared with controls. Cathepsin C has no impact.
Mice with knockouts in cathepsins B and S also show impaired vascularization and reduced microvessel density, whereas the absence of cathepsins L or C does not impair the angiogenic phenotype.

Control mice show a majority of micro-invasive carcinomas, with some adenomas and a few widely invasive carcinomas. The absence of cathepsins B, S or L shifts this back toward a more benign phenotype, with a lot of adenomas and some micro-invasive carcinomas but very few widely invasive carcinomas. Once again, the lack of cathepsin C has no impact.

These results suggest that cathepsins have both common and distinct functions in the islet tumor phenotype. Cathepsins B and L affect proliferation and all three of them affect apoptosis. In their absence, there is increased apoptosis and decreased proliferation. All three contribute to invasion and the invasive phenotype is impaired in all three knockouts.

Enzyme activity

To determine which cells express the cathepsin genes, the constituent cell types are purified by fluorescence activated cell sorting (FACS) into endothelial cells, innate inflammatory cells, Mac1/Gr1 positive cells and the tumor cells, along with a few stray fibroblasts.

By this analysis, cathepsin B is made in tumor cells, inflammatory cells and endothelial cells; cathepsin L seems to be highly expressed only in the tumor cells; and cathepsin S is expressed exclusively in the infiltrating immune cells. Cathepsin activity is not primarily in the tumor core but rather at the invasive fronts, which is consistent with its suggested role in invasiveness.

Another enzyme that has historically been associated with malignant phenotypes is heparanase. By RNA and protein analysis, the enzyme is up regulated in angiogenic islets and in tumors. Heparanase is expressed primarily in inflammatory cells, not in tumor cells or endothelial cells, despite the fact that it is expressed in many tumor cells in culture.

In control animals, tumors grow progressively for 10 to 13.5 weeks. In experimental therapeutic (preclinical) trials, a pharmacological inhibitor of heparanase called PI-88 impairs tumor growth and can slow down, albeit not stop, the growth of these tumors, arguing that heparanase contributes to tumor growth. Histologically, this inhibitor also impairs the invasive phenotype. Treated tumors show some micro-invasion but with a much better defined margin.

Not all proteases are important for invasion, however. For example, urokinase and the matrix metalloproteases (MMPs) MMP2 and MMP9 — also known as GelA and GelB — are not important for the invasive growth phenotype in this particular mouse model, as determined by gene knockouts and broad spectrum MMP inhibitors.

However, knockout mice and three different pharmacological inhibitors of MMPs do impair tumor growth, and they do so in significant part by impairing angiogenesis. Neutrophils and macrophages both produce MMP9, which helps to release vascular endothelial growth factor (VEGF) from matrix deposition and facilitate its association with receptors. Because MMP9 is pro-angiogenic, its absence impairs tumor growth, but is not pro-invasive.

This may explain the failures of MMP inhibitors in the clinic. These MMP inhibitors produces tumors that are, if anything, more highly invasive.
Promoting invasion

There are three angiogenesis inhibitors targeting VEGF-receptor signaling that are clinically approved: Avastin, the ligand-trapping antibody, as well as two tyrosine kinase inhibitors, Sutent and Sorafinib, which target VEGF receptor signaling. There are a large number of clinical trials seeking to expand the indications for these drugs. In general, all three produce transitory tumor shrinkage, delayed time to progression and a survival advantage. But inevitably, the period of response and clinical benefit is followed by relapse to disease progression.

One reason for this may be that effective angiogenesis inhibition can evoke a more invasive phenotype, perhaps a form of ‘evasive resistance’. Multiple inhibitors of VEGF-R signaling have been tested in this model and they produce similar effects to the ones seen in the clinic.

When mice are treated with a neutralizing antibody to VEGF-R called DC101, the tumors tend to be more invasive compared with sham-treated controls. A number of other angiogenesis inhibitors — MMP inhibitors, VEGF-R inhibitors, multi-targeted receptor tyrosine kinase inhibitors — also show increased invasiveness in response to anti-angiogenic therapy.

In a trial where mice are treated with a multi-targeted kinase inhibitor that hits VEGF-R in endothelial cells, platelet derived growth factor receptor (PDGF-R) in pericytes and fibroblast growth factor receptor (FGF-R) — which may be part of an evasive resistance that tries to escape the angiogenic blockade — the treated mice have a survival advantage, and live a couple of weeks longer than controls. They also show a dramatic reduction in vascularity.

In general, the tumors are smaller but are much more highly invasive.

Sutent inhibits VEGF-R and PDGF-R, which impairs angiogenesis and reduces pericyte coverage, stabilizing the tumor vasculature. Mice treated with Sutent show a substantial reduction in the vascularity and those vessels that persist have poor pericyte coverage. Once again, the tumors are highly invasive.

By becoming more invasive, tumors grow along and take over functional vessels in the tissue they’re invading. The interiors of such tumors are not substantively necrotic, indicating that they are surviving with a poor vasculature. Without access to treated human tumors, it is unclear whether tumors in people treated with Avastin or Sutent or Sorafinib also progress by these same mechanisms.

In summary, angiogenesis inhibitors have transitory benefit in the clinic and in this mouse model. Blocking VEGF up regulates other angiogenic factors and those can produce revascularization, mediated by other pro-angiogenic growth factors, such as FGF. But if some of those are knocked down, the tumors may be driven more toward invasion, enabling growth by cooption of normal tissue vasculature.

A combination of angiogenesis inhibitors or selective anti-inflammatory agents with inhibitors of invasion could prevent the pro-invasive effects of these proteases and produce more enduring benefits.

References
PART III: MECHANISMS OF METASTASIS

Richard Hynes
Cellular mechanisms contributing to metastasis

Ann Chambers
Novel imaging approaches for studying tumor metastasis

Jeffrey Pollard
Macrophages are a cellular toolbox that tumors sequester to promote their progression to malignancy

Wolf-Hervé Fridman
T effector/memory cells, the ultimate control of metastasis in humans

Kari Alitalo
Inhibition of lymphangiogenesis and metastasis

Shahin Rafii
Contribution of CXCR4 + VEGFR1 + pro-angiogenic hematopoietic cells to tumor oncogenesis
In the later stages of metastasis, after tumor cells have entered the vasculature, they must survive in the circulation, arrest at a distant site in the vasculature, extravasate and proliferate at the distant site. Selectivity in metastasis could be the result of mere plumbing — whatever is downstream; could be homing, or modeled on the seed and soil hypothesis of survival and growth. A popular model for metastasis is that some cells of the primary tumor are more metastatic than others. It’s also possible, however, that the bulk of a primary tumor evolves to a premetastatic state. Gene expression analysis of high and low metastatic variants reveals multiple cellular mechanisms contributing to metastatic spread, including genes involved in cell migration, tumor growth and tumor-stroma interactions. Host genes involved in tumor-stroma interactions are also important. Lymphangiogenesis, however, is not essential for lymph node metastasis, which has relevance for attempts to target metastasis by inhibiting lymphangiogenesis. Richard Hynes talked about these later steps in the metastatic cascade from the perspective of a cell biologist.

In the later stages of metastasis, once cells get into the circulation, they have to arrive at a distant site, proliferate and survive. This process is very inefficient, raising the question of whether it is stochastic or whether there are metastatic variants.

It seems clear, at least in many popular models of cancer, that metastatic variants do exist in the tumors and, most important, can preexist in the primary tumor. If such variants exist, how do they relate to the idea of cancer stem cells or cancer-initiating cells?

During the progression of a tumor, there may be points at which the tumor has good or poor prognosis that can be picked up by microarrays. What array analyses show depends on whether they are analyzed for things that pinpoint a poor prognosis or for differences between primary tumors and metastases.

In most cases, array data from human clinical samples don’t just sample the tumor. Up to 30% of what is sampled in some tumors is stroma, so signatures of a good or bad prognostic tumor may be the result of host cell activity, such as the influence of lymphangiogenesis, angiogenesis or the extracellular matrix (ECM).

The models for cell adhesion in the vasculature and for the recruitment of leukocytes and platelets to sites of inflammation or infection are very well understood. Most of the molecules that play important roles have been identified and many of them are being targeted therapeutically. Tumor cells seem to use these some of these same systems for their own goals.

In the case of infection or inflammation, chemokines and cytokines are activated and in turn activate endothelial cells and leukocytes. There is evidence suggesting that the expression of chemokines from tumor cells also affects where cancer cells form metastases.
Selectin significance

Selectin ligands, which are initiators in the inflammation cascade, slow cells down in the fast bloodstream, bringing them close enough to cytokines to get activated, thus allowing integrins, the main adhesion molecules, to stop the cells.

Tumor cells also frequently express selectin ligands, and expression of these ligands is one of the criteria that pathologists use to diagnose a tumor with poor prognosis. There is evidence that host selectins recognize the selectin ligands on tumor cells and help the tumor cells plug into this cascade of adhesion.

The presence of P-selectin on platelets or L-selectin on leukocytes both play roles in the metastasis of the tumors in xenotransplant models. There are also some data supporting a role for integrins.

Together the data suggest that this cascade is in play, perhaps in different situations with different tumors, supporting the idea that metastasis might be enhanced to places of injury or inflammation.

If C57BL6 adenocarcinoma cells are introduced into a C57 black mouse using a tail vein metastasis assay, glistening nodules of metastases appear in the lung, as scored by a variety of different techniques.

But if the experiment is repeated in a P-selectin or a L-selectin deficient mouse, fewer metastases appear. The change here is not in the tumor, but in the host. If both P- and L-selectins are knocked out, there are even fewer metastases. This has been shown with a number of different tumors.

These results suggest that the tumors, once they are in the blood, bind or interact with platelets or leukocytes, and that this interaction is mediated by P- or L-selectin, respectively. When tumor cells are injected into a wildtype mouse, it’s well established that the tumor often gets a coat of platelets and/or fibrin. In a P-selectin deficient mouse, platelets don’t bind to the cells. When the tumor cells cannot interact with platelets, it compromises their ability to metastasize.

The data that platelets enhance metastasis goes back a long way, although how they do so is unclear. There is some evidence, for example, that coating tumor cells with platelets protects them from NK cell killing in model systems.

Because platelets act as delivery bags for promoting wound healing, they are also loaded with growth factors such as the platelet derived growth factor and others and could be providing these growth factors and cytokines to the tumors.

They could also be providing tumor cells with adhesion molecules. These molecules could be providing enhanced adhesion through integrins, for example, helping tumor cells to adhere by bridging between them and the endothelium.
Picking up patterns

For a circulating tumor cell to be metastatic, the deciding factor could be in the arrest, it could be specific or non-specific, could be mechanical because of the size, a result of selective adhesion and it could be host-assisted or intrinsic to the tumor itself.

The tumors have to extravasate, and that presumably reflects migratory or invasive capacity, may well reflect tumor and host cell interactions, and could be affected by vascular injury.

Once the tumors are out, or even if they stay inside the vessel and grow, they have to survive and grow. This again could be tumor-intrinsic or require tumor and host cooperation.

To assess what it is tumor cells do differently that allows them to be metastatic, a pool of tumor cells are injected into mice. The resulting lung metastases are collected, allowed to grow, and then put back into mice to ask whether the process selects for cells that are metastatic. This method can be used to select for variants that are either high or poor for metastasis, comparing RNA and protein profiles of the two groups. If a particular gene’s expression goes up, it can be ramped up or down and the effects assessed.

By injecting human tumor cell lines into a mouse model, it is easy to identify whether a gene that ramps up belongs to the tumor or stroma. The analysis is done on the tumors, not on the cell culture, so that it picks up gene expression patterns in vivo. Using this method, comparing a whole series of different tumors from poorly metastatic to highly metastatic generates a list of about 150 genes that differ between the two groups.

These are consistent patterns, even between isolates from independent tumors. Of the 150 genes, about half are up regulated and about half down regulated. When this signature is compared with a set of human samples, the expression of these genes in some samples correlates, the expression in others is the reverse of what’s seen in the mouse samples, and there’s a third class that’s ambiguous.

Those patients whose signature looks like the mouse-derived signature have a worse prognosis than those whose signature does not correlate.

If all the human genes from the melanoma metastases are lined up in rank order of expression levels, and compared with the mouse signature, there is a very good correlation. The mouse hits are highly enriched in the genes that are highly expressed in the human tumors. So the mouse model is predicting something about the clinical prognosis.

Among the genes that are up regulated in highly metastatic cells are several that affect cell migration, such as RhoC [see Tak Mak, page 29], 4.1B and IQGAP-1, all of which have been shown to be causal. Others such as fibronectin and thymosin b4 also correlate but haven’t been proven to be causal.

There are also genes involved in tumor-stroma interactions and survival such as GPR56, TGF2 and others. Secreted extracellular proteins and membrane proteins — which might affect tumor-stroma interactions — comprise about half of the 150 genes, compared with 15-20% in the total proteome, so that fraction of the genome is enriched.
Finding a ligand

In the array data is a group of genes that is downregulated in the metastases as compared with the tumors from the poorly metastatic parentals. Among these is GPR56, an unusual G-protein coupled receptor.

The protein is downregulated in the metastatic tumors and, in general, the degree of down regulation correlates with the level of metastases. It is a member of a family of about three dozen G-protein coupled receptors, all of which have homology in their seven transmembrane segments with the secretin receptor.

The distinctive thing about the set is that they have long N-termini with adhesion motifs — cadherin or immunoglobulin domain, thrombospondin or EGF repeats and RGD motifs.

A highly metastatic cell line with low expression of GPR56 gives many metastases. In different experiments in immune incompetent mouse backgrounds, when GPR56 is over expressed, it suppresses metastasis. Overexpression of GPR56 doesn’t make much difference to tumor growth in vitro, but makes a big difference to growth in vivo.

In the opposite experiment, if GPR56 is knocked down in a poorly metastatic line, there are many more lung metastases and the mice die much faster. Here again, the tumors grow bigger in vivo, but not in vitro.

The difference between the in vitro and in vivo effects suggests that there might be an interaction with the tumor environment in vivo.

When the lung is serially extracted with an increasingly stringent detergents and the soluble fractions in each case are run on a gel and probed with the extracellular domain coupled to an Fc fragment of human immunoglobulin, a nice band of about 80-kDa that’s only soluble in SDS emerges. A series of gel filtrations followed by mass spectrometry reveals the protein to be tissue transglutaminase (TG2)5.

TG2 is a well-known crosslinking enzyme in the ECM and its 25-kDa C-terminus is bound by the N-terminus of GPR56. There are numerous reports in the literature of TG2 down regulation in tumors and metastases. Adding exogenous TG2 can suppress tumor growth. Conversely, tumor growth is enhanced in TG2 knockout mice.

In addition to its crosslinking activity, TG2 also binds to fibronectin and to integrins b1 and b3 and acts as a linker between them, creating another way for fibronectins and integrins to interact.

**Matrix crosslinking**

All these data suggest a model where TG2 binds GPR56 and b1 integrin and, through them, fibronectin, perhaps helping to promote binding and crosslinking of the ECM.

CD81, which is a tetraspannin, is also implicated in a trimeric complex with GPR56 and the trimeric G-protein Gq. TG2 may also activate TGF-b.
All of this might together control the crosslinking of the ECM and affect tumor progression or adhesion or signaling by one or more of these receptors. It’s as yet unclear whether TG2 binding to GPR56 is what produces the growth suppressive effect.

Mutations in GPR56 are linked to a mental retardation disease called bilateral frontoparietal polymicrogyria or BFPP, in which there is an excess of the cortex. GPR56 is highly expressed in neuronal progenitors and hematopoietic stem cells, so could be involved in keeping those under control. When it’s mutated, there is loss of that control, and an overabundance of neurons, leading to polymicrogyria. That would parallel GPR56’s action in metastasis, where it’s a growth suppressor. This picture supports the seed and soil hypothesis.

