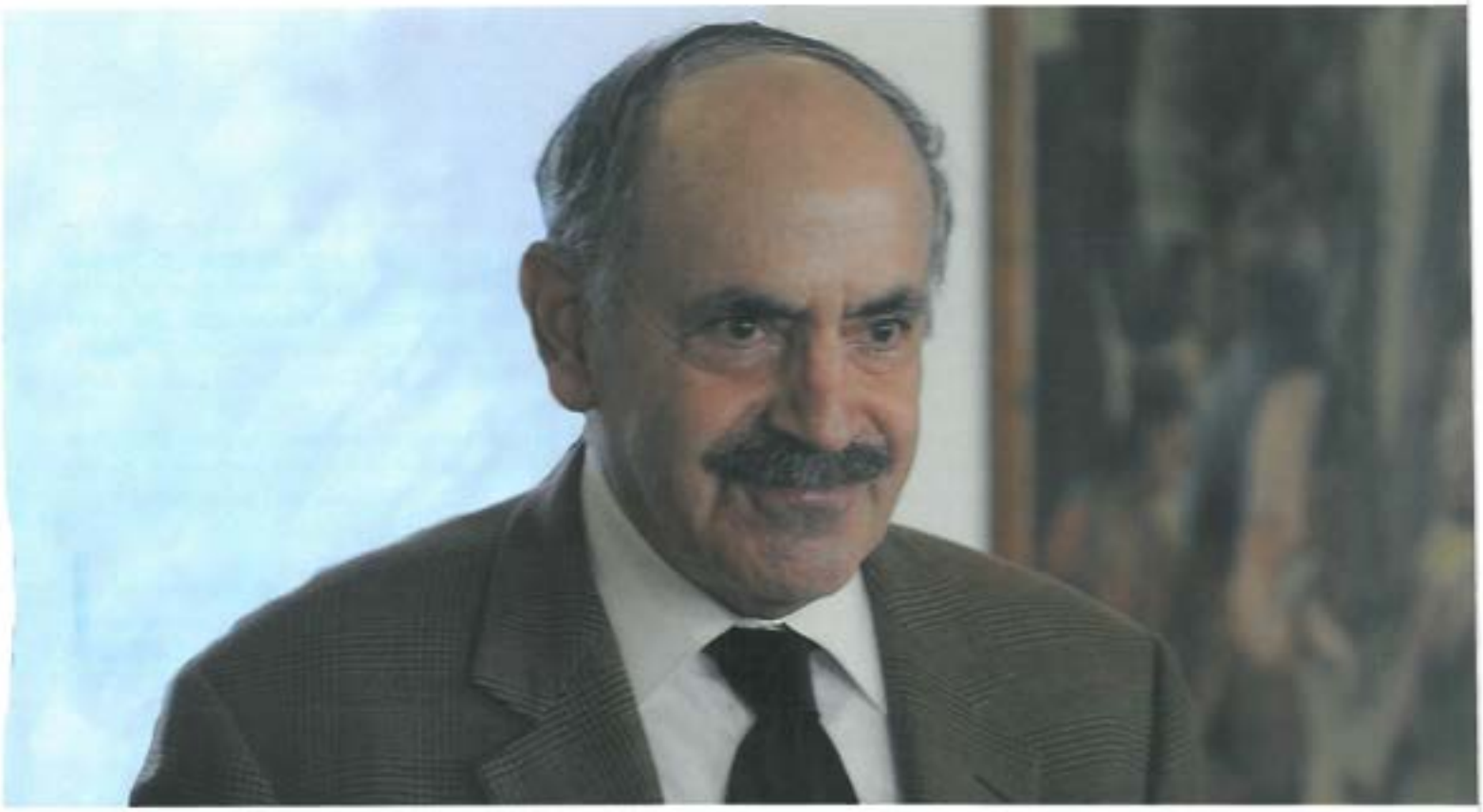
The Pezcoller Foundation

Journal



Summary

News from the
Pezcoller Foundation World

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We are grateful to prof. Robert A. Weinberg, for the kindness to let us publish his Korsmeyer lecture entitled "Stem Cells and the Epithelial-Mesenchymal Transition", given in Padua at VIMM Venetian Institute of Molecular Medicine on May 9, before the Award ceremony in Trento. We are glad to remind that Prof. Robert A. Weinberg of the Whitehead Institute for Biomedical Research Cambridge Center, Cambridge, MA is the prestigious recipient of the 2012 Pezcoller Foundation-AACR International Award for Cancer Research.

The annual Stanley J. Korsmeyer Lectureship has been started by the Pezcoller Foundation in 2006 in accordance with the AACR American Association for Cancer Research and the VIMM Venetian Institute of Molecular Medicine in Padua (Italy). The goal of this event is to honour the fundamental contribution of the late S. Korsmeyer who was the recipient of the Pezcoller Foundation-AACR International Award for Cancer Research in 2004. Although under heavy treatment for cancer, he presented his last European lecture at VIMM immediately before receiving the Pezcoller Award. Unluckily he passed away a few months later. Therefore we wish to remember Stanley Korsmeyer every year with a lecture given by the recipient of our Award.

The 2012 Symposium entitled "Molecular Basis for Resistance to Targeted Agents" took place in Trento last June and was very successful for the high level of the five sessions and for the large participation. As usual we gave also the "Pezcoller Begnudelli Awards" for the best poster to one deserving researcher: Dr. Hadas Reuveni of Novo Tyr Therapeutics Ltd, Tel Aviv, Israel.
We are also glad to present the next 25th Pezcoller Symposium which will be held in Trento on June 20-22, 2013 and will be entitled "Metabolism and Tumorigenesis".

The Pezcoller Foundation is pleased to announce a new Award: "the Pezcoller-Foundation-EACR Award and Lecture" which was presented for the first time in Barcelona at the EACR 22 meeting in July 2012.

New grants for young Italian researchers will be awarded by the end of the year. More details in the last page.

The 17th Pezcoller Foundation Seminar on "The Role of Pathologists in the Management of Neoplastic and non Neoplastic Lung Pathology" has been held in Trento on September 21-22. The seminar has been successfully attended by many pathologists.

Gios Bernardi MD
Editor and Pezcoller Foundation President Emeritus

Picture on front page:
Prof. Robert A. Weinberg

The Stanley J. Korsmeyer Lecture "Stem Cells and the Epithelial-Mesenchymal Transition"

Prof. Robert A. Weinberg

Whitehead Institute for Biomedical Research Cambridge Center, Cambridge, MA

The work in my laboratory has been focused on the development of human cancer. A model that is clearly embedded in our thinking is this description here of multi-step tumorigenesis, as it occurs in the context of the human colon and of colorectal carcinoma as first developed by Vogelstein and Kinzler in 1989. Here we see the multiple steps of tumour progression and the accumulation of distinct mutations in the genomes of cells as they are evolving toward high-grade malignancies **(1)**.

We kind of have an idea of how primary tumours are formed. But the challenge has been to understand what this last step is, the so-called "other alterations" depicted here that happen when primary cancer cells metastasize, when they disseminate to create life-threatening metastases, which are responsible for 90% of cancer-related mortality. In fact, even though these are so important clinically, we still understand relatively little about this process of metastatic dissemination, which is the focus of my talk today.

Here's what the histopathological progression looks like in the context of the actual colon; we see it here presented in great detail **(2)**. In fact, such multi-step tumorigenenesis occurs in a whole series of epithelial tissues throughout the body, which resemble one another and which give one the hope that the lessons that we learn from one carcinoma will prove to be applicable and generalizable to other types of carcinomas as well **(3)**.

In fact, we know many of the critical mutations occurring during primary tumor formation, but again we don't really understand what happens in the last step, which is what I will be focusing on today. We know that the order of mutations can be quite variable and that there are multiple paths through which cells can progress during their genetic evolution from normalcy to high-grade malignancy, in this case once again in the context of a colon **(4,5)**. And we understand much about the circuitry that operates inside cells and that becomes disrupted during the formation of primary cancers **(6,7)**, but again none of this really tells us much about how metastatic dissemination occurs -- what goes on at this last step.

One important clue that we have learned over the last several years in my own laboratory, and I assume in others, is perhaps unexpected, and that is the following: the identity of the normal cell of origin is an important determinant of the behaviour of the cancer cell that arises twenty, thirty or forty years later**(8)**. Some cells, if they generate primary tumours, will generate tumours that metastasize, other normal cells will generate primary tumours that do not metastasize. As a consequence, we believe that the differentiation program of the normal cell-of-origin - that is the precursor of all the neoplastic cells in the tumour - is really an important determinant of what happens in this last stage **(9)**. How and why this relationship operates in this way is something that is still obscure and the evidence why this operates mechanistically is still elusive.

Importantly, the invasion-metastasis cascade,

as it is portrayed here, is itself a process of multiple steps that is biologically highly complex. Here we see the steps, as first enunciated by Isaiah Fidler **(10)**, in which cancer cells invade locally, intravasate into the circulation, extravasate into the parenchyma of distant tissues, form micro-metastases and, with low probability, form macroscopic metastases. This last step is extraordinarily inefficient.: thirty per cent of women who are diagnosed with breast cancer on the day of diagnosis have thousands of micrometastases in their marrow, but only half of those women will ever develop metastatic disease, indicating that this final step is, fortunately for those women, very inefficient and only occurs at a very low probability.

The question is then how all this occurs. Indeed, the complexity of the invasion-metastasis cascade, which and is subsumed as the last step of multistep tumor progression rivals the complexity of the preceding steps that led previously to the formation of the primary tumour **(11)**. So the question is: are additional mutations required in order to empower cancer cells to successfully execute all of these distinct steps? How do all these cells acquire the distinct capabilities that in aggregate are required in order for them to successfully metastasize? **(12)**.

Here we see much from examinations of histopathological sections of tumours. This is a human tumour xenograft, a breast cancer, in which these experimentally transformed cells are grown in a mouse, as a xenograft **(13)**. Here are the breast cancer cells labelled with an antibody against cytokeratin, seen in red, which, as you know, is an epithelial marker. Out here are the blue nuclei of recruited mouse stromal cells, which have been brought into the tumour in order to provide physiologic support for the carcinoma cells; what is of greatest interest to us today are these green cells. These cells are of human origin, and they therefore by necessity derive from the implanted carcinoma cells. However, they have shut down the epithelial marker cytokeratin and instead express the mesenchymal marker vimentin. This behaviour suggests that they have undergone a profound change in cell phenotype. They have undergone what is called an mesenchymal-epithelial transition in which they have shut down pre-existing cytokeratin epithelial marker, an "EMT", and now express instead a mesenchymal marker **(14)**. Hence the term epithelial-mesenchymal transition.

In fact, the cells that undergo this EMT, moving from the epithelial to mesenchymal state, seem to be on the outside of this island of carcinoma cells, suggesting, but hardly proving, that they initiate the EMT program in response to signals that they are receiving from the nearby mouse stroma, which they previously recruited **(15)**. This localization is indicated here more persuasively in this same tumor but stained differently **(16)**. Here we see a tongue of carcinoma cells that expresses cytokeratin, outside are the recruited mouse stromal cells, and around this tongue you see a rim, a layer of cancer cells that have shut down cytokeratin expression; and here is another tongue of cancer cells. In this second image, the cells in the middle continue to express cytokeratin, but the cells on the outside, which are directly apposed to the nearby stroma,have now acquired the mesenchymal marker vimentin. These images show quite dramatically that the localization of these cells within the tumour, specifically their apposition to the nearby stroma **(17)**, is an important determinant of the behaviour of these cells, to the extent that we agree that the EMT is an important biological change in their behaviour. So it suggests, but doesn't prove, that close contact between the carcinoma cells and the surrounding recruited stroma seems to be the trigger that provokes an EMT, but this thinking is hardly proven by the evidence we see here.

Still, this gives us an important and simple insight: that the micro-environment of the cancer cells is important to understand the behaviour of the tumour as a whole **(18)**. This micro-environment cannot be understood by sequencing the genomes of the cancer cells. This can only be understood by understanding the signalling environment in which these cells exist, deep inside the tumour.

These days people like to sequence cancer cells genomes, but such sequencing doesn't give one any insight into processes, such as the one we see here. The EMT process, the epithelial-mesenchymal transition, is of ancient lineage. It was already invented in the pre-Cambrian and is preserved in virtually all modern metazoan phyla. It is choreographed by a series of transcription factors: I show six of them here, there are several more. Each of these transcription factors is in fact expressed in virtually all existing metazoan phyla. Here

we see SNAIL, TWIST, SLUG, SIP1 GOOSECOID and FOXC2 (19). TWIST in this case, which I'll refer to shortly, is expressed in the *Drosophila* embryo, where its expression preordains and determines the site of ingression, i.e., of gastrulation.

Many of these transcription factors were initially discovered in the context of the developmental genetics of other organisms. Years ago the *Drosophila* developmental geneticists came to the cancer funding agencies and they asked for money to support their research, promising that they would tell people something about how cancer developed. We cancer biologists knew better, because we knew that the developmental biologists were poor as church mice, and that they just needed some money to keep their laboratories going, and that their work would never yield anything useful for cancer biology. But we were wrong, because much of what I am about to tell you came through the route of discovering these various transcription factors, which operate at various stages of normal morphogenesis to enable EMTs to occur and are indeed critical parts of the morphogenetic process. Here's my favourite one, a sea anemone which is almost not even a metazoan and yet one of these transcription factors, SNAIL, is already expressed in that early and very primitive organism, indicating its antiquity of more than six hundred million years (20). In fact we can now understand that virtually all the metazoan phyla express these transcription factors, and that they are of very ancient lineage. Here we see that the arthropods already had this transcription factor, from whom we are diverged by 650-700 million years of separate evolution (21).

The epithelial-mesenchymal transition was first discovered by a woman named Elizabeth Hay, who had studied it in the context of embryonic development (22). When my laboratory started working on the epithelial-mesenchymal transition the developmental biologists thought of me as an intruder, who was moving into their territory! I realized only later that I actually did have a right to work on the EMT, because only recently did I understand that the woman who discovered this was lured into biology by her professor, who was my sister-in-law's father! So, now I have a direct connection with this process and a justification to work on it!

She described the fact that the EMT is a multi-faceted program, involving changes in motility, morphology, cell-cell associations, and the replacement of various cell-surface markers by one another. In fact the EMT program involves changes in the expression of more than 300 distinct genes. So, to state the obvious, we are making here an important association, hardly original to me, about this early embryologic program -- here we see it in the context of a sea urchin embryo where it operates to enable gastrulation and the formation of the presumptive mesoderm and endoderm (23). This early embryonic program is appropriated opportunistically by cancer cells, in order to enable them to acquire many of the cell-biological phenotypes that we ascribe to high-grade malignancy.

Here is the work of Jing Yang, in which she studied highly metastatic mouse breast cancer cells, which were able to metastasize from the site of subcutaneous implantation to the lungs in large numbers (24). These cancer cells normally express high levels of the TWIST EMT-inducing transcription factor. When these cells were deprived of TWIST, the primary tumour actually grew more rapidly, but the number of metastases was reduced by 85%, and the residual 15% of metastases that continued to form were found to never have lost TWIST in the first place. This allowed us to conclude that TWIST -- this EMT-inducing transcription factor -- is essential for the ability of these mouse breast cancer cells to metastasize. Granting that it is *necessary*, we do not know whether it is *sufficient*. In other words, if one were to express these EMT-inducing transcription factors in a previously non-metastatic cell, would this expression enable that cell to metastasize. That sufficiency has not yet been proven experimentally.

Still, given the complexity of the EMT program and of the role of TWIST, as I've just mentioned (25), one can now pose the question: How many steps of the invasion-metastasis cascade (here depicted slightly differently) could in principle be achieved by a cancer cell in the primary tumour that activates this previously latent cell-biological program (26).