Lymphangiogenesis within the tumor, at least in the system analyzed, is not contributory to metastasis. Another factor that could combat metastasis is the immune system such as NK cells, which can kill tumors.

When the vigorous LL/2 tumor is introduced subcutaneously into the C57BL/6 background, it hardly grows at all. But the tumors grow bigger in mice that lack selectins. Knocking out E-selectin doesn’t make much difference but knocking out P-selectin does. If all three selectins, P-, E- and L-selectin are knocked out, the tumors grow much bigger.

Putting in wildtype bone marrow cures that situation, suggesting that a bone marrow-derived cell is involved. Knocking down NK cells with an antibody or a transgene again gives many more and bigger tumors.

This suggests that the selectins on NK cells are helping the cells combat tumors. NK cells are recruited into tumors in wildtype mice but much less so in mice deficient in L-selectin or in all three selectins. NK cells appear to use selectins, particularly L-selectin, to target the tumors.

References

For successful metastasis, tumor cells have to travel through the circulation to get to a secondary site and arrive there in appreciable numbers. This may be less a consequence of some 'homing' to signal in the cancer cells and more a result of plumbing — the size of the cancer cell, the size of the blood vessel and the rate of blood flow from the primary site to the secondary site. At the secondary site, cells have to decide to proliferate or go into a dormant state, or some of them may die. Once in a micrometastatic state, the cells then either continue to divide, undergo apoptosis or become quiescent. All of these events are affected by a myriad factors, including the genetics and epigenetics of the cells, the microenvironment and cancer stem cells. Using sophisticated imaging experiments and animal models, Ann Chambers revealed the intricate mechanisms underlying the complex process of metastasis.

Metastasis is responsible for most of the deaths attributed to cancer, so any decrease in cancer mortality and morbidity requires the ability to better understand metastasis and how to prevent and treat it.

It’s important to match the animal model used to the clinical picture. Injecting a tumor into an orthotopic primary tumor site provides a model for neo-adjuvant therapy to treat primary tumors, which is not the primary approach in cancer treatment. To test drugs that would be used in a metastatic setting, a metastatic model is needed, in which the animal may be cured of its primary tumor, but there are micrometastases still amenable to treatment.

For metastasis to occur, plumbing is crucial. There are various circulatory systems — venous, arterial, hepatic and lymphatic. All of these systems matter. For example, cells leaving a primary tumor in the colon would travel via the hepatic portal system, be taken first to the liver leading, typically, to liver metastases from colon cancer.

With breast tumors, cells can travel via the venous system through the heart, and the first capillary bed they encounter is in the lungs. Breast cancer does give rise to metastases in the lung, but also in the brain, liver and bone, so there must be another way for the cells to travel.

Breast and other cancers can also spread by the lymphatic system, through which they can get to the lymph nodes and travel along lymphatic chains. But to get to the brain or bone, the cells have to return to the circulatory blood system.

Solid tumor cells are big and capillaries are little. That means that the first capillary bed the cells run into is a very efficient, albeit not perfect, filter that sifts out most of the cells.
In an experimental system that models the latter half of the metastatic process, cells are injected into the circulation using either the mesenteric vein to target them to the liver, the tail vein to target them to the lung, or the heart to target them to any arterial organ. The endpoint then is which organs end up with metastases.

Spontaneous metastasis requires an artificial primary tumor, perhaps by injecting the cells subcutaneously, injecting the mammary fat pad for a mammary tumor or other orthotopic sites for other primary tumors. Once the tumor grows, it can be removed surgically and the endpoint again is the presence of metastases in internal organs.

In the third method, transgenic mice have multiple primary tumors, which makes quantification difficult. The endpoint is whether these cells spread to other organs.

All three assays can test whether a gene or a therapy works, but don’t reveal how that happens or which step is affected by the gene in question.

To avoid misinterpretation and guessing at mechanisms, imaging modalities can help observe the process and ask address questions related to the progression rather than just to the endpoint.

**Seeing is believing**

*In vivo* optical video microscopy was developed to study circulation in a living animal in three dimensions over time, up to about four hours. In this method, a portion of some mouse organ is exteriorized and placed on the stage of an inverted microscope, so that the animal is on a stationary surface. The optics come from underneath the animal, allowing a glimpse into about the bottom 50-100 microns of tissue.

This method illuminates the metastatic process but is invasive, limiting the ability to go back and look over time. But high frequency ultrasound, microcomputed tomography and magnetic resonance for brain metastases confer the longitudinal ability to follow the process.

Another procedure helps keep track of where cells go and how many cells there are. This procedure is based on the way people monitor blood flow patterns. Cells are mixed with microspheres in a known ratio, usually 10 cells for every microsphere, providing a fixed reference of the original dose of the cells. If the ratio at a given point is down to five cells per microsphere, for instance, only 50% of the cells have survived. This method allows for careful cell accounting over time, which is labor-intensive, but very informative.

Video microscopy reveals that when breast cancer cells are injected into the mesenteric vein, the cells are big and get stuck in liver sinusoids, implying that where the cells are delivered is often determined by physical constraints. More than 85% of cells survive this process.

Cancer cells once they’ve extravasated also often interact with the outer surface of vessels. In a chick chorioallantoic membrane (CAM) preparation, which is a good model to observe extravasation, a fluorescent cell that has extravasated reaches pseudopodial projections around a pre-existing vessel. In the same model,
a melanoma cell three days after injection forms a crab-like structure on the outer surface of the vessel, a phenomenon also observed in the clinic. If these cancer cells make use of pre-existing vessels, that might affect anti-angiogenic therapies.

At the endpoint, various cell lines differ in their ability to form metastases. But some of the steps leading to the endpoint are consistent. In various models, for instance, more than 85% of cells consistently survive the initial arrest in a vessel.

But the proportion of cells that reach the organ, make the initial decision to grow and keep on growing, or remain dormant in secondary sites are all variables that determine whether there are more or fewer metastases.

**Divide and conquer**

One method to quantitate the number of cells at different stages is to inject B16F1 mouse melanoma cells into the mesenteric vein to target them to a mouse liver, along with little beads that can be tracked. Following the cells over time will then reveal what proportion of them are present as solitary cells, as little micrometastases that have made the initial decision to grow, or as persistent metastases that have grown bigger.

Using the bead method, about 1 in 40 of these cells make the initial decision to form micrometastases, and only about 1 in a 100 of those persist to form growing metastases. There are also a surprisingly large number of solitary, dormant cells. By 14 days, only about 0.02% of the initial inoculums have grown to form large metastases that would kill the animal.

The results have implications for microarray experiments because they suggest that the vast majority of cells in a metastatic population are functionally non-metastatic.

In summary, cancer cells shed from a primary tumor travel via the circulatory system — lymphatic or blood — are arrested at least in the hematogenous system by size restriction, initially and primarily but not exclusively. In any given secondary site, there is a spectrum of cells with some present as solitary dormant cells, some as micrometastases and others as vascularized, progressively growing metastases.

In terms of the ‘seed and soil’ hypothesis, seed and soil both matter. Cancer cells are ‘seeded’ and arrested wherever the circulation takes them. The circulatory patterns from primary tumor to various secondary sites affect how many cells are delivered as the initial insult to organs. Once the cells reach a site, there are various seed factors, such as whether the cells express certain oncogenes and receptors for ligands that may be present in the secondary site, or whether the cells are cancer stem cells. There also various ‘soil’ factors, such as growth factors and extracellular matrix molecules.

In a meta-analysis of published autopsy studies, in which 16 primary tumor sites are compared with 8 secondary sites, about two-thirds of the time, the metastatic burden is proportional to the blood flow rate from the primary sites to the secondary sites.

But in the remaining third of the pairs of tumor sites, there are discrepancies. About 20% of the time, there are ‘friendly’ interactions, by which an organ develops more metastases than expected just based on blood flow to that organ. For example, breast and prostate cancer metastases grow more favorably in bone than in other organs. In another 14% of the pairs, there are ‘hostile’ interactions, which generate fewer metastases than expected.

**Diet and dormancy**

What it means to be metastatic may be defined by the assay used. In a woman who has had her breast cancer removed, for instance, diet could affect metastatic outcome.
Genistein is a soy protein that’s a phytoestrogen and has been implicated in cancer. When human breast cancer cells are injected into the mammary fat pad in mice and the primary tumor is surgically removed, there is no evidence of metastasis in distant organs at the time of surgery. If the mice are then given either a controlled diet or one that contains genistein, mice fed genistein develop significantly fewer metastases, suggesting that changing the diet at the time of surgery could affect the metastatic outcome of cells that had been seeded before surgery.

Genistein is not a good therapeutic option for some tumors because it’s a weak phytoestrogen. But as proof of principle, the results show that animal models can test diet and other factors. More important, the results show that the metastatic fate of seeded cells can be altered by adjuvant therapy, diet and host factors, such as genotype and menstrual phase.

To better understand dormant cancer cells, the cells can be labeled so that they can be detected by imaging modalities. As the cell divides, the label is diluted, and the cells that don’t divide stay green.

When two different mouse mammary tumor cell lines are injected into the mammary fat pad, at the end of the experiment, large numbers of green cells persist in the organs. Clearly drug-marked green tumor cells can be recovered from the livers, suggesting that some of those cells at least are viable.

Because the cells retain label, they are presumably non-dividing and should be resistant to chemotherapy. If quickly growing tumor cells are injected into the mesenteric vein, allowed to grow in the liver and treated with doxorubicin, the tumor volume decreases, but there is no effect on the number of green cells in the organ.

When the same experiment is repeated with a slow growing cell line, however, doxorubicin chemotherapy has no effect on the green cells, but more important, it also has no effect on the outgrowth of metastases.

**High and low metastasis**

In a separate experiment with MDA-MB-231BR cells, a human breast cancer cell line selected for its ability to form brain metastases, little iron oxide particles are taken up by the cells in culture before they are injected. By magnetic resonance (MR), solitary iron-containing cells show up as black spots, and tumors when they get to be big enough appear as white spots.

Immediately after injection, the little black spots are in proportion to the number of cells injected and represent individual iron-containing cells. Over time, they gradually decline and by the three- or four-week end-point, about 94% of them disappear and white spots begin to appear. The metastases arise from less than 2% of the original inoculum.

Clinically, it would be important to know what keeps dormant cells quiescent, and whether killing them would be necessary once the metastasis is treated.

It has long been known that cloning metastatic cell lines can generate cells with high or low metastatic ability. This was interpreted as high and low clones preexisting in that population of cells. Instead, high and low metastatic clones could be generated during clonal expansion.
Luria-Delbruck fluctuation analysis was originally used to describe rates of mutation in bacteria that generate drug-resistant variants. Using the same type of analysis with cancer cells can identify clones of different metastatic abilities.

For example, from B16F1, a modestly metastatic melanoma cell line, a single clone is picked and then subcloned into a whole series of parallel clones, which are grown into either small or big population sizes. When they are grown to a large population size, some clones seem poorly metastatic and others highly metastatic.

But if the clones are grown only to a small total population size, with everything else constant, most of the clones are reproducibly non-metastatic. The rate is too fast to be a result of point mutations, suggesting a role for epigenetic events.

If a single B16 clone can give rise to metastasis, it implies that the one clone happens to be a cancer stem cell. But what is the stem-ness of various cancer cell lines? Is it proportional to how metastatic they are? Does the cancer stem cell theory apply differently to human tumors as opposed to cell lines? There are few clear answers to any of these questions.

References

Macrophages are abundant in the tumor stromal environment. Ablating the major macrophage growth factor, colony stimulating factor-1 (CSF-1), decreases the number of tumor associated macrophages in mouse mammary tumors and results in delayed tumor progression and a dramatic reduction in metastasis. Restoring macrophages to the mammary tumors by expressing CSF-1 rescues these phenotypes in mutant mice. Over expression of CSF-1 enhances tumor progression and doubles the rate of metastasis in wildtype mice. Tumor associated macrophages promote many critical processes associated with the progression to malignancy. Using intravital imaging of fluorescently labeled cells together with a novel micro-capillary invasion assay, Jeffrey Pollard demonstrated an obligate paracrine loop between macrophages and tumor cells that is required for tumor cell migration, invasion and intravasation. Macrophages also regulate the angiogenic switch that is associated with the transition to malignancy. In tumors, a high density of macrophages both enhances tumor cell invasion and intravasation and increases the number of target vessels through which the tumor cells escape into the vasculature.

There has been a strong emphasis in cancer research on the genetic changes that occur in a tumor cell, but there has been relatively less emphasis on cells — such as macrophages, fibroblasts, adipocytes and mast cells — and their interactions within this organ microenvironment.

However, tumor cells are not little islands, they interact with other cell types and there is a complex interplay between all these cells. Macrophages, in particular, are very important. It has become apparent from clinical studies that in about 80% of human adenocarcinomas, tumor-associated macrophage density correlates with poor prognosis.

In some cases, it’s an independent predictor of prognosis.

Macrophages are regulated by a growth factor called colony stimulating factor-1 (CSF-1), which acts through the cfms protooncogene, a transmembrane tyrosine kinase receptor. There is over expression of CSF-1 in many cancers of the reproductive tract, particularly endometrial, ovarian and breast cancer, and over expression correlates with poor prognosis.

In breast carcinoma, CSF-1 expression is prevalent in invasive tumor cells. In breast and endometrial cancer, there is also co-expression of the receptor on tumor cells, suggesting...
an autocrine reaction. However, there is no evidence of CSF-1R in mammary tumor cells in mice suggesting that this is not the case in this species. But more important, in breast cancer, there’s essentially a 100% correlation with leukocytic infiltrations and principally of macrophage infiltration. There are other macrophage chemoattractants such as CCL-2, whose over expression also strongly correlates with poor prognosis.

In a CSF-1 null mutant mouse, the effect is mainly on developmental systems and the rates of outgrowth of epithelial structures, for example in the pancreas and mammary gland. The effect is on rates of growth, rather than identity, suggesting a role for trophic and scavenging macrophages, rather than immunological macrophages. There are also less apparent immune defects in the mice than might have been expected.

These observations suggest that tumor-associated macrophages, recruited by CSF-1 and chemokines, accelerate tumor progression. The MMTV polyoma middle T model (MMTV PyMT) is ideal for testing this hypothesis because it has 100% penetrance, has many tumors formed at an early age, and has a high frequency of pulmonary metastasis. These mice are crossed with macrophage-deficient CSF-null mice.

**Macro effects**

The progression of a tumor can be characterized by morphology: there’s a benign hyperplasia, followed by an adenoma or a mammary inter-epithelial neoplasia, and two malignant stages, early and late carcinoma. These are relatively similar to human tumors by clinical pathology and protein marker expression, although there are some differences.

This characterization allows for staging the tumors as they progress in the two genetic backgrounds, the macrophage-replete and the macrophage-depleted backgrounds. The incidence of the tumors and their early growth is unaffected by the absence of the macrophages but the progression of the tumors to malignancy is much slower.

Macrophage-replete mice become metastatic by 14 weeks of age and by 22 weeks of age, when the mice have to be killed because of tumor burden, 100% of mice have lung metastases. But in the mutant, essentially none have any detectable metastases. By immunohistochemistry with aF4/80, a mononuclear phagocyte restricted antigen, there are large numbers of macrophages in the tumor stroma, which are depleted in the mutant animal.

In transgenic mice in which the MMTV promoter is used to drive expression of CSF-1, it is expressed only in the mammary gland and the salivary gland, where the promoter is also expressed. This expression does not correct the phenotypes in the CSF-1 null mutant mice, such as osteopetrosis, and thus this transgenic model allows for the determination of organ autonomous events.

CSF-1 is both chemoattractive and chemokinetic to macrophages and even in vivo, its expression recruits macrophages. If they are recruited to the tumors of CSF-1 null mice then tumor progression is accelerated such that at 18 weeks of age in the transgenic mice, all the tumors are driven to late carcinomas and a significant amount of metastasis can be detected in the lungs.

Thus in conclusion, in the presence of CSF-1, there is a large recruitment of macrophages into the stroma of the primary mammary tumor, the tumors progress rapidly and make pulmonary metastasis. When CSF-1
is ablated, tumor progression is delayed and there are no pulmonary metastases. If CSF-1 is added back, there
are once again many macrophages and the phenotype is rescued essentially, but not completely, to wildtype.
The only cells in this model that express CSF-1 receptor are macrophages, indicating that the effect is mediated
through macrophages.

**Complex crosstalk**

In the wildtype MMTV-PYMT mice, at eight weeks of age, some of the tumors have progressed into an early
carcinoma and there are areas of basement membrane breakdown with leukocytic infiltrates. In a macrophage-
depleted animal, in contrast, the tumors grow nicely, but there is no basement membrane breakdown and
no leukocytic infiltrates at this stage.

In the wildtype mice, there’s angiogenesis and collagen fibrillogenesis at the sites of basement membrane breakdown and the leukocytic infiltrate includes macrophages, mast cells and neutrophils. There’s crosstalk between the different immune cells within the micro-
environment. The tumors are beginning to either break out of the basement membrane or send signals so that the macrophages can help them break out. These tumor cells then migrate down through the stroma, get out into the circulation and become metastatic through the action of growth factors released by the macrophages.

Using intravital imaging of xenograft or PyMT tumors, the tumor cells can be seen using the collagen fibers as tram lines. The tumor cells delaminate, walk down collagen fibers, going about ten times faster on the fibers than they do through the matrix itself.

In a micro-invasion assay in which needles with growth factors can be introduced into tumors, the cells chemotax to the growth factor. If CSF-1 is loaded onto one of these needles, within five minutes, macrophages begin to move toward the bevel of the needle, where the growth factor is streaming out. Only epidermal growth factor (EGF) and CSF-1 cause recruitment of tumor cells and macrophages into the microinvasion assay needles.

EGF and CSF-1 recruit both tumor cells and macrophages and using either an EGF inhibitor or an antibody against the CSF-1 receptor blocks the effect. There’s a reciprocal expression of ligands and receptors on these two cell types: carcinoma cells make CSF-1 and carry the EGF receptor, and the macrophages express EGF and, by definition, carry the CSF-1 receptor.

In this paracrine interaction, the carcinoma cells produce CSF-1, which triggers CSF1-R signaling through a WASP pathway, causing podosome formation and migration and invasion. EGF binds to the EGF receptor, which then signals through N-WASP causing invadopodias. The consequence of this is cell migration and matrix remodeling and, eventually, tumor cell invasion.

**Building blood vessels**

*In vivo* confocal imaging of the whole tumor through the different stages can be used to build up three-
dimensional images of blood vessel formation. There’s a strong angiogenic switch with dramatically increased
density when the tumor reaches the malignant stage that occurs only in the malignant areas of the tumor.
This type of angiogenesis, in which the vessel structure starts from within the tumor and grows out, is different from that seen in xenografts, where a ball of cells is stuck into the flank of an animal and the vessels sprout in. This difference may in part explain why many of the available drugs don’t work.

Macrophages are recruited into the tumor stroma in the pre-malignant stages. As the tumor becomes a carcinoma, there are areas that are densely infiltrated by macrophages which tend to be avascular. In vascular areas, macrophages are essentially aligned along the vessels, with a very few in the tumor itself.

As tumors go from non-malignant to malignant stages, there is a recruitment of macrophages through CSF-1, MCP-1 (CCL-2) and SDF1. Angiogenic factors then trigger the formation of vessels, which drive the malignant transition. In late carcinoma, there are large infiltrates of macrophages.

In macrophage-depleted animals, there is a dramatic difference. In 18-week CSF1-null mice, the tumor may be enormous, but if they do not become vascularized they do not progress to malignancy. If tumors do progress to malignancy, there is an increase in recruitment of macrophages into the malignant area. In stage-matched tumors, the null tumors show about a 40% decrease in vascular area and vascular density compared with wildtype. When CSF-1 is added back, vascularity is rescued to wildtype levels.

These observations are all based on the mutant animal, which lacks CSF-1. In a wildtype background, which is more equivalent to a human breast cancer, CSF-1 is over expressed. Once again using the MMTV promoter, if CSF-1 is expressed early on in the mammary epithelium, macrophages are recruited into the hyperplasia, resulting in about a six-fold increase in macrophage density. Remarkably, these hyperplasias become completely hyper-vascularized.

At the eight-week stage, which is when tumors go into early carcinoma, 10-15% of wildtype tumors are malignant. But if CSF-1 is over expressed and the vascular switch is turned on early, more than 80% of tumors are malignant. Some of the tumors go on to late carcinomas at that stage and the metastatic capacity doubles.

**Metastatic role**

Using multi-photon imaging, macrophages are found abundantly in the stroma intercalating with and surrounding the tumor. They also align around the blood vessels. Some of the macrophages walk in an amoeboid movement through the stroma whereas others wobble backwards and forwards on the vessels.

These macrophages are important for tumor cell motility. In about 85% of the cases when a tumor cell moves, it’s next to a macrophage, whether the tumor cell is on the invasive edge or in the perivascular region. There’s more movement at the edge of tumors because there are many more macrophages there than in the perivascular regions, but perivascular macrophages have a higher specific activity in causing tumor cell movement.

Tumor cells also intravasate into a blood vessel about 85% of the time next to a cluster of perivascular macrophages, which are just on the outside of the vessels. In a macrophage-depleted animal, the number of circulating tumor cells is reduced proportionally with the number of peri-vascular macrophages, and this may be one of the reasons for the low metastatic rate.
Tying this in with the paracrine loop, a two-hour treatment of an EGF inhibitor, or a four-hour treatment with a CSF-1 neutralizing antibody, reduces the circulating tumor burden dramatically compared with wildtype mice that had not been treated.

A metastasis model in which tumor cells are injected intravenously can address whether macrophages are also important in the distant metastatic sites. In the mutant model, there is a dramatic effect on the seeding and growth of metastatic cells using the Met-1 cell line, which is a derivative of PyMT. The metastatic index, scored by histology of the lungs, is reduced in the absence of CSF-1. There’s a heterozygous effect on both seeding and persistent growth of the tumors, however, which is interesting.

Macrophages can also be depleted in other ways, for example using liposome-encapsulated Clodronate. Depleting macrophages in this way also has an effect on both seeding and persistent growth and dramatically reduces the metastatic index.

Fluorescence activated cell sorting reveals that in the lung, about 95% of the macrophage population is CD11c-positive and CD11b-negative. However, the recruited macrophages are CD11b-positive and Gr-1 negative.

If these CD11b-positive cells are depleted using a transgenic mouse in which the diphtheria toxin receptor is expressed from the CD11b promoter, thus making them susceptible to the toxin, the metastatic index is also blocked. Because there’s no primary tumor here, these are populations of macrophages specifically recruited when the tumor cells arrive at the location.

In summary, macrophages are independently required for tumor progression and for metastasis. There is a paracrine loop in which macrophages make EGF, which stimulates tumor cell migration and invasion, and tumor cells make CSF-1, which activates macrophages. The tumor cells and macrophages move together. Breaking this paracrine loop blocks invasion and intravasation. Macrophages are also involved in angiogenesis and can almost double vessel density.

During metastasis, a unique population of recruited macrophages appears to be involved in seeding and persistent growth of tumor cells in distant sites. This might be important clinically because over expression of CSF-1, EGF and VEGF correlates with poor prognosis in human breast cancer, suggesting that the paracrine loop and the effects of macrophages on angiogenesis may present therapeutic targets.
Chronic inflammation has been associated with tumor growth and metastasis, but no definite role has been attributed to lymphoid infiltrates. There is strong evidence of the importance of the natural anti-tumor adaptive immunity in human cancer. Based on analysis of patient samples from human colorectal cancer rather than on mouse models, the presence of mature antigen-presenting dendritic cells and of T lymphocytes at the tumor site is associated with a favorable clinical outcome. The beneficial effect of the adaptive immunity may persist throughout tumor progression, up to stage III. The type, density and location of immune cells, the ‘immune context’, has a prognostic value that is superior and independent to those of the classical tumor classification systems. Wolf H. Fridman presented evidence that the local immune reaction may play a crucial role in controlling tumor invasion and in determining clinical outcome.

VELIPI, a combination of venous emboli (VE), lymphatic invasion (LI) and perineural Invasion (PI), is a mark of early invasion as detected by pathologists. The notion that VELIPI in the primary tumor is associated with clinical outcome of colorectal cancer has been disputed.

More interestingly for immunologists, the quality and quantity of immune reaction, which is dubbed the ‘immune context’ at the primary tumor site, must be evaluated for its influence on early invasion, or on VELIPI status.

The cohort used to address these issues is a set of 959 colorectal cancer samples that have been followed clinically since 1986. This is a classic cohort of colorectal cancer, based on which there are several factors that do not influence disease-free survival or cancer recurrence.

For instance, the sex or gender of the individual from whom the sample was taken and the localization of the tumor — whether the tumor is in the right column, the left column, the transverse column or the rectum — don’t influence disease-free survival and overall survival. But the less the tumor is differentiated, the worse the tumor is, although this difference doesn’t reach significance. The number of lymph nodes analyzed also makes no difference in outcome.

However, there are several factors that do influence survival and these are analyzed in the clinic for prognosis. For instance, the larger the tumor at the T stage, the worse the prognosis. At the N stage, the presence of lymph node invasion has a strong impact. The metastatic stages and the Dukes classification —
UICC 1, 2, 3 and 4 or A, B, C, D; A being small, non-invasive tumors, B local invasion, C lymph node involvement and D metastasis — have a strong effect on clinical outcome and disease-free survival.

There is a good distribution of different tumors at the different stages so that large groups of tumors can be analyzed for their response to various factors, particularly immunological factors.

**Pinning down a prognosis**

There is a strong prognostic factor associated with the presence of a few cells in the vasculature or the lymphatic system, whatever the other parameters of the tumor. This can be illustrated by a Kaplan-Meir curve with tumors that show either no signs of early invasion, or that show one or more components of VELIPI.

Tumor-associated events, T and M classification, tumor size, lymph node invasion and metastasis all have a heavy impact on prognosis. To assess whether the immunological microenvironment of the tumor can also influence prognosis, information on the immune infiltrate, gene expression and regulation, protein interaction, the various cell types in the tumor, localization and functionality of these cell types are all entered into a bioinformatics computer model. This is then mixed with 20 years of histopathological and clinical data from these large cohorts.

The experiments are based on immunohistochemistry and tissue microarray analysis on 959 paraffin-embedded tumors, 515 frozen tumors and 39 fresh tumors to analyze living cells in these tumors. When RNA is extracted from tumors and gene expression analyzed by real-time Q-PCR, analysis of gene expression in 75 tumors that have good quality RNA reveals that there is no difference in the expression of inflammatory markers such as interleukin 8 (IL-8) and vascular endothelial growth factor (VEGF).

This is true whether the tumors are VELIPI-plus or VELIPI-minus or, more important, relapse-plus and relapse-minus, in which the primary tumor has already been taken out. There is also no difference in expression of the immunosuppressive markers IL-10 and TGF-b, of FoxP3 and other genes.

Instead, the differences in gene expression between these tumors are almost all related to adaptive immunity. In tumors that show no signs of early invasion or relapse, there are higher levels of Th1 cells, T-BET, interferon-g and IRF-1. There is no difference in the expression of GATA-3 (see Werb, page 21), which is a marker of the Th2 population, indicating that only Th1 cells are important in this phenomenon.

**Killing fields**

It is well known that Th1 cells induce cytotoxic T cells. The markers for cytotoxic T cells, CD8, granzyme B and granulyzin, mediate killing by cytotoxic T cells and NK cells. The higher the level of expression of these genes in a tumor, the better the clinical outcome.

If tumor cells are extracted from the 39 fresh tumors and analyzed by fluorescence activated cell sorting with antibodies against all 65 possible parameters related to the immune system — T cell markers, activation markers, migration, differentiation and memory cells — there is again a clear difference between VELIPI-plus and VELIPI-minus patients.
The tumors that show early signs of invasion lack something that is present in VELIPI-minus patients. Closer analysis reveals that the only significant difference is the presence of T cells: CD3 cells, and both CD4 and CD8 cells. There is no difference in the numbers of macrophages, NK cells or NKT cells and a small, but not significant, difference in the expression of B cells.

T cells and B cells begin as naïve cells. When they encounter an antigen, they undergo a maturation step that transforms them from early memory cells to intermediate and late memory cells. A series of markers can differentiate these various levels of T-cell maturation.

Tumors carry a few naïve T cells. But memory cells which have seen an antigen — albeit not necessarily a tumor antigen — are differentially present in VELIPI-minus and VELIPI-plus populations. Among CD8 cells, the highest difference is borne by the T effector memory cells: those cells that are capable of killing, have seen the antigen and have retained a memory of the antigen.

Based on tissue microarray of large series of tumors, patients who are VELIPI-minus, with no lymph node involvement and no detectable metastasis, have significantly higher numbers of CD45RO+ memory cells. The difference is quantitative and the local immune reaction controls the ability of the cells to emigrate. When the immune reaction is low, metastatic cells can leave the tumor and invade the lymph node. Or the reverse could be true: when metastatic cells begin to leave the tumor, the immune reaction changes. It’s not clear which of the two steps occurs first.

In disease-free, recurrence-free, metastasis-free tumors, the difference is highly significant. Memory T cells, and in particular T effector memory cells, correlate with the absence of early metastatic invasion and improved clinical outcome in colorectal cancer\(^1\).

Adapt or die

To analyze more thoroughly the immune reaction at different stages of the tumor, a quantitative PCR assay can detect whether there are clusters of immune genes that are expressed. Of genes associated with inflammation, adaptive immunity and immunosuppression, only the adaptive immunity cluster is strongly associated with relapse rates. Only 20% of tumors that express these genes at high levels relapse compared with 80% of tumors that don’t express the genes.

Within a tumor, lymphocytes are present in three regions: in the center of the tumor, at the invasive margin, and in lymphoid islets, where the immune reaction goes on. A combined analysis of the lymphocytes at the center of tumor and at the invasive margin can help predict clinical outcome in colorectal cancer.

Having a large number of CD3 cells, CD8 cells, granzyme B expressing cells or memory cells in the center of a tumor makes for a better clinical outcome. The same is true at the invasive margin.
If the two analyses are combined, there is an even more significant impact. If CD3 cells, CD8 cells, granzyme B expressing cells and CD45RO cells are present in high numbers at both the tumor center and at the invasive margin (Hi-Hi), the prognosis is better than if there are low numbers of cells at both sites (Lo-Lo). If the cells are in high numbers at the center and low at the invasive margin (Hi-Lo), they behave significantly differently than if they are low at the center and high at the margin (Lo-Hi), suggesting that both parts have a role to play. The center of tumor seems to be more important for small tumors and the invasive margin more important for larger tumors.

Compared with tumor classification by the usual TNM categories, Dukes’ staging A,B,C,D or UICC 1,2,3, the level of adaptive immune response can predict poor clinical outcome even in small tumors — or at least, those that appear non-invasive.