Here I present a speculation, which in fact by now is close to being proven (27). I believe that if a cancer cell would activate this EMT program in a primary tumour, it would be able to execute all of these steps of the invasion-metastasis cascade, except the last one, that is, the one of colonization, which

presumably involves the adaptation of cells from one primary tissue, such as the breast, to a micro-environment that is otherwise alien and familiar, for example, a breast cancer cell adapting to growth in the liver or the brain, the lungs or the bone marrow. This involves a difficult adaptation program, which would not seem to be within the powers of the EMT. Still, if this notion is sustained, and I believe that it shortly will be, this suggests that cells within the primary tumour are already genetically competent to disseminate; they just need activate their latent EMT program **(28)**. This means that the actual act of physical dissemination does not require additional mutations beyond those that were previously acquired during the formation of the primary tumour.

Indeed there is correlative support here, for example the work reported by Vogelstein and Markowitz several years ago, where they studied the development of human colorectal adenomas, adenomatous polyps, into primary carcinomas. That process, they deduced by sequencing the genomic DNA of colorectal adenomas and carcinomas, took approximately 17 or 18 years, this time being required for the polyp to evolve into carcinoma. But once the carcinoma formed, within a year or two, there were already metastases apparent in the liver, suggesting to me at least, that the formation of these metastases did not depend on additional rare mutations beyond those that were sustained during the formation of the primary tumour. This model of primary tumor formation and metastasis remains to be proven rigorously, but if this thinking is sustained, it obviously leads to great conceptual simplification of the entire process of metastatic dissemination. So perhaps we can one day understand all this, at least these steps, in terms not of additional genetic alterations, but instead in terms of epigenetic changes in the transcription program of primary cancer cells.

I now wish to switch topic quite dramatically, but of course gracefully! Here are two different types of human breast cancer cells, both experimentally generated **(29)**. What I would like to point out is that even though these two cell populations have an identical set of introduced transforming oncogenes, when these cells were implanted in mice, they exhibited dramatically different abilities, differing abilities to seed tumours in those mice. This is a topic which seems unrelated to the EMT that I was describing before, but you will see the relationship momentarily.

In the case of one of these populations, a million cells needed to be implanted in a mouse in order for one primary tumour to form. In this other case, between 10 and 100 cells sufficed to generate a primary tumour **(30)**. This profound difference in tumour-initiating ability, as depicted here, has an important logical consequence, which is: there must be at least two distinct kinds of cancer cells in each of these cell populations, those that are capable of seeding a primary tumour and those that are not capable of doing so **(31)**.

We can refer here to the work of Michael Clarke and Muhammad Al-Hajj, who described the following paradigm, which describes the hierarchical organization of cells in various epithelial organs. One imagines in general that this hierarchical organization includes self-renewing stem cells up here, which divide asymmetrically, transit-amplifying cells, which increase exponentially and post-mitotic differentiated cells **(32)**. The trait of self-renewal is however confined to these cells, self-renewing stem cells and transit-amplifying or "progenitor" cells **(33)**. This same scheme from the normal tissue has been appropriated by Michael Clarke with Muhammad Al-Hajj to describe how cells in a breast cancer are organized: that within a cancer there are self-renewing stem cells, there are transit-amplifying cells, and there are post-mitotic differentiated cells **(34)**. Within the context of a tumour these self-renewing stem cells have the ability to seed a new tumour and are therefore called tumour-initiating cells. In contrast, the great majority of genetically identical cells in the tumour do not have that tumour-initiating ability, meaning that they have lost the ability to seed a new tumour.

The same scheme is applicable to the question of metastatic dissemination and the formation of metastases. Because if this cell succeeds in physically disseminating to a distant tissue, it is at least *qualified* to seed the new tumour in the distant tissue because of its tumour-initiating capability **(35)**. Being qualified to do so doesn't mean that it is guaranteed, but it is at least qualified to do so. The bulk of these cells conversely in the primary tumour, even though they may have an identical set of oncogenic mutations, have given up tumour-

initiating ability and therefore even if these cells were physically to disseminate to distant tissue, they would not be qualified to seed a new metastatic colony, at least that's how the logic operates in this case. In the work of Al-Hajj et al. of eight years ago **(36)**, they demonstrated that, using FACS analysis, a small sub-fraction of cells, 200 of these could form a new tumour while 20,000 of the bulk cells in the tumour could not do so -- all of these cells coming from the same tumour cell population. This demonstrates quite dramatically that the cells within a single tumour population reside in distinct states of differentiation.

Workers in my own lab used two cell-surface markers that were first developed by Al-Hajj et al., CD24 and CD44, in FACS analysis to fractionate breast cancer cells or normal mammary epithelial cells into two populations **(37)**. Using the Al-Hajj et al. criteria, we see here that the 24 high/ 44 low cells reside in the position of non-stem cells and conversely the 44 high and 24 low sit in the position of stem cells. In this case, we are observing the behaviour of immortalized human mammary epithelial cells. If this identification were correct, then here we are observing the behavior of a small minority population of stem cells that is able to maintain itself in a Petri dish, not just in the complex micro-environment of the stem cell niche.

In any case, people in my laboratory -- Sendurai Mani, Mai-Jing Liao and Wenjun Guo, -- were interested in the following question: What happens if there is a connection between the first topic that I discussed, EMT, and cancer stem cells. And of course when someone says two unconnected things are actually connected, usually such a person is a bit over-ambitious or trying to make extremely rapid advances in his or her career. In any case, they decided to ask the following question: what would be the effect of either SNAIL or TWIST expression on these cells, i.e., if these cells were forced experimentally to undergo an EMT **(38)**? In fact, they found, if they put either SNAIL or TWIST into these mammary epithelial cells, these cells underwent a morphological change **(39)**. Here you see the epithelial cobblestone morphology in monolayer culture and here you see two populations of cells which indeed look very mesenchymal. What was most interesting, was when they switched their focus to the behaviour of these cells after they had gone through an EMT: The 24 low-44 high population, once it was forced to go through an EMT, either through the ectopic expression of SNAIL or TWIST, exhibited an en masse migration from the non-stem cell to the stem cell state, at least as identified in this FACS analysis **(40)**.

This suggested that one of the consequences of moving through an EMT was, at least at this level of analysis, entrance into a stem-like state. Here is a more compelling experiment to my mind. Here Sendurai Mani and Wenjun Guo separated these populations of stem cells by fluorescence-activated cell sorting into a a putative stem cell population and a non-stem cell population **(41)**. They then prepared mRNAs from these two sub-populations of cells and examined the expression of various messenger RNAs in these two sub-populations **(42)**. What they discovered was that the putative stem cells express one to hundredth of the keystone epithelial marker E-cadherin mRNA and conversely the putative stem cells express, on a per-cell basis, between 80- and 120-fold higher levels of the following mesenchymal markers: E-cadherin, Vimentin and Fibronectin; of additional interest, the putative stem cells express between 2- and 120-fold higher levels of SNAIL, TWIST, SIP1 and FOXC2, these all being EMT-inducing transcription factors.

So, this leads to a rather bizarre conclusion. That is, if one takes these data seriously, epithelial stem cells express naturally a series of EMT-inducing transcription factors. This is a part of their normal transcriptional repertoire and is not forced in any way by experimentation. For me this was a counter-intuitive notion, because to my mind it would be far more reasonable that epithelial stem cells should express epithelial markers. But instead here, at this level of analysis, the epithelial stem cells appear to be quite mesenchymal. So this suggests that these cells are actually mesenchymal and that when they differentiate, only then do they acquire epithelial characteristics **(43)**. Again, this is the most immediate conclusion of what I've just showed to you.