For example, tumors that are Hi-Hi in CD45RO+ cells have a good level of non-recurrence even with lymph node involvement. Perhaps more interestingly, even stage 1 tumors that lack an immune infiltrate, which would otherwise be treated only by surgery, progress to invasion and metastasis.

These results could change treatment of colorectal cancer. For example, using immune information, it may be possible to predict which patients will rapidly relapse and therefore which patients should be treated more aggressively.

These same results were also observed in an independent series of 119 patients and in a smaller series of 69 patients. With a multivariate Cox analysis of the usual prognostic factors and the immune pattern factor, the only factor that remains highly significant is the immune associated factor. Other factors lose significance at that level.

**Immune infiltrate**

In studies with non-small cell lung cancer, tumors contain all kinds of dendritic cells (DCs), including Langerhans cells, CD1A, interstitial and plasmacytoid DCs and macrophages. However, the vast majority of these DCs are immature and are not capable of inducing an immune response, although they may be capable of inducing tolerance.

Mature DCs are present in the lymphoid islets with DC LAMP, in close contact with T cells. These lymphoid islets behave as a germinal center. There are follicular DCs touching the B cells which proliferate wildly, suggesting that at the contact of the tumor, there is a very strong immune reaction. It’s not clear whether this reaction is against the tumor or against the vasculature, however. The number of mature DCs in these sites correlates with the number of CD3 cells and the number of B lymphocyte CD20 cells.

Most of the cells in the lymphocye gate are T cells by immunofluorescence. In contrast to the DCs, the T cells and the B cells are mature. The T cells at the contact of the tumor are T memory cells, CD3, CD45RO+, and again, as in colon carcinoma, Th1 cells are in close contact with the tumor cells.

Apart from Th1 cells, there are also cells with the full cytotoxic potential. These CD8 cells have perforin, granzyme A and B, TIA-1, and some of them have the CD56 marker. So in the tumor, close to these lymphoid islets, there is all this immune machinery needed to deal with the first metastatic cells that want to leave
the tumor. There is a very diverse repertoire of T cells in the tumor, with no apparent selection of a given T
cell response.

The immune pattern — high or low density of mature DCs, memory T and B cells, Th1 and cytotoxic T
lymphocytes — in lung cancer is independent of the age of the patient, gender, smoking history, tumor
histological type and tumor stage and differentiation. High numbers of mature DCs correlate with high
numbers of T effector/memory cells, B memory cells and favorable prognosis.

After four years with lung carcinoma — which is enough follow up because these cancers are very aggressive
— 95% of those who have the right immune pattern survive versus 44% without. The rate for disease-free
survival with no distant metastases is 88% versus 51% for those who lack the right immune pattern. Overall
survival is 80% versus 35%, suggesting that this immune pattern is a major controller of metastasis and of
clinical outcome.

Whether the tumor is small or large, the right immune pattern can combat cells that go out and colonize
distant sites. In the absence of those mechanisms, even the cells of very small tumors will not be controlled,
will leave the tumor, invade and metastasize. An immune reaction is going on in lymphoids islets that may
induce a central memory robust and persistent enough to take care of the small lumps of metastatic cells
that circulate before they can establish and kill the patient.

In conclusion, the type, density and location of immune cells, the 'immune context', has a prognostic value
that is superior and independent to those of the classical tumor classification systems. The time to recurrence
and overall survival time would be mostly governed by the state of the local adaptive immune response.
This novel paradigm may lead to revision of the current indicators of clinical outcome and, more important,
may help identify the high-risk patients who would benefit from adjuvant therapy.3

References
Inhibition of lymphangiogenesis and metastasis

A report on a lecture by
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The induction of new blood vessels is an important mechanism that promotes tumor growth and metastasis. The lymphatic vascular system offers another conduit for the spread of tumor cells, functioning as an exit route for immune cells from tissues. In the past few years, regulatory molecules and markers specific for lymphatic endothelial cells have been identified. The Prox1 homeobox transcription factor and the FoxC2 forkhead transcription factor, both of which may be implicated in tumor progression, distinguish lymphatic vessels from blood vessels. Vascular endothelial growth factor C (VEGF-C) is essential and VEGF-D dispensable for lymphatic vessel development in embryos. Both stimulate VEGFR-3 and VEGFR-2, which also induce adult lymphangiogenesis. Antibodies directed against VEGFR-3 show significant inhibition of tumor growth in several xenograft models. Blocking the Notch signaling pathway also leads to widespread endothelial VEGFR-3 expression and excessive angiogenesis, which is inhibited by blocking VEGFR-3. Kari Alitalo suggested that VEGFR-3 could provide a target to complement current anti-angiogenic therapies.

The lymphatic system provides a drain. Whenever fluid extravasates from pressurized blood vessels and accumulates in interstitial tissue, not all of that fluid can escape back to the veins and capillaries. Part of the fluid that is retained in the tissue becomes problematic for metabolism, so animals from zebrafish to mammals have developed a system that can drain this fluid back to the blood vascular system.

In order to carry out this main task, lymphatic vessels have to differ greatly from blood vessels. Whereas blood capillaries have adherence junctions between the endothelial cells, lymphatic capillary endothelial cells have valve-like junctions and are joined literally by cadherin buttons.

When interstitial pressure rises, these vessels do not collapse despite having very little structural integrity. They don’t have a basal lamina, for example, but have so-called anchoring filaments, which exert a pulling force when interstitial pressure rises.

Many types of leukocytes, for example activated antigen-presenting cells, are attracted to the lymphatics via chemokine-induced signals secreted from the lymphatic endothelial cells. This is a regulated process. Tumors exploit and abuse the lymphatic system for metastasis.

The lymph vessel system in mammalian embryos develops from the anterior cardinal vein and other large veins. Differentiated cells in the endothelium of the vein that are marked by Prox1 transcription factor start...
a sprouting process. When the gene for vascular endothelial growth factor C (VEGF-C) — which is expressed in a paracrine fashion in the nearby mesenchymal type of tissue — is deleted, the sprouting process is totally inhibited.

Prox1 is a cell type-specific transcription factor, involved in *Drosophila* in inhibiting the self-renewal of one of the daughter cells in the neuroblast. Deleting Prox1 triggers tumors in *Drosophila*. Prox1 is also involved in maintaining the mitotic potential of longitudinal glia.

In mammalian embryos, deleting Prox1 is embryonically lethal. If only one copy is deleted, only 1 in 30 mice survives. Prox1 is also required for elongation of lens fiber cells, and is involved in hepatocyte and lymphatic vasculature differentiation.

About 70% of colorectal carcinomas, but not other tumors, over express Prox1. The same is true in APCmin mice. In SW480 colorectal cancer cell line, only 2% of the cells, the round tumorigenic cells, express Prox1, whereas the 98% of flat cells don’t, suggesting that it may be the tumor stem cells in these cultures that express Prox1.

If Prox1 is down regulated, the round cells revert to adherent phenotype and stay like that for three to four weeks. Eventually, some of them die. In these down regulated cells, cell cycle inhibition markers and many of the differentiation markers go up.

In human colon carcinomas, high levels of β-catenin expression correlate with up regulation of Prox1. Prox1 is downstream of β-catenin and when β-catenin is down regulated, Prox1 is also down regulated. Prox1 is expressed in the mouse small intestine in the +4 position above the Paneth cells, the putative location of stem cells in the intestine.

Knockouts of both Prox1 and VEGF-C inhibit lymphangiogenesis. VEGF-C works through VEGF receptor 3 (VEGFR-3), stimulating the lymphatic endothelium to sprout. Unstimulated cells in the absence of VEGF-C undergo apoptosis.

### Embryonic origin and development

In normal embryonic tissues, lymphatic endothelial cells make what is called lymph sacs, from which additional sprouting occurs in a centrifugal fashion through various parts of the body. Most tissues become penetrated by lymph vessels. This is followed by a maturation phase.

![Figure 2](image.png)

**Figure 2**

VEGF induces dilation of lymphatic vessels but no sprouting lymphangiogenesis.

The FoxC2 transcription factor is important for valve generation and also regulates the pattern of smooth muscle coating of the large lymphatic vessels. These draining vessels do not take up the fluid, but transport the fluid further to the lymph nodes and on to the circulation. All of this happens after mid-gestation in the embryos. Prox1 in particular appears to be very important.

In culture, there do not seem to be any spontaneous inter-conversions between blood and lymphatic endothelial cells.

The gene expression transcripts of the two types of endothelial cells differ by...
Perhaps just 1% to 2%. Among the differences, Prox1 appears to be important. When vascular endothelial cells, which do not express endogenous Prox1, are transfected with Prox1, the homeobox transcription factor is able to down regulate about 40% of the blood vascular endothelial specific genes and up regulate about 30% of lymphatic specific genes.

In the early embryo, Prox1 up regulates the expression of VEGFR-3. This gene is also expressed in the blood vascular endothelium but at this point, its expression starts to be down regulated from the blood vessels. In adult tissues, it's expressed mostly in lymphatic endothelial cells. The VEGF-C and VEGF-D ligands for the receptor have been isolated and characterized.

The ligands are secreted as pre-pro-proteins, requiring at least one proteolytic clipping for activation of receptor binding. After that clipping, they bind to VEGFR-3 and transduce signals for sprouting, migration, proliferation and survival of lymphatic endothelial cells. After the second proteolytic cleavage, these ligands can also bind to VEGFR-2, the main signal transducing receptor for blood vessels, suggesting that they could also have pro-angiogenic functions in some scenarios.

In a heterozygous knock out of VEGF-C, skin lymphatics develop poorly and the mice are born live. They develop lymphedema and swelling of the paws. The homozygous deleted embryos develop swelling, lymphatics fail to develop, and the mice die before term. When the skin is hit with an adenovirus vector expressing VEGF-C, lymph vessels start to sprout. They start generating new vessels and within 14 days, a very dense lymphatic network develops.

If lymph nodes are taken from a red fluorescent mouse, engineered with an adenoviral vector over expressing VEGF-C and transplanted back, virgin nodes develop in the new site in the axilla of the mouse. This induces nice plumbing of these lymph nodes in about 70% of the mice. If those mice are inoculated with new tumors, mimicking tumor recurrence, there the transplanted lymph nodes provide a sieving mechanism whereby the vessels trap the tumor cells and thus block further metastases.

**Blocking metastasis**

In contrast, VEGF doesn’t induce lymphatic sprouting. In a transgenic mouse that over expresses VEGF-A in the skin, compared with wildtype vessels, there’s hyperplasia of the lymphatic vessels, but no new vessels. In the same mice, there is abundant angiogenesis of blood vessels, however.

When a VEGF-C transgene is cloned under the insulin promoter and the transgenic mice are mated with mice that express the SV40 antigen in the islets, a third of these mice come up with lymphatic metastases in the nearby draining lymph nodes, whereas lymphatic metastases are seldom found without VEGF-C overexpression normally. Xenografts with human tumor cells engineered for VEGF-C over expression also show increased lymphatic metastasis.

The VEGF-C knockout has shown that this pathway is important in embryonic lymphangiogenesis.
The pathway can be blocked using various tools, including an inhibitor consisting of a soluble receptor, which is a fusion of the ligand binding domain with the Fc portion of the human immunoglobulin heavy chain. There are also blocking antibodies against VEGF-C and VEGF-D and a second class of inhibitors targeted towards VEGFR-3.

The best tools currently are the blocking antibodies against VEGFR-3, which a collaboration may soon bring into clinical trials. Several different tyrosine kinase inhibitors also inhibit this pathway.

If the so-called VEGF-C/D Trap, the soluble VEGFR-3, is over expressed, starting at E15 in the transgenic mouse, the lymphatic endothelial cells are driven to apoptosis because they don’t survive in the absence of their ligands. The mouse is alive and has lymphedema but otherwise is seemingly normal. In the gut of that mouse, for example, 95% of the lymphatic vessels are missing compared with control.

In the adult mouse, VEGF C/D Trap given via adenovirus homes to the liver and produces the soluble receptor, which penetrates all tissues. Growing lymphatic vessels associated with tumors are again driven to apoptosis, but mature lymphatic vessels are saved and persist.

Lymphatics do not penetrate deep into the tumor. There’s good evidence that only the lymphatics in the tumor periphery are functional because they collapse inside the tumor as a result of the interstitial pressure.

Typically, in mice that have been treated with VEGF C/D-Trap, there is no detectable metastasis. Using a variety of models, including xenografts and transgenic tumors, typically about two-thirds of metastasis is inhibited late into tumor development. Different tumors seem to require a different dosage, in some cases requiring very high levels of the soluble protein, but in each case so far, metastasis has been considerably delayed.

Metastatic spread

Tumor cells implanted in the ear produce endogenous VEGF-C, which triggers sprouting of lymphatic vessels in the tumor periphery. These vessels become very leaky. If dextran is injected into the skin, it is taken up by the lymphatic capillaries and carried by the collecting lymphatics deep into the skin. However, dextran leaks out from lymphatic capillaries within the tumor, staining the tumor stroma.

When this tumor is treated with the soluble receptor, the lymphatics now have a smooth surface. The numbers of tumor cells within the lymphatic vessels would be fewer in this mouse. If fluorescent dextran is injected into the vicinity of this tumor, it’s taken up by the lymphatic vessels and carried away, but the tumor is almost black because there is no leakage. So the therapy blocks the sprouting and the leakage, by sealing the endothelium.

There’s also a second process. When the ligand is extravascular, the cells sense a gradient and migrate upwards of the gradient. But if the ligand comes from the tumor intraluminally, the cells undergo mitogenesis in the vessel wall. When the tumor is treated with VEGFR-3 signaling inhibitors, the collecting lymph vessel size goes down via inhibition of DNA synthesis and mitogenesis.

One model to study further steps in the transgenic model is the keratin 14-VEGF-C over expression model, where a hyperplasia emerges late in embryonic fetal development.

If a two-stage chemical carcinogenesis is used on adult mice, the mice that come up with tumors have greatly increased lymphangiogenesis in the draining lymph nodes. There are a lot of lymphatic marker-positive cells within the lymph node structure even before cells have metastasized, indicating that there might be a wash down of the growth factor all the way into the lymph nodes, which might induce trophic changes before the tumor cells arrive.
When carefully quantified, there is some statistical significance. If VEGF-C is over expressed, there is a statistically significant increase in sentinel lymph node metastasis. There’s also an increase in the distal lymph node metastasis and lung metastasis.

Among mice that don’t over express VEGF-C, the mice that have lung metastases are exactly the same ones that have distal lymph node metastases, suggesting that cells from the squamous cell carcinoma get to the lung, presumably via the lymphatics and not via the blood circulation.

Screening a number of tumor xenografts reveals that when lymphangiogenesis is inhibited using the blocking antibody against VEGFR-3, tumors are slightly smaller by luminescence imaging. The volume and weight is also significantly smaller in 60-70% of tumors.

When the blocking antibody is combined with low doses of avastin, there seems to be a trend toward additional effect. Blood vessel density also appears significantly decreased in the treated tumors. There is a clear increase in hypoxic and necrotic cells.

**Blood vessel sprouting**

Although VEGFR-3 is down regulated after the embryonic period, it’s re-expressed in the tumor vessels. When VEGFR-3 is removed from mouse embryos, blood vascular remodeling fails. There’s no hierarchical development of small vessels, arteries and veins, and the mice eventually die. If the soluble VEGFR-3 is used in Zebrafish, blood vessel development is again severely disturbed in the developing embryos.

In post-natal angiogenesis, sprouting vessels are clearly positive for VEGFR-3. An antibody that blocks VEGFR-3 inhibits sprouting and reduces the vascular area, suggesting that VEGFR-3 is involved in post-natal angiogenesis in the retina.