Work of our collaborator at that time, Kornelia Polyak, looked both at human breast cancers and normal mammary epithelial tissue **(44)**. She works at a cancer hospital, the Dana-Farber Cancer Institute and I work more in a machine shop, at MIT, where we do not have access to these tissues. What she

discovered was the following: if she compared the gene expression patterns of normal mammary epithelial cells from a reduction mammoplasty and breast cancer cells, the non-stem, CD44 low cells looked quite similar to one another, and if she looked at the CD44 high stem cells from breast cancer and normal mammary glands, they also looked quite similar to one another, not quite identical. This was the first indication of a theme that has expanded in subsequent years, which is the following: that the stem cell program in a breast cancer is very similar to the stem cell program in a normal mammary gland. In other words, when a breast carcinoma develops, it is not as if the breast carcinoma invents a new stem cell program or assembles a new program. Instead, it appropriates a pre-existing normal stem cell program from the normal tissue -- a theme to which I will return.

But still, this adds to the list of changes that accompany an EMT, the additional trait of acquired self-renewal, that is, entrance into the stem cell state **(45)**.

If one looks from the standpoint of a cancer patient, then one comes to appreciate that the EMT is doubly dangerous for the patient. Because, first of all, it allows physical dissemination and secondly, it allows the disseminating cells to become self-renewing, which, I argue, is a prerequisite to the subsequent ability of the disseminating cells to successfully serve as founders of new metastatic colonies **(46)**.

Still we felt the burden -- the onus -- to generate a more compelling biological proof that the EMT actually generates epithelial stem cells **(47)**? And here I refer to the work of Wenjun Guo, who used a technique that has been in use for more than 50 years, which is used to gauge the stemness of mammary epithelial cells.

Importantly, I'm switching from human breast cancer to normal mammary epithelial cells in the mouse. Thus, I'm switching from neoplastic to normal, and from the human to the mouse, doing so with the faith, which I will show you is justified, that the mouse model teaches us much about the human condition.

In the context of a mouse, one can do the following manipulation **(48)**: Take a young female mouse, remove all of the endogenous mammary epithelial stem cells from its mammary stromal fat pad, and then create what is called a "cleared mammary fat pad". Into that stromal fat pad one can then implant candidate stem cells and if these stem cells really are functional, they can regenerate an entire mammary ductal tree, which is indistinguishable from a normally-formed mammary ductal tree, except for the inability of the ectopically implanted tree to form a connection, an anastomosis with the nipple.

Here is what the normal mammary gland looks like, formed under normal morphogenic processes and here is what an engrafted mammary gland, made by Wenjun Guo, looks like. This ability to form an entire mammary gland is a rigorous and stringent test of stemness. In fact, when the mouse becomes pregnant, these implanted cells produce milk, just like the endogenous gland would. He has now used a set of cell-surface markers that Jane Visvader, an Australian biologist, developed -- no longer CD44 and CD24, as in the human case, but now CD49f and CD24. According to Visvader, here are the stem cells and here are the non-stem cells **(49)**. And in fact, when he implanted this sub-population here, indeed the cells in this compartment generated a mammary ductal tree and they looked mesenchymal in mono-layer culture, whereas the cells over here failed to generate a mammary ductal tree. These structures in the latter are only veins and lymphatic ducts, and the non-stem cells look epithelial in monolayer culture.

So I no longer need to speak in tentative terms about these as "putative" or "candidate" stem cells, because here they are proven definitively to be functioning as stem cells. When Wenjun Guo looked at these isolated stem cells, compared with the non-stem cells, he discovered that when he looked at messenger-RNA, using RT-PCR, the putative stem cells over-expressed the SLUG EMT-inducing transcription factor by a factor of 36 relative to the non-stem cells **(50)**. So, here once again is the preferential expression of an EMT-inducing transcription factor once again in stem-like cells. Here we see, in the context of a cross-section of a normal mouse mammary duct **(51)**, that there are cells down here, in the nuclei of which one observes the expression of the SLUG EMT-inducing transcription factor. This means that the expression of this EMT-inducing transcription factor is not

just found in developing embryos and during wound healing or during malignant progression, but is also found in a normal homeostatically maintained tissue, in which presumably these cells are close to being stem cells, although that relationship is not proven by this micrograph. Here in a more recent work he purified the stem-like cells from a normal mouse mammary gland better and in this case there was a 120-fold over-expression of SLUG and here you can see more clearly that in these abluminal sites there are cells that naturally express SLUG, ostensibly because they are stem cells, but again that is hardly proven.

So Wenjun Guo undertook to organize a horse race **(52)**. He took one population of mouse mammary epithelial cells and transiently expressed in them for a period of four or five days the SLUG transcription factor and then turned it off. In another population in parallel, he had an empty control vector which he turned on and off, and therefore those cells never experienced SLUG. After generating these two cell populations, he mixed them together and introduced them into a cleared mammary stromal fat pad. The idea here was to see which of these two implanted populations would preferentially succeed in generating a mammary gland. Here I would just mention that one population of cells was labelled with red and one with green, and if there is an overlap together they generate yellow. One day, after implanting these mixed populations, the two were present in equal number **(53)**. One week later the population that had experienced SLUG transiently earlier was present in about twice the amount. Most importantly, seven weeks later the cells that had experienced a brief period of SLUG expression formed a mammary ductal tree, whereas the cells that had not experienced experimentally induced SLUG expression formed only this rudimentary mammary duct, testifying to the presence of small numbers of naturally existing stem cells in the unmanipulated cells. Here one can see that the mammary epithelial cells that experienced SLUG expression were vastly more effective in repopulating the mammary gland than the cells that had not. So this represents the beginnings of a rigorous and stringent proof that the EMT program is causally connected with entrance into the stem cell state.

Here there is another experiment that Wenjun Guo undertook. In this case, he used the mammosphere assay in three-dimensional culture in vitro **(54)**. The formation of these colonies in three-dimensional culture *in vitro* is a surrogate assay for stemness *in vivo*. So, cells that are able to form these mammospheres are also able to form mammary epithelial ducts when implanted in vivo. In this *in vitro* assay, if he deprived normal mammary epithelial cells of the mouse of SLUG protein, by shutting down SLUG RNA expression, doing so with one or another shRNA vector, then these cells lost their ability to form mammospheres and thus their ability to function as stem cells. So this suggests that the maintenance of the stem-like state, as judged by this assay, was necessary for the maintenance of the mammary stem cell state and, as I showed you before, expression of SLUG was sufficient to induce these mammary epithelial cells to enter into the stem cell state.

The only problem with the latter conclusion is that it was wrong **(55)**, and it was wrong for the following reason: Wenjun Guo separated the normal mammary epithelial cells of the mouse into three sub-populations using criteria that Visvader had previously developed. In fact, if the basal cells of the mammary gland were induced to express SLUG, they exhibited a slight increase in mammosphere-forming potential and thus in stemness; this testifies to the fact that these basal cells naturally have stem-like properties, even without the ectopic expression of SLUG. The luminal progenitors from this compartment, if they were forced to express SLUG transiently, now exhibited a great increase in their mammosphere-forming potential. That was certainly reassuring. However, the critical experiment is shown down here: when Wenjun Guo took mature fully differentiated mammary epithelial cells and put them in mammosphere culture, they form no mammospheres, and when they were forced to express SLUG, they also formed no mammospheres. Therefore SLUG was unable on its own to induce these cells to enter into stem-like state **(56)**.

So Wenjun Guo undertook to look for additional transcription factors that could collaborate with SLUG to generate mammospheres or organoids, as they are

called here. He went through a set of these transcription factors and discovered one of them, called SOX 9, which others had shown in the context of neural crest formation, is able to collaborate with SLUG or its cousin SNAIL in the formation of a neural crest **(57)**. Sure enough, when SOX and SLUG were transiently co-expressed together, now he could generate significant numbers of mammospheres.

This was most reassuring, because it began to allow him to draw a map of how cells in a mammary gland are organized. Fully differentiated mammary epithelial cells require transient expression of these two transcription factors in order to become stem cells. Because Luminal progenitors naturally express SOX 9, they just need SLUG to get up here. Other progenitor cells need that naturally express SLUG need SOX 9 to get up here. So this suggests a rather simple genetic roadmap from a differentiated state to a stem cell state **(58)**. And whether this one day will be useful for those who practice regenerative medicine, I cannot say.

Here is perhaps the most dramatic piece of evidence that Wenjun Guo produced **(59)**. Here he took 10,000 normal mammary epithelial cells implanted in cleared mammary stromal fat pad and nothing happened. Here he took as few as 100 hundred cells that had transiently, briefly, expressed SLUG and SOX 9 under the control of an inducible promoter and now these 100 cells formed this very nice mammary ductal tree, indicating a minimum 100-fold increased representation of epithelial mammary stem cells. So, in fact we grew increasingly bold in thinking that there was an intimate interconnection between the epithelial-mesenchymal transition program and residence in the epithelial stem-cell state.

In fact when he went back to human breast cancer and he deprived them of either SLUG or SOX 9, now these human breast cancer cells lost their ability to form tumours, much like the normal mammary epithelial cell in the mouse lost SLUG and SOX 9 and lost the ability to form mammary ductal trees **(60)**. This begins to suggest that these same transcription factors that are responsible for orchestrating the stem cell state in the normal murine mammary gland, are also responsible for the cancer stem cell state among human breast cancers as well.

Here are some questions we are left with **(61)**. How do cells get into the epithelial stem cell state normally? Is the EMT program the main road, the main highway for entering into the stem cell, or is it only a side door? Are there other ways of getting in there? Are there other key regulators for entering into the stem cell state? This last one is a question we cannot answer at present. It could well be that five to ten years from now we come to recognize that the epithelial stem cell program and the EMT are part of one unitary machinery; we only have different names for them until now because of the history of how we discovered these processes in mammalian cells.

Another unanswered question here is: are the lesson that we have learned here from studying mammary epithelial cells applicable to other types of epithelial cells, elsewhere in the body? One might think that they have different ways of organizing their stemness. It is my intuition on the basis of work in the literature, scattered pieces of evidence, that in fact much of what I've told you today is applicable to epithelial cells in other organs of the body, including the neoplastic derivatives of these epithelial cells.

Another question is: Does the EMT shed light on non-epithelial cell types?For example, let's think of a whole series of non-carcinomas, non-epithelial cancers. Does the EMT have any relevance in understanding their acquisition of a malignant phenotype. In the case hematopoietic and connective tissue tumours, such as osteosarcomas, at present there is no evidence to suggest that the EMT is relevant. Interestingly, in the context of neuroectodermal tumours, like glioblastomas, there is a suggestion, strangely enough, that the EMT transcription factors are associated with evolution to high-grade malignant state. To the extent that this is validated, this could be rationalized, *a posteriori*, as a consequence of the fact that the neuroectodermal tumours also derive from an epithelium -- in this case the epithelium is the neural ectoderm of the early embryo. But again, at this stage this is a speculation.

What are the contextual signals that induce a cell to activate its endogenous EMT program? In general we believe in living tissues this does not happen spontaneously; instead, it happens only in response to signals that cells receive from the local

tissue microenvironment. If you look at an actual human tumour, do cancer cells normally pass all the way through the EMT, going from a fully epithelial to a fully mesenchymal state, as depicted here? Or is it more natural and more common that cancer cells go only partially through an EMT, activating mesenchymal markers while retaining a whole series of epithelial markers.

Here's one interesting insight that Christine Chaffer generated recently **(62)**. I don't have time to go into her data, but she did a series of experiments in which she demonstrated that CD44 low cells, which are non-stem cells, can in culture and in vivo spontaneously go into the CD-44 high stem-like state -- in other words, a spontaneous de-differentiation **(63)**. She studied a whole series of cells, and what she discovered was that when she took human mammary epithelial cells that had been immortalized with telomerase enzyme in monolayer culture, one-half percent per cell generation spontaneously entered into stem cell state **(64)**. When she looked at cells that additionally had acquired the early region of SV40 tumor virus, his number doubled and it doubled again when a RAS oncogene was further introduced into these cells. If you follow the work that she has just done and the other kinds of experiments that I've just demonstrated to you **(65)**, you can begin to conclude that the diagram that I showed you earlier needs to be revised. That is to say, that there has to be an additional arrow which goes from the progenitor transit-amplifying state back into the stem-cell compartment state **(66,67)**. What induces this spontaneous de-differentiation is not clear at present.

It does however have interesting implications for the entire field of clinical medicine. Here I'm referring largely to the work of Piyush Gupta, who began some of this work while in my lab as a graduate student and then continued as a post-doc in the laboratory of Eric Lander. This is the situation that operates in many human carcinomas at present. We have stem cells in grey and the-non stem cells that coexist as the majority population in the same tumour. Following current treatment with chemotherapy or radiotherapy the tumour is debulked and there is only a small residue of cells that can no longer be observed **(68)**. The oncologist declares victory, much as do American Presidents when they are standing on the decks of aircraft carriers....you are too young to remember that...we once had a President named Bush....wrong audience for this one! Subsequently the tumour re-grows, generating a clinical relapse. Now the tumour re-grows because these cells that show increased resistance to therapy are able to re-grow and regenerate the tumour. Obviously this creates great difficulties. Here is one line of evidence for that. Here we see the epithelial cells that are responsive to chemotherapy or monoclonal antibodies, whereas these mesenchymal stem-like cells are resistant and there is much justification to believe that this situation occurs in many types of human tumors **(69)**. Therefore, Piyush Gupta and Tamer Onder asked the question: could they find agents that could specifically kill the cancer stem cells, rather than the non stem cells. To do so, they generated large numbers of stem cells using the EMT program, as first accomplished here by Tamer Onder **(70)**; the great bulk of this work that I will now show you was undertaken by Piyush Gupta, after he left my laboratory.

Here is the outcome of his work **(71)**. With Lander he screened 16,000 compounds to look for agents that specifically kill the cancer stem cells, not the non-stem cells. Here you see the behaviour of two commonly used therapeutic agents, doxorubicin and paclitaxel. Here you see that the non-stem cells in blue are killed at a lower drug concentration than the stem cells in red, in the case of both of these drugs. The non stem cells are about 10-fold more sensitive to killing by these two conventional chemotherapeutics. Gupta found 22 different compounds in this screen that preferentially killed the stem cells rather than the non-stem cells. He studied only two of them because they were available in large amounts, salinomycin and abamectin. Interestingly these are both anti-parasitic compounds and their mechanism of action is unclear to us. But you see nonetheless if we focus on salinomycin that it kills the stem cells at a 6-to-8-fold lower drug concentration than the non stem-cells. In fact, salinomycin is used in chicken feed to prevent parasitic infections in chickens. Therefore, if you would like some salinomycin, maybe you can get small

amounts of it by going to Kentucky Fried Chicken, KFC; this might be very good for you or, at least, for the owners of Kentucky Fried Chicken!

Here you see a testimony to salinomycin and its effects. Here is a population of cells, 5% stem cells 95% non-stem cells treated with paclitaxel. After the therapy, in vitro, 70% of the surviving cells are in the stem cell position, 30% in the non-stem cell position. In other words the non-stem cells were preferentially killed off, as I argued before. Here are the effects of the KFC compound: After therapy 0.2% of the cells are in the stem cell position, 99.8% of them in the non stem cell position. So you see a dramatic difference in their specificity. This is not to say that salinomycin is about to go into the oncology clinic, but only to indicate that, in general, one should have reasonably straightforward means -- experimental strategies -- for identifying compounds that specifically kill the cancer stem cells. That may in turn offer a new type of therapeutic advantage.

But is this on its own the "answer to cancer" **(72)**? If we kill the cancer stem cells what might we anticipate? Here is one outcome of killing off cancer stem cells **(73)**. You get rid of the cancer stem cells and with time the entire tumour disappears, because the source of the self-renewal in the tumour has now been eliminated, that is the stem cells. But instead, I think that an alternative scenario is more likely **(74)**. Once again, one gets rid of the cancer stem cells with compounds that specifically kill the cancer stem cells, but now, among the surviving non-stem cells they regenerate de novo new stem cells, as I showed to you before. If this scenario is sustained, it suggests that the only way that one can hope to have clinically durable responses against solid tumours is to target on the one hand the cancer stem cells and on the other the non-stem cells. Targeting only one population or the other seems to me in the long run not to have the prospect of yielding a durable clinical response, which is what we all hope for.