What’s more, when VEGFR-2 specific ligand VEGF-E is over expressed under the keratin promoter in mouse skin, there are many more vessels and these abnormal vessels express VEGFR-3.

Several recent papers have noted that when DLL4 Notch signaling is inhibited, there is hyper sprouting in the retina that is not productive for angiogenesis. DLL4 normally instructs adjacent cells not to become tip cells, allowing only one cell to sprout at a particular location.

The tip cell up regulates DLL4, which binds to a Notch receptor in the adjacent stalk cells, and instructs them not to sprout. If DLL4 is inhibited, sprouting occurs everywhere and the process becomes ineffective. VEGFR-2 is known to be up regulated in the tip cells and down regulated in the stalk cells.

When the mice are treated with a g-secretase inhibitor, which blocks Notch cleavage, Notch no longer goes to the nucleus, and there is VEGFR-3 expression everywhere in the retina. Notch thus seems to control the expression of VEGFR-3. Blocking with VEGFR-3 specific antibody significantly inhibits hyper sprouting more than does a VEGFR-2 specific antibody, suggesting that the VEGFR-3 pathway is more intimately associated with angiogenic sprouting.

In the tumors, where there is hypoxia and necrosis, PECAM-1 stains the vasculature, but not the sprouts or tip cells. Double staining with VEGFR-3 antibodies reveals VEGFR-3 expression in the sprouts, which emerge...
from the PECAM-positive blood vessels. There are many sprouts in the tumor as it grows, but disappear when inhibit with the soluble receptor.

Besides inhibiting lymphangiogenesis and metastasis, using the antibody might also inhibit angiogenesis. Recent experiments in collaboration with ImClone show a dramatic synergistic effect of VEGFR-3 and chemotherapy.

References
Pro-angiogenic hematopoietic and vascular cells can be mobilized from the bone marrow to contribute to angiogenesis during tumor growth or acute vascular injury. The variability in the incorporation of marrow-derived cells depends on the tumor’s growth phase, aggressiveness and the degree of vascular trauma. Overall, luminal incorporation of the hemangiogenic progenitors is highest in the early phases of rapidly growing tumors or acute vascular injury. Co-recruitment of VEGFR1+CXCR4+ hematopoietic cells conveys signals that support incorporation and differentiation of VEGFR2+ endothelial cells into functional neo-vessels. Stromal derived factor-1 provides cellular road maps for the localization of these cells to the vascular niche and subsequent mobilization into the circulation. The number of engrafted SDF-1 recruited endothelial progenitors and the magnitude of demand for neo-angiogenesis determines the extent of luminal incorporation of endothelial cells. Shahin Rafii explains that targeting CXCR4 in conjunction with VEGF-A receptors may provide an effective means to block growth and metastatic potential of hemangiogenesis-dependent tumors.

Within the hematopoietic system, there are cells that can be anti-angiogenic and anti-tumorigenic and those that can be pro-angiogenic and pro-tumorigenic. Similarly, macrophages can also under certain conditions support angiogenesis and tumor growth and under other circumstances act in an anti-angiogenic, anti-tumorigenic manner.

CXCR4, a chemokine receptor for stromal derived factor 1 or SDF1, and vascular endothelial growth factor receptor 1 (VEGFR1), a tyrosine kinase receptor for VEGF-A, together provide the mechanism for hematopoietic cells to stabilize and induce angiogenesis.

In the model system used, when CXCR4 and VEGFR1 are activated, hematopoietic cells function as pro-angiogenic and pro-tumorigenic and the cells can establish a pre-metastatic niche.

Published data have shown that when SDF1 and VEGF-A are upregulated, this hits the bone marrow endosteal zone where, through activation of CXCR4 and hematopoietic cells, they induce mobilization of the cells into the circulation. Some of these cells are CXCR4/VEGFR1 hematopoietic cells, some are progenitors, some are bonafide monocytes and macrophages, and others are platelets. These cells release a novel factor called angiomodulin, allowing vessel stability and tumor angiogenesis to take place. They also initiate the pre-metastatic niche.
CXCR4 and VEGFR1 are expressed in 20% of this cell population. Both of these receptors are also expressed on platelets and megakaryocytes.

By releasing VEGF-A, tumor cells engage VEGFR2, which is exclusively expressed on endothelial progenitors. VEGFR1 activation turns on VEGFR1/CXCR4 hematopoietic cells, called hemangiocytes, which are pro-angiogenic hematopoietic cells. CXCR4 predominantly produced by platelets also turns on these cells.

When primary tumors are implanted, before the tumor arrives in the lung or in metastatic niches, clusters of CXCR4 and VEGFR1 are formed. By day 16/18, tumor cells arrive in this cluster, and full grown metastasis takes place. If these genes are knocked out, there is no distant metastasis.

It seems that the tumor cells release factors that remodel collagen and fibronectin, which allows the CXCR4/VEGFR1 cells to establish themselves. This combination of fibronectin and CXCR4/VEGFR1 forms a pre-metastatic cluster when the tumor eventually homes.

This is an interesting concept, because it helps define why certain tumors spread to certain sites. It's possible that the specificity of soluble factors produced by these tumors dictates the formation of pre-metastatic niches and tumor metastases. For example, if the conditioned medium for B16 melanoma is injected into mice with Lewis lung carcinoma (LLC), the LLC tumors, which usually don't metastasize to kidneys or oviduct, now begin to metastasize to those sites.

**Blocking metastasis**

This begs the question, what are the factors that allow tissue specificity to take place?

Tissue-specific activation of chemokines, such as S100, seems to dictate this specificity. The primary tumor, by elaboration of VEGF-A and tumor necrosis factor (TNF), turns on recruitment of the signaling cascade, which activates CXCR4 and VEGFR1.

These factors establish themselves within pre-metastatic clusters. Selectively in the lung, the cells produce S100-A9, which is a chemokine selectively expressed in the lungs. This remodels collagen and fibronectin and allows tumor metastasis to take place. In this model, S100-A9 is not produced in other tissues and as such, there is no metastasis at those sites.

If this is correct, blocking VEGFR1 and CXCR4 should abolish the formation of pre-metastatic niches and prevent metastasis. A VEGFR1 neutralizing antibody is not enough on its own, nor is blocking CXCR4 alone. But if both are knocked out, pre-metastatic clusters don’t form and distant metastasis is abrogated.

The release by tumor cells of specific factors, such as VEGF-A, TNF and perhaps TGF-β, promotes the recruitment of CXCR4/VEGFR1 hemangiocytes. The cells that localize to pre-metastatic niche can form colonies. These cells include progenitors, macrophages and a small subset are bonafide hematopoietic stem cells. S100 chemokines are selectively induced in the lungs and selectively allow the pre-metastatic niche to form in the lungs. If the chemokine is blocked, metastases don’t form in the lung, but do in other tissues.
Assembling vessels

The magnitude of SDF1 seems to modulate the extent of mobilization of these cells. K-Ras mutated stroma also increases SDF1 production and mobilization of a lot of these cells. Inhibition of SDF1 seems to block mobilization of CXCR4/VEGFR1, thereby abrogating the establishing of the pre-metastatic niche.

This provides a nice genetic model to understand which released factors cause vessel assembly to take place and how pre-metastatic niches form. Using a genetic screen, one factor isolated is angiomodulin, a 30 kDa protein, which localizes predominantly to tumor vessels and seems to play a role in vascular remodeling.

This gene in zebrafish is not duplicated but is homologous to both human and mouse forms. When morpholinos are made against this angiomodulin and put into zebrafish, they completely abrogate the vessel remodeling.

By confocal microscopy in wildtype mice, intrasomatic blood vessels migrate and approach the top blood vessels, but in these zebrafish, the migration of the blood vessels is abrogated. The vessels have mispatterning defects, and they don’t assemble very well, suggesting that angiomodulin plays a role in blood vessel assembly.

Zebrafish also provide a great genetic model to understand the interaction between two different cell types. If a low-dose morpholino is introduced against VEGF, it doesn’t induce a knockout. Low dose morpholino of angiomodulin doesn’t induce any manifestation of angiogenesis. But if the two non-effective low doses are given together, there is a resulting phenotype, suggesting again that these two interact genetically. Angiomodulin appears to bind SDF1 and VEGF-A and is a molecular chaperone that presents these two molecules to their receptors.

A knock in/knock out mouse, in which the native promoter of angiomodulin derives the pro expression of lacZ, shows that angiomodulin is predominantly expressed on embryonic vessels, but shuts off in the adult formation. When the tumor is implanted, tumor vessels induce the expression of angiomodulin both on endothelial cells and hematopoietic cells. The adjacent normal tissues don’t express angiomodulin, however.

Similarly, although the normal liver does not express angiomodulin, the metastatic liver expresses high levels of angiomodulin, both on the vessel and on hematopoietic cells. Tumor growth is significantly abrogated in angiomodulin knockout mice, underscoring the molecule’s importance.

Role for platelets

VEGF-A expression is induced by hypoxia. Where does SDF1 come from? Malignant tumor stroma is believed to be epigenetically programmed to produce SDF1 (see Weinberg, page 37), but in this model, VEGF-A hits platelets through VEGFR1, up regulates SDF1 and allows mobilization to take place.

A series of experiments reveals how platelets are essential for pre-metastatic niche formation (see Hynes, page 61). In mice lacking thrombopoietin or TPO, a principal growth factor for platelets, the level of platelets is decreased by 95%. This profound decrease in platelets prevents tumors from growing. Giving TPO to these knockout mice increases the number of platelets, and tumor growth is enhanced.
In the TPO knockout mice, counting residual tumors shows that angiogenesis is very defective, the areas of necrosis are increased and vessel density is significantly decreased. The metastatic lesion is non-existent in the lungs or kidneys of these mice, suggesting that when these pre-metastatic niches are formed, the vessels are very thrombotic. They recruit a lot of platelets, which provide an environment by releasing VEGF and other growth factors for establishing a pre-metastatic niche.

Based on this model, there may be two pathways. VEGF-A hits the platelets, produces SDF1 and recruits these cells to the vasculature. These CXCR4/VEGFR1 cells, by releasing angiomodulin and other factors such as proteases, allow angiogenesis to take place. Thrombospondin and PML seem to temporize this pathway.

In conclusion, CXCR4/VEGFR1 cells are pro-angiogenic hematopoietic cells that establish tumor vessels by secreting angiomodulin. Angiomodulin is induced in tumor vessels and functions as a molecular chaperone, presenting VEGF-A and SDF1 to their receptors, VEGFR1 and CXCR4. The balance of pro-angiogenic (SDF-1, VEGF-A) and anti-angiogenic factors [Thrombospondin-1, -2] by platelets dictates the extent of tumor neo-angiogenesis and metastasis.

Various groups have shown that these VEGFR2 endothelial progenitors seem important for certain tumors to get mobilized from the bone marrow and contribute to angiogenesis. VEGFR1/CXCR4 hematopoietic cells may provide instruction for this process.

In Id1 -/+ Id3-/- knockout mice, there is an angiogenesis defect and tumors do not grow. If you transplant the tumors into wildtype mice, they become angio-competent, because bone marrow-derived hematopoietic cells and endothelial cells are recruited to the tumor and allow the tumor to grow.

**Imaging development**

The contribution of these endothelial progenitors seems to be very specific to rapidly growing tumors, the early phase of tumors and also during acute tissue remodeling, such as after a heart transplant.

But studying endothelial progenitors in adults is very difficult because evidence shows that in addition to the bone marrow, organ-specific endothelial progenitors exist in organs. With bone marrow transplants, for instance, endothelial progenitors may not appear to contribute, but that may not be the case because there are organ-specific endothelial cells.

To study these endothelial cells from organs, a new, very elegant model uses non-approved human embryonic stem cells developed at Cornell. Human embryonic stem cells can be instructed with different growth factors to develop embryonic bodies and for huge tissue explants, some of which can have a functional myocardium beating and neuronal tissue and function almost like a flattened three-dimensional embryo. These hearts are very vascular, making it a good model to study angiogenesis.

A lentivirus with a VE-cadherin promoter driving reporter GFP is expressed only in the endothelium and not in smooth muscles, fibroblasts or mesenchymal cells. The formation of embryoid bodies and the endothelium can be seen over time using real-time confocal microscopy.
Beyond nine days, multiple tissues are formed. By day 11, intestinal and other cavities form, revealing how the vasculogenic foci give rise to a vasculogenic plexus and angiogenic foci, as it happens during embryonic development. By day 18, there are areas of hematopoiesis, with blood cells moving into blood vessels and neuronal tissue with blood vessels. As this progresses, very interesting structures form, such as an aorta-like structure with endothelial cells traveling through it.

This also provides a model to study tumor angiogenesis. Using the lentivirus with human embryonic stem cells, if human teratoma are introduced into mice, and then the mice are injected with lectin to interrogate the specificity and functionality of the vessels, human GFP endothelium is seen surrounding functional vessels. A lot of the endothelium is perivascular, but some also become intravascular.

To get rid of mouse endothelial cells, antibodies to VEGFR2 and VE-cadherin, two molecules that are absolutely essential for angiogenesis, are used. These are specific for mice and don’t react with human molecules.

Fully humanized tumor vessels are then generated. As lectin is injected, which shows functionality of these vessels, GFP-positive human vessels, stained with human CD31, have functional blood going through them. At a maximum, about 20-30% of fully humanized CD31 positive GFP-positive functional human vessels fuse to mouse endothelial cells.

References

PART IV: CANCER STEM CELLS

Paolo Comoglio
Invasive growth: a MET-driven genetic program for cancer and stem cells

Hans Clevers
Wnt and Notch cooperate to maintain proliferative compartments in crypts and intestinal neoplasia

Owen N. Witte
Progression of prostate cancer from normal tissue stem cells
Metastasis follows the inappropriate activation of a genetic program termed invasive growth, which is a physiological process that occurs during embryonic development and post-natal organ regeneration. There is growing evidence to indicate that invasive growth is also executed by stem and progenitor cells, and is usurped by cancer stem cells. The Met proto-oncogene, which is expressed in both stem and cancer cells, is a key regulator of invasive growth. Paolo Comoglio presented recent findings which indicate that the Met tyrosine-kinase receptor is a sensor of adverse micro-environmental conditions, such as hypoxia, and drives cell invasion and metastasis through the transcriptional activation of a set of genes. The early invasive-metastatic response includes the generation of a niche by transcriptional regulation of genes of the coagulation cascade. The late response includes activation of self-renewal genes such as Notch. Invasive growth and metastasis survival can be hampered therapeutically by targeting Met.

Invasive growth is not a program designed specifically for invasion and metastasis but is a normal physiological program intended for embryo development, wound healing and organ regeneration. This normal program is often turned on inappropriately by cancer cells, which exploit the process for their own survival.

The physiological program for invasive growth must be controlled by a signaling system. There are at least two families of molecules that may be responsible: scatter factor or Hepatocyte Growth Factor (HGF), which has long been known, and the relatively new semaphorins. These proteins are apparently dissimilar on a structural basis but are evolutionarily related. The semaphorin domain, which characterizes this class of signals, is a piece of the receptor for scatter factor.

Only certain growth factors can induce invasive growth, or at least scattering, in vitro. For example, the epidermal growth factor or EGF induces many things including cell movement, but does not induce scattering. Liver progenitor cells, which carry the EGF receptor, are stimulated by physiological concentrations of EGF to migrate and divide, but the cells do not scatter.