Finally, I finish by mentioning the work of Christina Scheel, who is interested in the following problem: Here are two populations of mesenchymal cells that were generated in the lab **(75)**. These cells became mesenchymal under the action of the TWIST, EMT-inducing transcription factor. These cells became mesenchymal because they spontaneously went from the epithelial to the mesenchymal state and remained in that state for weeks and months afterwards. That behavior provoked the question: how do these cells remain stably within the mesenchymal state? How do they know how to maintain their state, their stable residence in the mesenchymal stem-like cell state **(76)**?

This question provoked the following speculation: they do so via autocrine signalling **(77)**. I would show you all the experimental evidence, but time does not allow me to do so. Suffice it to say that she examined the culture medium in which stem cells were grown. There she found that the culture medium around stem cells was very different from the culture medium around non-stem cells, and that in the mesenchymal stem cells there were three kinds of signalling programs that became activated, involving canonical Wnts, non-canonical Wnts, and TGF-beta **(78)**.

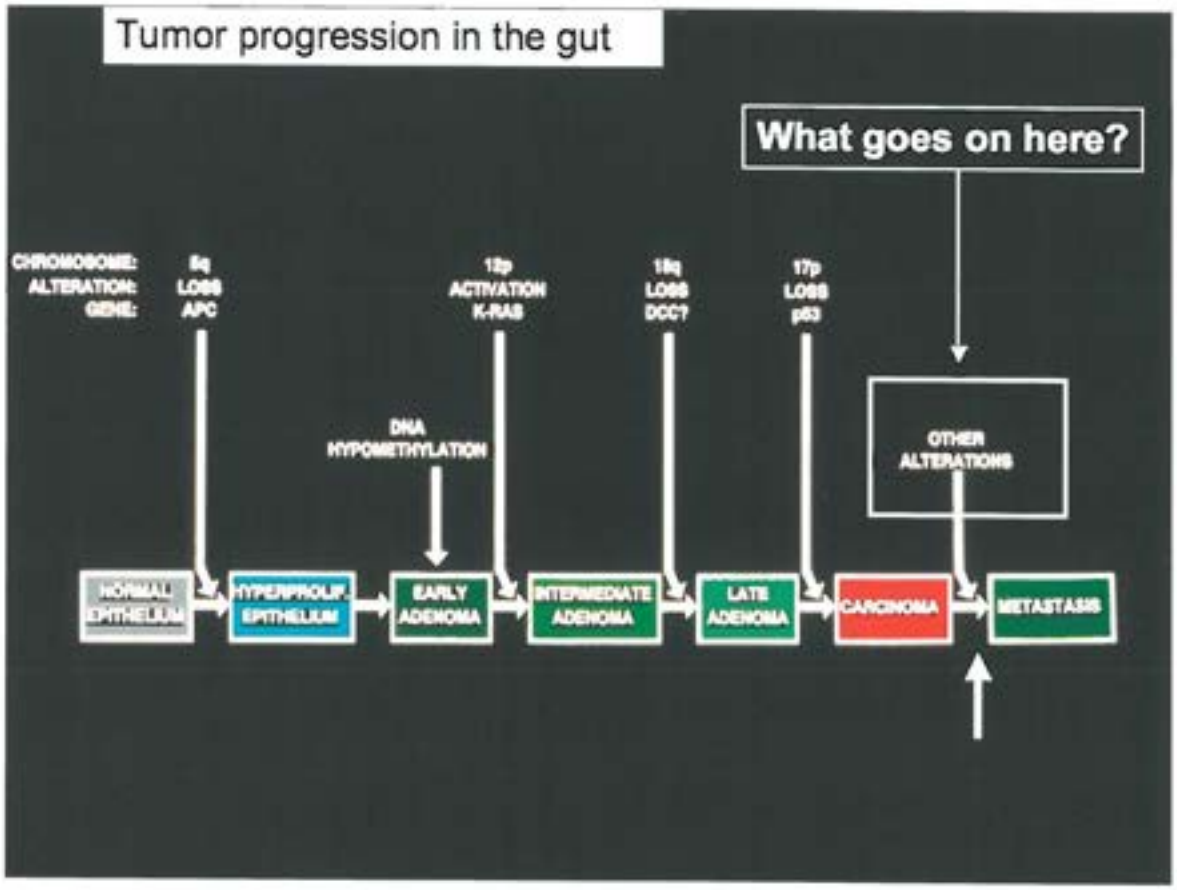
I will illustrate and summarize her work with the following cartoon **(79)**. Mesenchymal cells maintain their residence in the mesenchymal stem cell state through the continuous firing of three distinct autocrine signalling pathways. They produce large amounts of TGF-beta, they produce large amount of canonical Wnts and non-canonical Wnts. These signals conspire to ensure that these cells maintain their long-term residence in that state in a stable fashion. Epithelial cells on the other hand are quite different. Epithelial cells make TGF-beta, but that TGF-beta is ambushed, antagonized by large amounts of bone morphogenetic proteins (BMPs) that these cells make, which antagonize TGF-beta signalling at the intracellular level. Epithelial cells make canonical Wnt proteins, but these canonical Wnt proteins cannot operate in the epithelial cells, because they are ambushed by two different Wnts antagonists, called here DKK1 and SFRP1 that antagonise Wnt signalling.

I once again have a proprietary interest here, because my mother always called me *Dickkopf*, which is a German expression that is not too flattering and implies someone who is irrevocably stubborn! But I digress! So, if we go from the epithelial to the mesenchymal state, we see the following changes. The BMPs disappear, and now TGF-beta signalling can operate. DKK1 and

SFRP1 disappear and now the canonical Wnt signalling can operate. And now these mesenchymal cells start to make non-canonical Wnt proteins. So, this allows the stable maintenance in one state or the other over an extended period of time. What we don't understand is how cells go from one state to the other state -- how they make that transition and what are the mechanistics that enables such a transition.

This remains a mystery for us, but it now appears possible that these signalling pathways may be shared in common by a variety of EMT programs.
So to finish here, here are the unusually good-looking people who did all this work **(80)**! Here they are Photoshopped together over a period of years. I already mentioned some of their names.
Now I say once again Ringrazio per tutto!

1.



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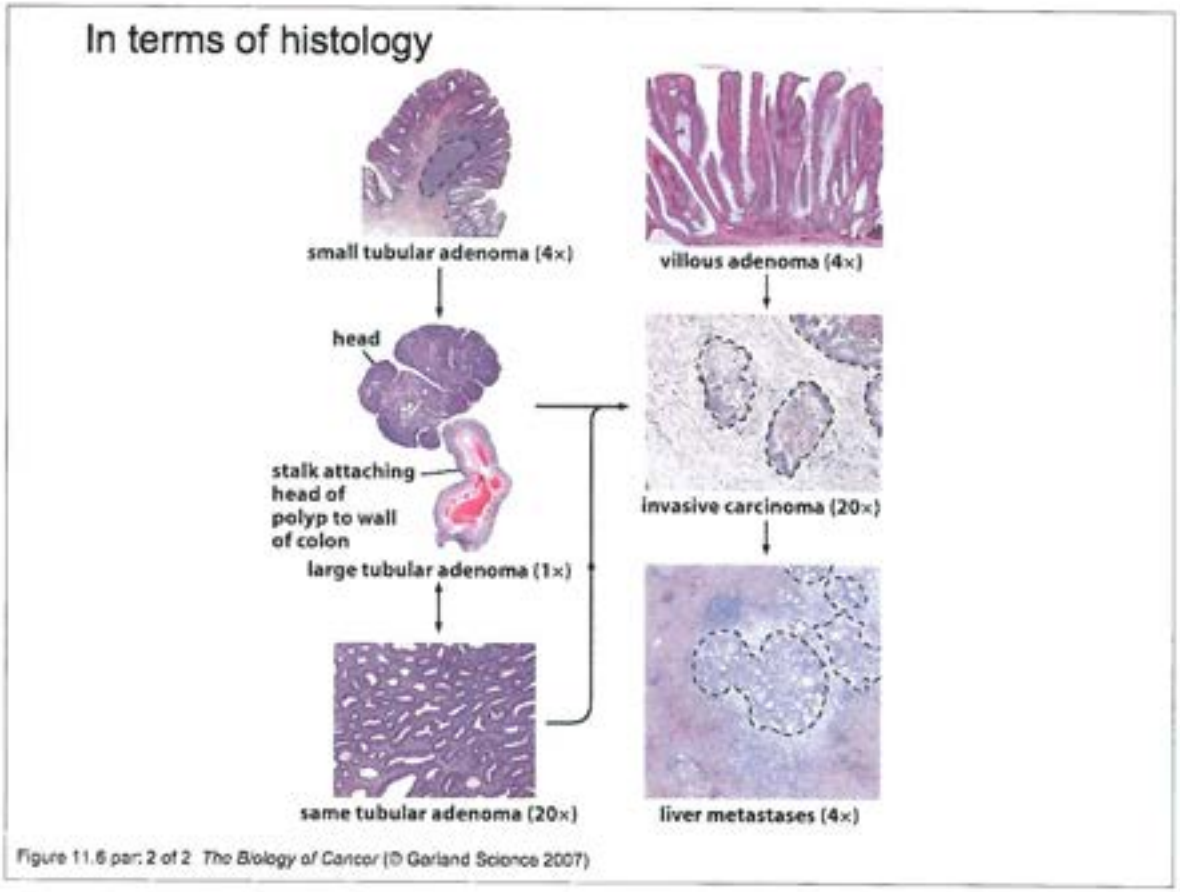