In contrast, early liver progenitors, which can give rise to either hepatocytes or bile duct cells, start to move when stimulated with sub-nanomolar concentrations of HGF. The cells then detach, become independent, highly motile and, most important, they survive anoikis. Anoikis is a well-known phenomenon by which cells that lose cell-cell contact or cell-matrix adhesion die by apoptosis. It’s a safety mechanism to prevent inappropriate dissemination of cells during embryogenesis.
The functional Met receptor is the prototype of the invasive growth receptor family: it has a semaphorin domain of 500 amino acids, a Met-related sequence (PSI, a plexin semaphorin, integrin motif) and a G-P rich immunoglobulin-like domain of 400 amino acids. There is also a short hydrophobic transmembrane sequence, and a juxtamembrane domain that contains a serine which, when phosphorylated, shuts off the kinase. The cytoplasmic catalytic domain is endowed with tyrosine-kinase activity, which increases about 100-fold when the receptor dimerizes.

Invasive growth is such an important physiological program that it cannot be regulated by a single gene. There is a family of Met-related receptors that includes Ron, which is often present in tumors that don’t express Met. Ron and Met are structurally related in the cytoplasmic domain.

These receptors sit in functional structural complexes. In the absence of the ligand, Met is associated with a splicing variant of CD44, which is both storage for the ligand and a link to the cytoskeleton. Met also functionally interacts with plexins, which are the receptors for semaphorins. The integrin-α6β4 is also involved in the case of the Met receptor — and other integrins in case of Ron.

**Combinatorial assembly**

When HGF binds at the receptor and the receptor dimerizes, there is first activation of the kinase by trans-phosphorylation, and then phosphorylation of a tail docking site (called "the super site"), which binds to p85 and PI3-kinase at one site and GRB2 at the other. GRB2 is upstream of Ras, which is responsible for growth. Through this pathway, there’s transient MAP kinase activation, which is responsible for proliferation.

Met does not only induce proliferation. The full program requires adaptors that have been identified, and that contains multiple tyrosines phosphorylated by Met. One is the cytoplasmic domain of the integrin-α6β4, which contains three sites that upon phosphorylation by Met generate specific binding for SHP2, a phosphatase that dephosphorylates SRC and activates it. Activated SRC, in turn, phosphorylates specific sites on a second adaptor, GAB1, which contains more sites for PI3K, GRB2, PLC-g and CRCK. This creates sustained MAP and PI3-kinase activation and is responsible for cell invasion and for apoptosis protection.

The program does not end until STAT3, an important transcription factor, is recruited. STAT3 binds to tyrosine in the tail of Met and is phosphorylated. Phosphorylated STAT3 generates a site with higher affinity for the SH2 domain of another STAT3 molecule, leading to the formation of a dimer. This dissociates from the receptor, migrates to the nucleus and triggers cell differentiation. Epithelial cells invade the surrounding matrix and make branched tubules with a lumen inside. The whole process is called branching morphogenesis.

HGF induces invasive growth while EGF does not because although the signal transducers are all the same, the specificity lies in the combinatorial assembly of receptors. The Met receptor matches with integrin-α6β4, and both of them combine with plexin. There may be other factors that have not yet been discovered. It’s not the receptor that generates the intracellular signal, but this receptosome, which induces concomitant activation of multiple signaling pathways.
There is a biological response, a factor that induces it, and a selective signal transduction mechanism, so there should also be genes that are specifically or selectively turned on or off in the 'Met transcriptome'.

When a piece of bloody tumor is compared with a piece of the so-called surrounding normal tissue, it’s the genes that are turned on in the cancer epithelial cells that are of interest to measure. But in a tumor, epithelial cells comprise less than 20%; the remaining 80% are macrophages, fibroblasts, endothelial and other cells.

To overcome this problem, a clonal cell line is derived from a single cell to minimize variability. The cells are grown in an artificial extracellular matrix (ECM) made up of collagen and glycans. Under these conditions, HGF scatter factor in nanomolar concentration induces scattering and branching morphogenesis. The same cells grown in the very same conditions are used as control, and given just serum, EGF, or nothing.

When HGF-stimulated hepatocyte progenitors are compared with EGF-stimulated cells, more than 1,150 genes appear to be turned on and about 250 genes are differentially regulated. Although genes are turned on by both growth factors, the pattern of activation is different.

Patterns of expression

When gene expression is compared at various times after the ligand is given — at 1 hour, 6 hours and 24 hours — a number of ‘immediate early’ genes are turned on by HGF, but not by EGF, after 1 hour and are suddenly shut off after 6 hours.

‘Delayed early’ genes turn on only after 6 hours and disappear at 24 hours. ‘Late’ genes, which are mostly machinery, are turned on late and stay on. They code for proteolytic enzymes, migratory molecules, actins, actin-associated proteins and proteins that prevent apoptosis.

Interestingly, there are some genes that are biphasic, that are turned on and off, which can be grouped by functional annotation. There are 11 genes involved in ECM chromatin remodeling; 21 cytokines that are early genes and 27 apoptosis genes, most of which prevent apoptosis. There are also genes for cell cycle, transcriptional factors, cytoskeleton, metabolic genes for protein synthesis, 104 transcriptional factors, other factors for signal transduction, metabolism and 316 unknown genes.

Surprisingly, the Met oncogene regulates most of the 25 known genes of the blood clotting cascade that turn soluble fibrinogen into fibrin. This is an important mechanism of the machinery that drives invasive growth.

Conventional transgenics are a suitable model to study only the 3-4% of cancers in which the genetic lesion is inherited. In the vast majority of tumors, there are sporadic mutations where a few cells, that have activated (mutated) oncogenes, are surrounded by normal cells. To generate a mouse model that is closer to human sporadic cancers, a lentiviral vector that can integrate genes into adult non-dividing cells, including stem cells, is used.

As expected, using a specific promoter, the liver develops foci of pre-neoplastic cells that express the human activated Met oncogene introduced with the vector. At the end of the experiment, some of these foci turn into a liver carcinoma. Surprisingly, however, 100% of the mice at very early pre-neoplastic stages show a thrombo-hemorrhagic syndrome. The lesions have an artery wall with a big clot inside and no sign of inflammation.

Interestingly, this phenotype precedes the overt transformation of the cell. The onset of thrombosis, induced by the activation of a Met oncogene, is between two and four weeks — at the same time as clonal expansion, but before the cells look cancerous. The cells begin to look like cancer at 12 weeks and by 20 weeks mice have overt cancer.
Between 10% and 15% of patients, especially those with pancreatic cancer, show up with no sign of tumor, but with clots and hemorrhages. In 1865, Armand Trousseau described this syndrome as Migrans thrombophlebitis, or Trousseau’s syndrome, a sign of occult malignancy. As a sad anecdote, Trousseau diagnosed himself with stomach cancer and died of the disease. Interestingly, the most activated gene downstream of Met is plasminogen activator inhibitor 1 (PAI-1), a key player in coagulation. In the literature, PAI-1 has been repeatedly associated with cancer onset and progression. A second gene is COX-2, a robust metabolic gene that synthesizes the coagulation factor thromboxane.

In conclusion, both cancer cells and normal cells have a signaling system that is turned on by its ligand, and this leads to the transcriptional activation — among others — of PAI-1, COX-2 and the other 25 genes of coagulation. The cells produce and release in the nearby pericellular space coagulation factor that convert fibrinogen into fibrin. This pro-coagulant activity leads to “fibrin nesting”, which was described by Virchow decades ago, and functions as a niche for stem cells or a scaffold for anoikis protection and cell migration.

**Invasive program**

Invasive growth seems to be a physiological program that is useful for stem cells. Invasive growth is observed at the very early stage of embryonic development. Embryo cells are driven either by an autocrine pathway, in the case of ectodermal cells that produce HGF, express the Met receptor and migrate to form the mesoderm; or in later stages by a paracrine system. An example is the migration of myoblast stem cell or myoblast precursor, from myotomes toward the tip of the limb buds, following a gradient of the ligand, HGF, produced there. In the literature, there is evidence that in post-natal tissues, Met is a functional marker of stem or progenitor cells. This is clear in the liver, pancreas and bone marrow and may be the case in the gut. There is very little Met in adult differentiated epithelial cells. Most of the Met receptor is expressed in a site that suggests association with stem or progenitor cells.

Met is not present in *Drosophila*, but when an activated Met transgene is introduced into *Drosophila*, it transcriptionally regulates the Notch pathway, via Delta. Activated notch induces HES-1 transcription and represses Met. This negative feedback loop is particularly interesting since invasive genes have to be turned “off” once cells reach the distant site. They stay and don’t migrate anymore.

Moreover via the late activation of Notch the invasive growth program turn “on” stemness genes so that the cells can be clonogenic. In terms of chronology, Met is switched off after several hours. That’s consistent with the inactivation of Met in a distant site and activation of self-renewal genes in that distant site for clonogenic purposes.
Hypoxia response

Inappropriate activation of the invasive growth program may generate an aggressive phenotype that gives invasion and metastasis. When Met is activated by mutation, the tumor becomes invasive. Mutations are found in 5-10% of human cancers. However, as a rule, Met over expression in primary tumors is the driving force that activates invasive growth, and this accounts for 50% of gastrointestinal-tract tumors, 70% of thyroid, and a significant subset of prostate and mammary tumors.

The Met promoter has five hypoxia response elements and one AP1 binding site, which provide the perfect machinery to respond to hypoxia. In experimental tumors and in human cancer, the Met protein is produced in response to low oxygen concentrations.

In an in vivo experiment with a growing tumor, if Met is switched off by an inducible shRNA driven by a tet repressor, the tumor still grows, but is no longer metastatic. If the same experiment is repeated with Met allowed to operate for 45 days, the mice have already established lung metastases. When Met is switched off by the inducible tet repressor, metastasis disappears. This is a bright example of “oncogenic addiction” and strongly suggests that Met could serve as a target for therapeutic approaches.

There are several Met inhibitors, although not many of them work. However, there is an antibody that has a unique mechanism of action. The antibody is a partial agonist but surprisingly, it induces cleavage of the extracellular site, which then becomes a “decoy” further impairs ligand binding.

Using the cDNA for this antibody in a gene therapy approach, cancer cells synthesize and assemble the antibody, which binds with very high affinity and down regulates the Met receptor from the cell surface, ultimately inhibiting anchorage-independent growth in vitro, and metastasis dissemination in vivo.

In summary, invasive growth is a genetic program of stem cells, usurped by cancer stem cells. It is triggered by specific signals, transduced by an oligomeric receptor complex of Met, plexins and integrins. Met expression is induced by unfavourable micro-environmental conditions, such as hypoxia.

The early invasive-metastatic response includes the generation of a niche by transcriptional regulation of genes of the coagulation cascade. The late response includes activation of self-renewal genes such as Notch. Invasive growth and metastasis survival can be hampered therapeutically by targeting Met.

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Mutations in the Wnt pathway components APC and β-catenin induce sustained complex formation of the co-activator β-catenin with TCF transcription factors. The resulting transactivation of TCF target genes is believed to represent the primary transforming event in colorectal cancer. Inhibition of β-catenin/TCF activity restores the differentiation program in colorectal cancer, despite the presence of multiple other mutations. The Notch pathway also controls cell fate along the crypt-villus axis. Upon conditional blocking of the common Notch pathway, there is a rapid, massive conversion of proliferative crypt cells into post-mitotic goblet cells. Stem cells in the colon are believed to be at the +4 position. However, six tiny cells, called the crypt-based columnar cells, appear to better fit the bill. The cells can be identified by a single marker, the GPR49 protein, and make all the cell types of the colon. Hans Clevers presented the first formal proof that GPR49 is a single marker for adult stem cells in the intestinal tract.

The gut epithelium is divided into crypt compartments and villi. It is an ideal system to study stem cells, which are thought to be somewhere near the bottom of the crypt. The same processes that control the extremely rapid cell renewal in the gut also deregulate it and cause cancer of the gut and colon.

It takes only about four to five days for the cells at the bottom of the crypt to go through the entire cycle of proliferation, differentiation and apoptosis. Stem cells that are either quiescent or steadily cycling give off daughter cells that occupy most of the crypt. When the cells reach the top of the crypt, they either become absorptive cells or secretory lineage cells. Of the latter, Paneth cells can reside at the bottom of the crypt for up to eight weeks.

There are no molecular differences between the small intestine and the colon, but the epithelium is very different histologically. The surface of the colon is flat with crypts, but no villi. Stem cells have never been studied in the colon, but are believed to be somewhere near the bottom of the crypts. Proliferative cells, which are mixed in with the goblet cells, start proliferating near the bottom, move up and die at the top.

Wnt signaling is crucial in the colon. The TCF-1 gene, cloned a long time ago, has two partners. One of the partners, groucho, turns TCF into a repressor of transcription and the other, β-catenin, turns it into an activator of transcription.

At the heart of the canonical Wnt cascade is β-catenin, which is synthesized at a steady rate in every cell of the body. The protein is translated and immediately captured by a complex that leads to its rapid phosphorylation and subsequent degradation by the proteasome. As a result, β-catenin never reaches significant levels of cytoplasmic expression.
In the nucleus, TCF is bound by groucho. There are multiple grouchos in the genome which potently repress a tissue-specific transcriptional program. TCF target genes in the gut are totally different than those in the kidney or the lung so findings from one organ are not applicable in the others.

When Wnts occupy their receptors, they somehow block the complex, so b-catenin is never phosphorylated, accumulates and some of it goes into the nucleus. In the nucleus, b-catenin replaces groucho, binds TCF and turns TCF transiently into a transcriptional activator.

**Gut signals**

Up to 90% of colon cancers are believed to harbor APC mutations. In those cancers, constitutively high levels of b-catenin occupy TCFs, and the pathway is locked into the on state. Point mutations in the target sites for the kinases will also render b-catenin indestructible, resulting in constitutive levels of b-catenin.

In the gut, there are four TCF/Lef relevant genes. When the APCmin mouse, which already carries one mutant allele at high frequency, loses the second allele, cells accumulate b-catenin. In these cells, b-catenin activates TCF4 and instructs the cells not to differentiate, but to slowly proliferate in a fairly undifferentiated state.

There are higher levels of b-catenin near the bottom of the crypts, where physiological Wnt signaling occurs. There is a gradient of decreasing nuclear b-catenin about halfway up the crypt, which correlates with the presence of proliferative cells.

Of the 20 Wnts in the mouse genome, a number of them are expressed in a gradient near the bottoms of the crypts. When TCF4 is knocked out, the mouse lives to about birth, suggesting that these Wnts do signal and have a function. The gut normally has pockets of cells with prospective crypts that are cycling. The TCF4 knockout mice have villi, but they don’t have pockets of proliferative cells, implying that Wnt is needed to establish crypts.

Wnt is also needed to maintain crypts. When the dickkopf1 protein — which occupies Wnt receptors and blocks them — is expressed in patches in the gut by the gut-specific Villin promoter, there is no proliferation in those patches, implying that Wnt is needed to maintain proliferative crypt compartments throughout life.

The Notch pathway is also required. When Notch is activated by its ligand Delta, it is cleaved by the g-secretase enzyme and other proteases. The intracellular domain goes to the nucleus, where it binds a transcription factor called CSL. There’s only a single CSL in the genome, so a floxed CSL allele allows the deletion of all Notch signaling in a tissue.

The downstream effectors of the Notch pathway are conserved between worms and men. The first tier of transcriptional repressors is Hes1, a crucial gene in the gut. Hes1 is translated in the cytoplasm, goes back into the gut and then inhibits the expression of a second target gene called Math1.

Normally, only secretory cells stain positive for Math1. But when CSL is deleted in an adult mouse, all Notch signaling is blocked and as a result, all crypt cells become Math1 positive.
If goblet cells are stained for Pas, a chemical stain for mucus, about one in ten villus cells is Pas-positive. But when Notch is blocked, all the proliferative cells in the crypt are converted in a few days to Pas1-positive goblet cells. These mice die within the first few days of blocking the Notch pathway because they lose proliferation.