Figure 11.6 part 2 of 2 *The Biology of Cancer* (© Garland Science 2007)

3.

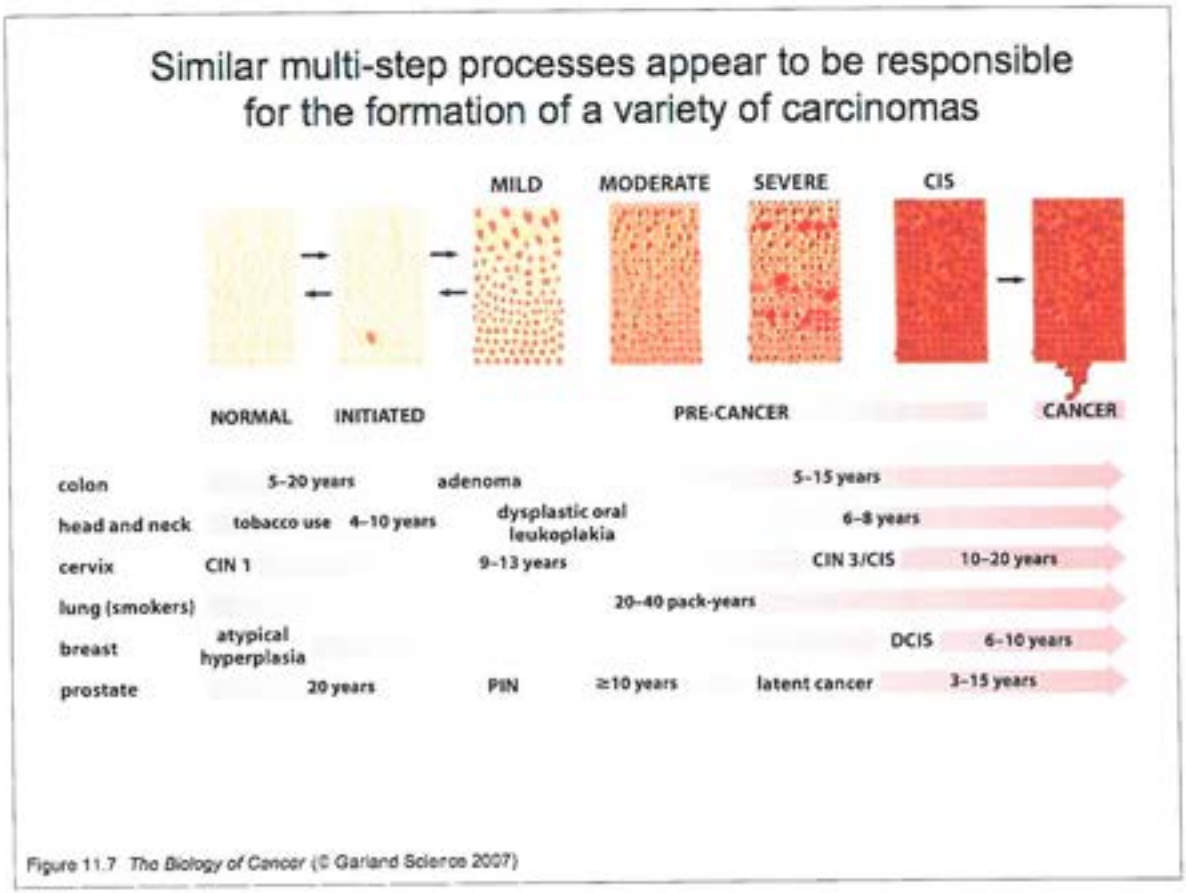


Figure 11.7 *The Biology of Cancer* (© Garland Science 2007)

4.