In summary, crypts require two potent signals, Wnt and Notch. If Wnt signaling is taken away, all the cells become enterocytes and if Notch signaling is lost, cells stop proliferating and turn into goblet cells. Wnt and Notch together maintain proliferative cells.

Crypts and cancers

Through TCFs, Wnt directly activates hundreds of target genes in a tissue-specific manner. In crypts, Wnts maintain crypts and progenitors, and those two phenomena are likely to be linked.

In various experiments using dominant negative TCFs, inducibly deleted b-catenin, TCF4 knockout mice, or APC deletions, microarrays can reveal which genes are up regulated. In each case, there is a relatively short list of about 500 genes. Comparing the lists reveals an overlap of 350 genes that appear to be activated by the Wnt pathway.

Colon cancers have an activated Wnt pathway and when it is blocked, the cells immediately switch to a differentiation program. This implies that despite all the other mutations in these colon cancer cells, the initiating mutation is a Wnt pathway mutation. Even 30 years later, the cells appear to be dependent on this initiating mutation because when it is blocked, the cells differentiate and die five or six days later.

For example, EphB2 is known to be up regulated in colon cancer. In an adenoma of the human colon, there’s a gradient of this Wnt target gene in the crypts. Its levels are high at the bottom and taper off halfway up the crypt, but in the cancer, it’s ubiquitously expressed. Although all these genes are co-expressed in human colon cancers and in mouse adenomas, however, they have slightly different expression patterns in the crypts. Most of the genes, such as c-Myc, are expressed in a gradient, high just above the Paneth cells and then tapering off toward the top of the crypt. Most of the 250 genes turn out to be proto-oncogenes and cell cycle regulators, S-phase, M-phase proteins.

Wnt drives cells into proliferation and inhibits the differentiation of these cells. Myc appears to be a bit special. In colon cancer cells, when the Wnt pathway is blocked, but Myc is stably expressed, the cells keep proliferating, suggesting that they only need Myc at endogenous levels to actively divide.

If Myc is knocked out using the inducible Cre-mouse, the mouse is entirely negative for Myc for the first few days after deletion. However, after about a week, large hyperplastic crypts that stain positive for c-Myc begin to appear, suggesting that some cells escape. Reacting to the absence of enough crypts, they undergo crypt fission, divide sideways and rapidly fill out the epithelium, replacing the Myc-negative crypts. In about 20 days after c-Myc deletion, the gut looks normal and the crypt is again c-Myc positive.

If both APC and Myc are deleted, the cells have extremely high levels of b-catenin. The mice live between three and five weeks and then develop adenomas that are Myc-positive. This again implies that Myc is a crucial...
component of the APC mutant phenotype in the gut, which makes sense given the role of Myc amplifications in colon cancer.

**Stem cell signs**

Some of the 350 gut-specific Wnt targets are expressed in colon cancer. These genes are expressed in crypts and might be stem cell markers. The current dogma is that stem cells reside at position +4, based on label retention assays which indicate that cells at that position rarely cycle.

There are several genes that mark this +4 cell, the first proliferative cell above the Paneth cells. Sox 4, which has an embryonic phenotype, is expressed in the circle of cells directly above the Paneth cells. If Sox4 is deleted using a floxed allele, the +4 cells seem to be dying on day 1, day 2, day 6 and even on day 20. That suggests that the cells at +4 come from somewhere else, arrive at this position, and apparently require Sox4 to survive. If anything, the +4 cells appear to be a Paneth cell precursor, but not the gut stem cells.

There are two or three other genes that are not expressed at the +4 cells, but rather in about six tiny cells per crypt. Based on genetic marking studies, some researchers in the 1970s and 1980s had estimated that there are six independent stem cells per crypt so the number fits. These cells, dubbed crypt-based columnar cells, have elongated nuclei and look like tiny cylinders sitting between non-dividing Paneth cells.

The marker for these cells is a gene called GPR49, which has a large extracellular domain consisting of 18 leucine repeats. The closest homologs are the LH receptor and the FSH-R, although no definitive ligand has yet been identified.

Mice with a null mutation in GPR49 have an unpleasant phenotype, with something wrong in their lower jaw and the tongue. These mice swallow air and explode because they blow up their stomachs when they try to drink.

If an IRES-lacZ sequence is knocked in just upstream of the last exon, which encodes all of the seven transmembrane domains, it creates a null allele, which reveals that the gene is expressed in crypt-based columnar cells, but not in +4 cells. GPR49 is also expressed in the colon, where stem cells have never been defined.

GPR49 is a cancer gene. Small nests of cells in an adenoma of an APCmin mouse express the gene, unlike other markers that are expressed in the entire tumor. CD133 has been suggested as a marker for cancer stem cells in colon cancer, but some 10% of tumor cells express it. The GPR49 antibody in contrast picks up a small subset, maybe 1%, of those cells. When those cells are removed, the population no longer has stem cell activity.

**Single marker**

To prove that these are stem cells, a knock in with GFP, an IRES sequence and an inducible Cre-estrogen receptor, which can be activated with a single shot of tamoxifen, is used. When gut sections are viewed by confocal or two-photon microscopy for a day or two, the crypt-based columnar cells stain express GFP.
If the knock in is crossed with a promoter in the Rosa26 reporter sequence with a stop sequence and a lacZ reading frame, the combination mouse is white. When tamoxifen is shot into the mouse, it activates the Cre, and the presumed stem cells turn blue.

The stem cells appear to be dividing, generating offspring in a five-day cycle, which is unexpected. At 60 days, there are 12 full self-renewal cycles, with Paneth cells, goblet cells transit amplifying cells, enterocytes and enteroendocrine cells. The cells are long lived and make all the cell types of the colon, so they must be stem cells by definition.

In the stomach, the literature suggests that stem cells should be in the isthmus. But GPR49 is expressed at the bottoms of the glands, rather than halfway.

GPR49 appears to be a single marker for multiple adult stem cells, including in the retina, brain, intestinal organs, hair follicles and memory glands. This provides the first formal proof that stem cells exist in all these tissues. The cells divide about every 24 hours in small intestine. When adenomas are irradiated, these are the cells that survive, as cancer stem cells would be expected to do.

Based on the fact that the gene was originally picked up as a cancer-related TCF target gene, and that it appears to be expressed on cancer stem cells populations, GPR49 is also a cancer stem cell marker. According to the literature and several web-based databases, GPR49 is expressed on several tumors including in tissues, such as liver, that normally don’t express the gene.

References
Progression of prostate cancer from normal tissue stem cells

A report on a lecture by
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The capacity to isolate prostate stem cells is essential to explore their role in prostate development and in prostate cancer, the biology of which is poorly understood. Owen Witte and his colleagues have developed in vitro prostate colony-forming and sphere-forming assays to measure stem cell activity. The assays help identify cell surface markers that can enrich for prostate stem cells. These cells can self-renew to form spheres for multiple generations, and can differentiate to produce prostatic tubule structures containing both basal and luminal epithelial cells in vivo. The remarkable similarity in cell surface profile between the prostate stem cell and other types of stem cells suggests these markers are conserved amongst stem cell populations. Observations from the assays are also applicable to the study of both human prostate cancer and to normal prostate development.

Understanding the role of bcr-abl and the Philadelphia chromosome has helped develop targeted treatments for chronic myeloid leukemia (CML). In particular, the ability to manipulate and expand the specific cells of the hematopoietic system, add genes and allow the cells to advance to leukemia has allowed research on the disease to progress.

In contrast, there is very little known about the molecular and cell biology of human prostate cancer. One reductionist approach is to find the cells in the prostate that can generate a new organ and that can serve as targets for oncogenesis.

The goal, in this case, would be to try to define the principles for changing a normal prostate epithelial stem or progenitor cell into a prostate cancer cell; to define or at least highly enrich for stem-like cells in the prostate; and, finally, to study how embryonic patterning pathways may change the outcome of self-renewal, the numbers of cells and their susceptibility to transformation.

There are many clinical aspects of prostate cancer that don’t have a defined molecular explanation. For example, the disease often presents in a multi-focal manner. It’s not uncommon when a man is diagnosed with prostate cancer to find some, but not all, biopsies positive, often on opposite sides of the prostate. The cancer also goes through a preliminary or precursor lesion of prostatic intraepithelial neoplasia (PIN), which eventually progresses to frank carcinoma. As in breast cancer, there are many different histological subtypes with basal type luminal types among them.

When a prostate tumor metastasizes, which it does regularly, it goes typically to lymph nodes and to bone, where an osteoblastic phenotype is most commonly, although not exclusively, found.

The tumors have a variety of patterns of expression of cytokeratins that suggest cancer progression from basal, to intermediate — sometimes called transient amplifying phenotype — and on to a luminal epithelial cell type. This process of going through stages of differentiation is similar to that seen in CML.
AKTing on prostate cancer

Nearly 20 years ago, scientists observed that a core of epithelium can be dissected away from the mesenchyme of the mouse urinogenital sinus, digested and used to make mesenchyme preparations. Little fragments of the mesenchyme, mixed with little pieces of epithelium, and stuck back under the kidney capsule can give rise to growth reminiscent of the original prostate.

To address questions about prostate cancer, the cells are disassociated, used to make cell preparations that can be cultured for seven to ten days to expand them in vitro, and then mixed with tissues derived from either embryonic or adult sources, in this case from a β-GFP actin mouse.

When planted together in a collagen gel under the kidney capsule, those dissociated preparations give rise to very nice grafts filled with prostatic secretions. These tubules or globules show the growth initially of a solid mass followed by the production of a lumen. They are clonal in origin and have at least two recognizable subtypes, a basal type marked with a member of the p63 family and a luminal type, labeled with the androgen receptor (AR).

Can this structure be disturbed and turned into a cancer? How many genes would it take? What combinations would be most effective? To begin to answer these questions, one logical first step is the AKT/PTEN pathway. There is a lot of evidence associating the loss of function of PTEN and the increased activity of AKT with human prostate cancer. A number of mouse models have been created to show that perturbations in this pathway can eventually give rise to cancer in the animals.

When AKT is added by a lentivirus vector and activated by the addition of a myristoylation signal, PIN lesions are generated under the kidney capsule in a period of six to eight weeks. There are initially no abnormal growths outside of the tubular structures, suggesting that the organ structure forms first, and then the hyperplasia grows inside. Adding normal tissue doesn’t suppress this, nor is there a trans effect of the cancerous tubules on the neighboring normal tubules.

To advance the abnormal PIN lesions to more flagrant carcinoma, AKT is used along with AR, which is involved in much of prostate biology. Surprisingly, the combination of just these two genes, AKT and over expression of AR, drives the pathology all the way to frank carcinoma with an accompanying loss of the tubular like structure — although they do not progress further to metastasis.

A role for stroma

A surprising number of other growth factors, however, do not lead to cancer. One reason for that may be that when these fresh epithelial tissues are subjected to activated forms of many genes, they either undergo apoptosis, or senesce or fail to thrive in this system. Increasing the dose of the gene can sometimes overcome that so a whole series of signal transduction pathways are being investigated for their ability to trigger prostate cancer.
In these experiments, a change within the epithelial tissue itself is what leads to the cancer phenotype. But stroma is also very important in cancer. To identify whether single genetic changes would be sufficient to lead to this type of phenotype, fibroblast growth factor (FGF) is a good choice because it plays a crucial role in prostate development.

Artificial activation of the FGF receptor using a chemical dimerization technique can lead to PIN and carcinoma, but only after a period of nearly eight months to a year. But if epithelial cells are mixed with mesenchyme, which is infected with a vector that expresses FGF10, the result is striking. The simple introduction of this one growth factor gives rise high grade PIN and low grade carcinoma, with a histology very similar to that seen in basal type of human prostate carcinoma.

Diluting down the mesenchymal cells infected with FGF10 reveals that this is a regional effect, suggesting that it plays a role in the multifocal activation of early cancer lesions in the prostate.

When FGF10 is added into this mixture, there is also a dramatic increase in AR levels. If FGF10 from the stroma is combined with AKT in the epithelium, it generates wall to wall carcinoma — and, again, an increase in AR. This suggests that there may be specific pathways that activate a co-partner, making it more potent.

Finally, if grafts are labeled with an RFP epithelium so that they can be isolated cleanly away from the other mesenchyme, then mixed with wildtype epithelium and wildtype urogenital sinus mesenchyme, what transfers is a mixture of phenotypes. There are some normal tubules because of the wildtype epithelium, but also some abnormal things derived from the original epithelium. Exposure to the growth factor appears to have allowed something else genetically to happen in sub-portions of the cells that can then be serially transplanted.

**Marking stem cells**

For cells to qualify as stem cells, the general dogma is that they must be multipotent and how multilineage, proliferate extensively, be replication quiescent, niche dependent and have long term regenerative capacity. But there is much disagreement about the extent to which the cells must display each of these criteria.

There’s another unique criteria for prostate cells. If a rodent prostate is physically or chemically castrated to remove its source of androgen, the gland will involute, lose its biomass and shrink. But if the androgen is added back, the gland will regrow, presumably by mobilizing a population of stem cells, which have been localized to the proximal region near the urethra. Some of these cells are thought to move out, become transit amplifying cells and move to the tips of the glands, where they undergo extensive DNA replication and eventually fill out the gland. This cycle can be repeated a remarkable number of times, more than 25 times in one paper.

The prostate-specific Probasin promoter driving expression of luciferase can be used to repeat the above experiment and show that the cells used to generate the tubular structures also regrow the gland through androgen cycling in a similar manner, suggesting that the model system is relying on a cell with similar characteristics to those of the prostate stem cell.
Sca-1, or stem cell antigen 1 marks stem cells and another antigen, PSca, or prostate Sca, marks transient amplifying cells. Using Sca as a physical separation agent, in this case by magnetic beads, regenerates more tubules in the Sca-positive fraction. There are also other data, including its presentation during embryogenesis, that suggest that it defines a stem-like pool\(^4\).

Using Sca-1, and a sphere assay with a matrigel containing a high concentration of laminin — which is a ligand that might drive stem cell renewal in the prostate — helps find other markers for the different sub-components of the prostate.

One marker that identified by this approach is CD49f, also known as integrin \(\alpha\)-6. A combination of Sca-1 and CD49f can highly enrich the stem-like population, which live in the proximal region of the prostate\(^5\). The only cells that score in the sphere assays are also LIN-minus. These Lin-, Sca++ CD49f+ cells can regenerate the tubule-like structure.

Using only CD49f as a marker to separate out the stem-like cells, introducing activated AKT generates a large increase in the size of the mass, suggesting that enriched stem cells can serve as a target for cancer.

But CD49f-minus cells also show the same degree of aggressiveness in terms of the carcinomas. This could be because of contamination with CD49f-plus cells, or because the CD49f-minus luminal cells can also be a target for this gene, but at a slightly lower efficiency.

**Sphere surprise**

One surprise from the sphere assay, is that at an early stage, the structures have a marvelous structure with an outside, a middle and an in between. Certain markers segregate in an interesting way in these spheres. For example, all the p63 positive cells align along the outer layer of the spheres.

In another experiment, if the P-Sca promoter is used to drive GFP, the only GFP-positive cells are towards the interior as the cells move away from what is a basal-like layer. This is a useful artifact of cell culture because there are different cell populations during the growth phase of the spheres. If androgen, or testosterone in this case, is added to these cells, it changes the morphology of spheres from relatively solid to relatively hollow.

The addition of androgen seems to stabilize the AR rather than being degraded in the absence of androgen.

These spheres can therefore be used as a surrogate for *in vivo* experiments. For example, the polycomb protein Bmi-1 is prominently expressed in the earliest stage of prostate epithelial development. It’s been shown to be over expressed in a variety of cancers and previously published data suggests that Bmi-1 might influence stem-like populations.
Bmi-1 is expressed during the progression from normal tubules to PIN lesions with AKT or carcinoma with combined AKT and AR. In the sphere assays, knocking down Bmi-1 reduces the size and numbers of spheres and blunts the re-plating ability of the cells. Conversely, adding more Bmi-1 increases the size and re-plating efficiency of the cells. Adding Bmi-1 also induces the invasion or hyper-proliferation of basal-like cells into the center of the sphere. In the in vivo model over expressing Bmi-1 results in fewer surviving tubules. But among the ones that do grow, a subset progress to hyperplasia or carcinoma.

All of these observations are applicable to the study of human tissue, whether it is cancer tissue or normal human tissue isolated by surgery or autopsy for other reasons. All of the tools developed in the mouse are also applicable in the human.

References

1. ABBREVIATIONS
Terms in *italics* are defined elsewhere in the Abbreviations.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>EMT</td>
<td>epithelial-mesenchymal transition</td>
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<td>HGF</td>
<td>hepatocyte growth factor or scatter factor</td>
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<td>LMS</td>
<td>lung metastasis signature</td>
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<td>MMP</td>
<td>matrix metalloprotease</td>
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<td>MSP</td>
<td>macrophage stimulating protein</td>
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<td>PyMT</td>
<td>polyoma Middle T antigen</td>
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<td>VEGF</td>
<td>vascular-endothelial growth factor</td>
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<td>EGFR</td>
<td>receptor for VEGF</td>
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<td>green fluorescent protein</td>
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<td>MMP</td>
<td>matrix metalloprotease</td>
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<td>NCAM</td>
<td>neuronal cell adhesion molecule</td>
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<tr>
<td>TGF-b</td>
<td>transforming growth factor beta</td>
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2. GLOSSARY
Terms in *italics* are defined elsewhere in the Glossary.

- **adenoma** a benign tumor that results from excessive growth of *epithelial cells*. Adenomas are not capable of *metastasis* or *invasion*, but may become *malignant*, at which point they are called *adenocarcinomas*.

- **adenocarcinoma** a malignant tumor that originates from *epithelial cells*. It may first present as an adenoma.

- **alleles** different variants of a gene found in the normal population; as individuals carry two copies of each gene, one on each of a pair of chromosomes, they may have identical or different alleles (see also *wildtype*).

- **angiogenesis** the formation of new blood vessels.

- **anoikis** a form of *apoptosis* induced by anchorage-dependent cells detaching from the surrounding extracellular matrix.

- **apoptosis** the orderly program of biochemical reactions leading to cell death and degradation.

- **benign tumor** lacking the properties of a *malignant* tumor, meaning that these tumors are not capable of *invasion* and *metastasis*.

- **carcinogenesis** the process by which normal cells are transformed into cancer cells.

- **carcinoma** any cancer that originates from *epithelial cells*. Carcinomas are malignant and are capable of *invasion* and *metastasis*.

- **chemokine** a family of small *cytokines* or proteins secreted by cells.

- **cytokine** molecules released by cells in response to infection or injury that stimulate inflammatory or healing responses.

- **domain** region of a protein with a specific function, such as the binding to receptor or kinase activity; sequence in a gene coding for such a region.

- **endothelial cells** cells that line the entire circulatory system, they are a subset of *epithelial cells*.

- **epithelial cells** cells that line both the outside (skin) and the insides of various organs, including the lungs, the gastrointestinal tract, the reproductive and urinary tracts, and make up the exocrine and endocrine glands.
exon section of the DNA sequence in a gene that codes for a particular part of a protein.

gene construct artificially produced DNA sequences for specific proteins, inserted into cultured cells or mouse embryonic stem cells for experimental purposes.

hematopoiesis formation and development of blood cells.

hyperplasia excessive proliferation of cells within an organ or a tissue; in contrast to neoplasia, this is a physiological response to a stimulus and remains subject to normal regulatory control.

hypoxia a deficiency of oxygen in cells or tissues.

invasion the process in which cells break away from the primary tumor and penetrate surrounding tissues.

inhibitory RNA artificially produced messenger RNA that inactivates transcription from a gene by blocking the intrinsic RNA

kinase an enzyme that phosphorylates proteins, a part of the biochemical cascades involved in intracellular signaling.

knock-out mice in which one or both alleles of a gene have been experimentally deleted by using a non-functional gene construct, resulting in the loss of the protein under investigation.

knock-in mice in which a gene construct carrying a specific mutation has been inserted, either to inactivate a specific protein or to test the consequences of the mutation on the protein or the pathway in which it is involved.

lymphatic system a complex network of organs, nodes, ducts, tissues, capillaries and vessels that produce and transport lymph fluid from tissues to the circulatory system; a major component of the immune system.

lymphangiogenesis the formation of lymphatic tissue, including the production of white blood cells.

macrophage cells that engulf and digest cellular debris and pathogens and stimulate the immune system to respond to pathogens.

malignant transformation the process by which cells escape from normal controls on cell division and apoptosis to replicate continually; malignant cells often acquire destructive properties, such as secreting toxins or becoming metastatic.

metastasis the process of cells leaving the primary tumor and migrating through the lymph and blood systems to establish secondary tumors elsewhere in the body.

monoclonal antibodies selected to recognize a specific sequence on the target protein or antigen.

morpholino a molecule used to modify gene expression by blocking small regions of RNA.

necrosis accidental death of cells and living tissue that is less orderly than apoptosis.

neoplasia an uncontrolled and disorderly proliferation of cells to form a tumor, which may be either benign or malignant.

oncogene a mutation in a gene involved in regulating cell proliferation or cell death that leads to neoplasia and maybe to malignant transformation.

phosphorylation the addition of PO_4^- groups to a protein to activate or deactivate their interactions with other proteins in a signaling cascade (see kinase).

proto-oncogene a normal gene that can become an oncogene due to mutations or increased expression.
senescence  the state in which differentiated cells have lost the ability to divide.

stroma  the connective, non-functional supportive framework of a biological cell, tissue or organ.

transcription factor  protein that regulates the transcription of a gene into protein

transgenic  mice that carry gene constructs expressing either a high level of an endogenous protein or a human protein; often used as models for human disease.

tumor  an abnormal growth or mass of tissue, usually a result of neoplasia, that can be either benign or malignant.

tumorigenesis  the formation of a tumor as a result of uncontrollable reproduction, often caused by oncogenes.

tumor suppressor  gene coding for a protein that promotes apoptosis or suppresses cell division and therefore prevents cells from becoming neoplastic or malignant.

wildtype  gene of interest with no known mutations; animal carrying such a gene.

xenografts  human tumors grafted into immunocompromized mice.
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Participants
in the group picture
The Fondation IPSEN, created in 1983 under the auspices of the Fondation de France, has two objectives: the distribution of knowledge and encouraging the exploration of emerging areas of research.

**Contributing to the development and distribution of knowledge**

One mission of the foundation is to promote interaction between researchers and clinicians by creating ‘crossroads’ and forums for fruitful exchanges. Today, with the extreme specialization of knowledge and the increasing mass of information that many find difficult to decipher, such exchanges are indispensable. For this to be effective, the foundation has focused on some of the crucial biomedical themes of our time: the spectacular developments in neuroscience and the scientific study of cognitive mechanisms, the challenges of neurodegenerative pathologies, the omnipresence of genetics and molecular biology, the growing field of endocrine interactions, and the problems of aging populations and theories of longevity. More recently, activities have expanded into two areas that are exciting for both their medical and fundamental challenges and that are currently in a phase of rapid development: cancer and the vascular tree.

Another goal of the Fondation IPSEN is to initiate, in partnership with the specialists and institutions involved, discussions and exchanges on the major scientific challenges of the future. Rather than trying to provide definitive knowledge, or to replace the work of large research organizations, the aim of these discussions is to emphasise multidisciplinary approaches at the boundaries of several disciplines, an approach that is essential for understanding the complexity and originality of human beings and their pathologies.

To fulfil these commitments, the foundation organises several series of international Colloques Médecine et Recherche, as well as funding awards to encourage research and publishing reports on its meetings. For each of these activities, the foundation brings together partners from the scientific and clinical world, who can independently report on the current state of knowledge and discuss the main issues in the areas on which the foundation has chosen to focus.

Over the past twenty-four years, the Fondation IPSEN has established its place in the scientific and medical landscape and intends to continue to be at the forefront in forming links, initiating multidisciplinary exchanges and contributing to the spread of knowledge, with time, intelligence, good will and above all, the collaboration of leaders in current biomedical research.
The Colloques Médecine et Recherche series

The Colloques Médecine et Recherche were created in 1987, with the first series dedicated to Alzheimer’s disease. Its success stimulated the establishment of other several dedicated series: neurosciences, longevity, endocrinology, the vascular tree and cancer. Meetings in each series are held annually, bringing leading international specialists together to present their most recent work, sometimes even before publication. Through these meetings, the Fondation IPSEN has over the years developed a large, international network of experts.

By focusing on emerging fields of knowledge, the meetings have supported the development of many new topics and have impacted on scientific advances in areas such as gene therapy and stem cells in the central nervous system, the role of cerebral amyloidosis in neurodegeneration, the contribution of genetic factors in resistance to disease, the benefits of neuronal grafts, biological markers of Alzheimer’s disease, apolipoprotein E, brain-somatic cross-talk, relationships between brain and longevity, hormonal control of cell cycle to name a selection.

The series are organized around topics where active research is having or is likely to have a major impact on our knowledge:

• Alzheimer’s disease – Since 1987, this topic has been explored at annual meetings that have followed or even anticipated the development of the new field of ‘alzheimerology’, which has gone beyond histology and neurochemistry to establish the underlying pathological mechanisms.

• Neurosciences – Started in 1990, this series of conferences has both enabled the identification of the major themes to emerge in this area and has supported not only the remarkable expansion of the neurosciences in the past fifteen years but also the effort to integrate its subdisciplines, from molecular mechanisms to human cognition.

• Longevity – Launched in 1996, this series examines the challenges and paradoxes of medicine by focusing on a positive aspect, cases of exceptional resistance to the effects of aging, rather than on disease. The evolution of research dedicated to aging into research dedicated to longevity represents a remarkable development in this field.

• Endocrinology – Established in 2002, this series examines the involvement of the endocrine system in the integration of all bodily functions. One example is the recent discovery of many hormones important in the control of metabolism, such as leptin and ghrelin. As aspects of brain-somatic crosstalk, such topics have impacts far beyond studies of hormones and the endocrine organs.

• Vascular Tree – This new series, begun in 2004, aims to examine the various steps that lead to development of the vascular system, its growth in harmony with that of other organs, its degeneration, death and the possibilities for its regeneration. A new vision is emerging of blood vessels not as simple ‘pipes’ but as living, complex organs with interactions throughout the body.

• Cancer Science – Three annual experts meetings have been organized in collaboration with Inder Verma and the participation of remarkable leading opinion makers in the field. Challenging topics (Can Cancer be Treated as a Chronic Disease?, Are inflammation and Cancer Linked?, Metastasis and Invasion) have generated outstanding discussions among the participants. The 2008 meeting will deal with Metabolism and Cancer.

New Partnerships

Long ago, the Fondation IPSEN has developed partnerships with international institutions and organisations, to encourage cooperation between experts in various disciplines. These partners include: the World Health Organisation (WHO), the Fondation Nationale de Gériatrophologie (FNG) and Harvard University.
Three new partnerships were implemented in 2007:

- First with the **Salk Institute** (La Jolla) and **Nature Publishing Group**. This partnership consists of a series of annual meetings on “Biological Complexity”. The inaugural event in January 2007 focused on *Transcription diseases*. The 2008 meeting will deal with *Genes, Circuits and Behavior*.

- Secondly with **Cell Press** and the **Massachusetts General Hospital**. This series, “Exciting Biologies”, has been initiated in October 2007, with a meeting entitled *Biology in Motion*.

- And finally with **Nature Publishing Group** on the general theme of “Emergence and Convergence”, four meetings a year will be organized in various domains. The New York Academy of Sciences has hosted the first of the series in October 2007, *Small RNAs in Development, Immunology and Cancer* and the second one has been held in Seattle, *Genome Evolution and Structural Variation*.

**Awards to Encourage Research**

The *Fondation IPSEN* awards prizes to researchers who publish remarkable, pioneering studies. Currently, four awards are given annually:

- **The Neuronal Plasticity Award** has been given each year since 1990 to three researchers working on the same theme: Albert Aguayo, Anders Björklund and Fred Gage; Ursula Bellugi, Wolf Singer and Torsten Wiesel; Philippe Ascher, Kjell Fuxe and Terje Lomo; Per Andersen, Masao Ito and Constantino Sotelo; Mariano Barbacid, Yves Barde and Hans Thoenen; Jacques Melher, Brenda Milner and Mortimer Mishkin; Friedrich Bonhoeffer, Cory Goodman and Marc Tessier-Lavigne; Antonio Damasio, Richard Frackowiak and Michael Merzenich; Heinrich Betz, Gerald Fischbach and Uel McManus; Masakazu Konishi, Peter Marler and Fernando Nottebohm; Tomas Hökfelt, Lars Olson and Lars Terenius; Albert Galaburda, John Morton and Elizabeth Spelke; Arturo Alvarez-Buylla, Ron McKay and Sam Weiss; François Clarac, Sten Grillner and Serge Rossignol; James Gusella, Jean-Louis Mandel and Huda Zoghbi; Ann Graybiel, Trevor Robbins and Wolfram Schultz; Mary Kennedy, Morgan Sheng and Eckart Gundelfinger; Nikos Logothetis, Keiji Tanaka and Giacomo Rizzolatti.

- **The Endocrinology Award**, first given in 2002, has been received by Wylie Vale, Robert Lefkowitz, Pierre Chambon, Tomas Hökfelt, Roger Cone, and William Crowley.

- **The Jean-Louis Signoret Neuropsychology Award**: since 1992, the recipients have been Eric Kandel, Jacques Paillard, Rodolfo Llinas, Steven Kosslyn, Alfonso Caramazza, Jean-Pierre Changeux, Emilio Bisiach, Joseph LeDoux, Joaquim Fuster, Stanislas Dehaene, Deepak Pandya, Utah Frith, Antonio and Hanna Damasio, Marc Jeannerod, Faraneh Vargha-Khadem, and Alvaro Pascual-Leone.
• The Award for Longevity, created in 1996, has been bestowed on: Caleb Finch, Vainno Kannisto, Roy L. Walford, John Morley, Paul and Margret Baltes, Justin Congdon, George Martin, James Vaupel, Linda Partridge, Sir Michael Marmot, Cynthia Kenyon, and David Barker.

International Publications

Books summarizing of the conferences organised by the Fondation IPSEN are published in English and distributed by international publishers:

• Research and Perspectives in Alzheimer’s Disease [Springer, 24 titles]
• Research and Perspectives in Neurosciences [Springer, 15 titles]
• Research and Perspectives in Longevity [Springer, 5 titles]
• Research and Perspectives in Endocrinology [Springer, 6 titles]
• WHO/Ipsen Foundation series [Springer, 7 titles]
• Brain and Mind Collectio

In addition, since 1986 the Fondation IPSEN has published more than 190 issues of Alzheimer Actualités, a newsletter dedicated to Alzheimer’s disease; in 1993, a bi-annual journal, the Bulletin du Cercle de Neurologie Comportementale was started; and in 2005, the first of two series of annual reports on the conference dedicated to Cancer Science and the Vascular Tree appeared. The foundation also has widely distributed information in various forms to the medical professions and families of patients, as well as producing teaching films that have received awards from specialized festivals.