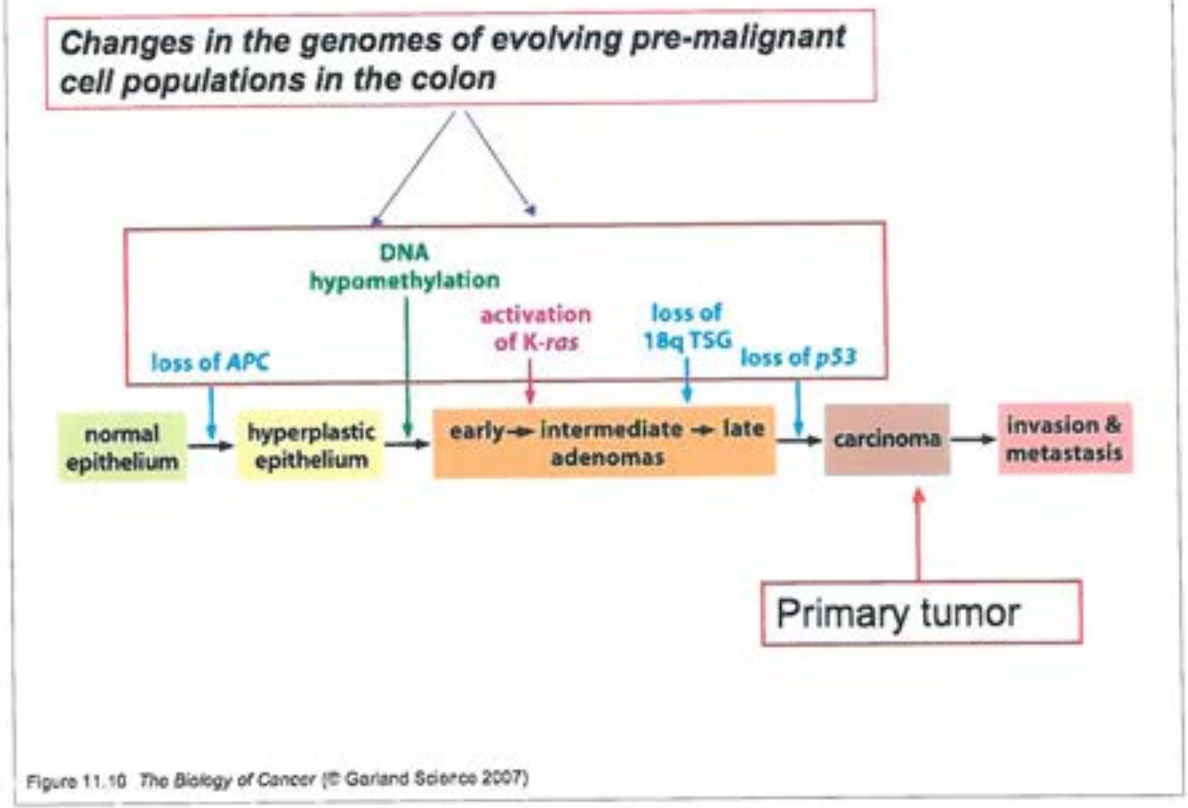
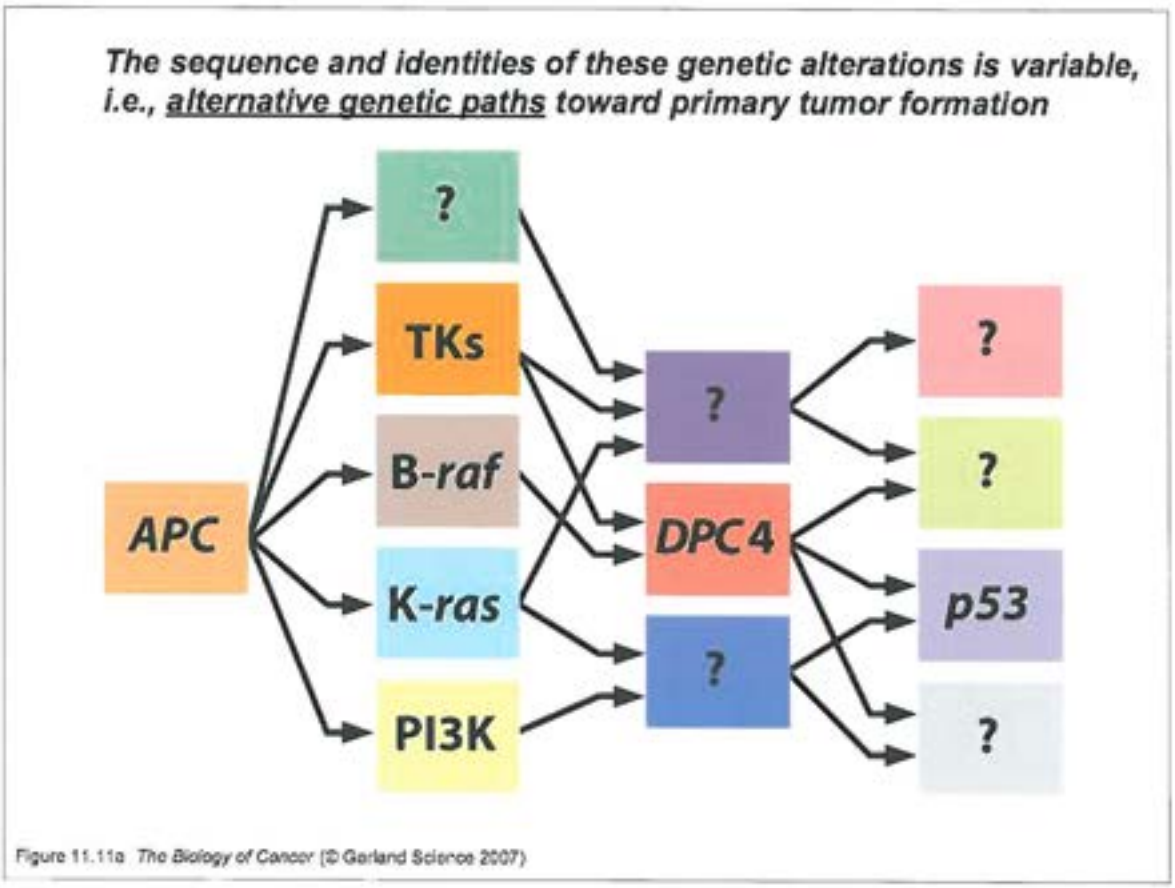
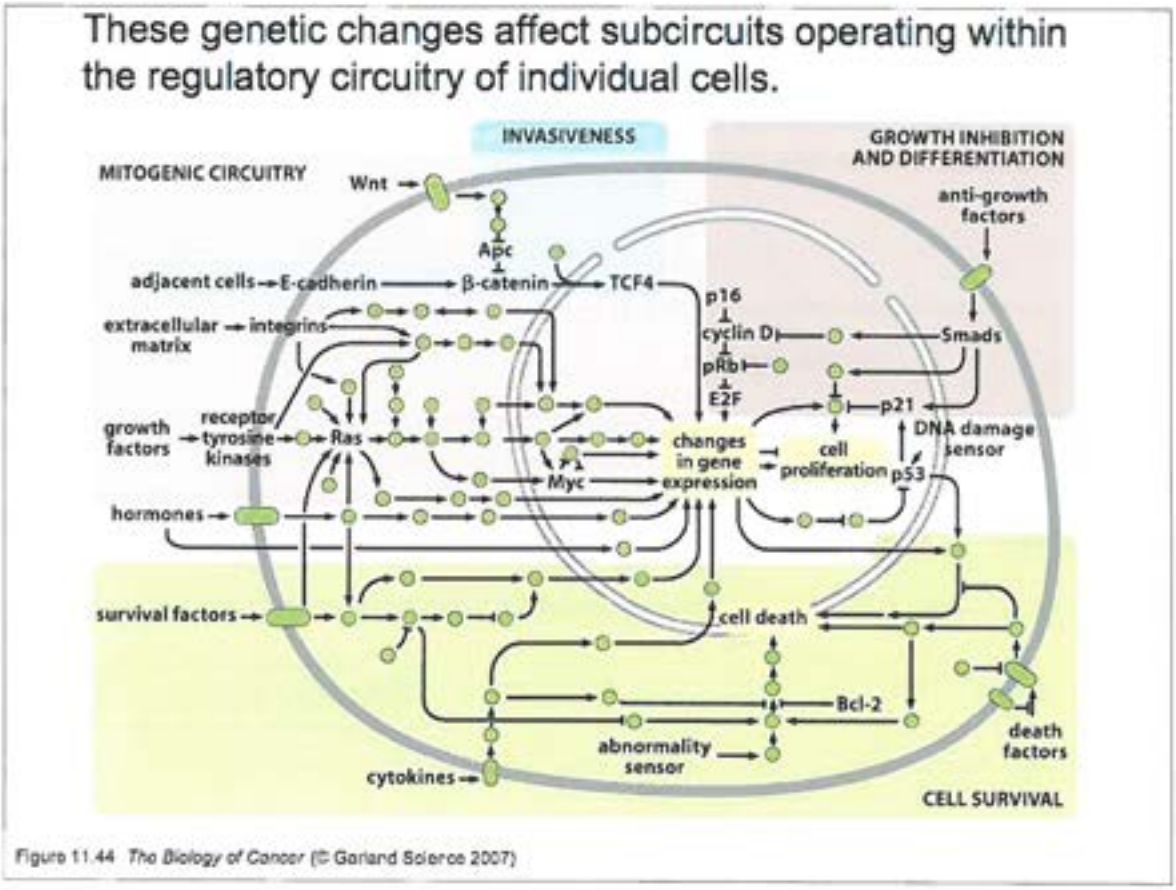


Figure 11.10 *The Biology of Cancer* (© Garland Science 2007)

5.


The sequence and identities of these genetic alterations is variable, i.e., alternative genetic paths toward primary tumor formation
APC
?
TKs
B-raf
K-ras
PI3K
?
DPC4
?
?
?
p53
?
Figure 11.11a The Biology of Cancer (© Garland Science 2007)

6.


These genetic changes affect subcircuits operating within the regulatory circuitry of individual cells.
INVASIVENESS
GROWTH INHIBITION AND DIFFERENTIATION
MITOGENIC CIRCUITRY
Wnt
Apc
adjacent cells
E-cadherin
β-catenin
TCF4
p16
cyclin D
pRb
E2F
anti-growth factors
Smads
extracellular matrix
integrins
growth factors
receptor tyrosine kinases
Ras
Myc
changes in gene expression
cell proliferation
p21
p53
DNA damage sensor
hormones
survival factors
cell death
Bcl-2
death factors
abnormality sensor
cytokines
CELL SURVIVAL
Figure 11.44 The Biology of Cancer (© Garland Science 2007)

7.

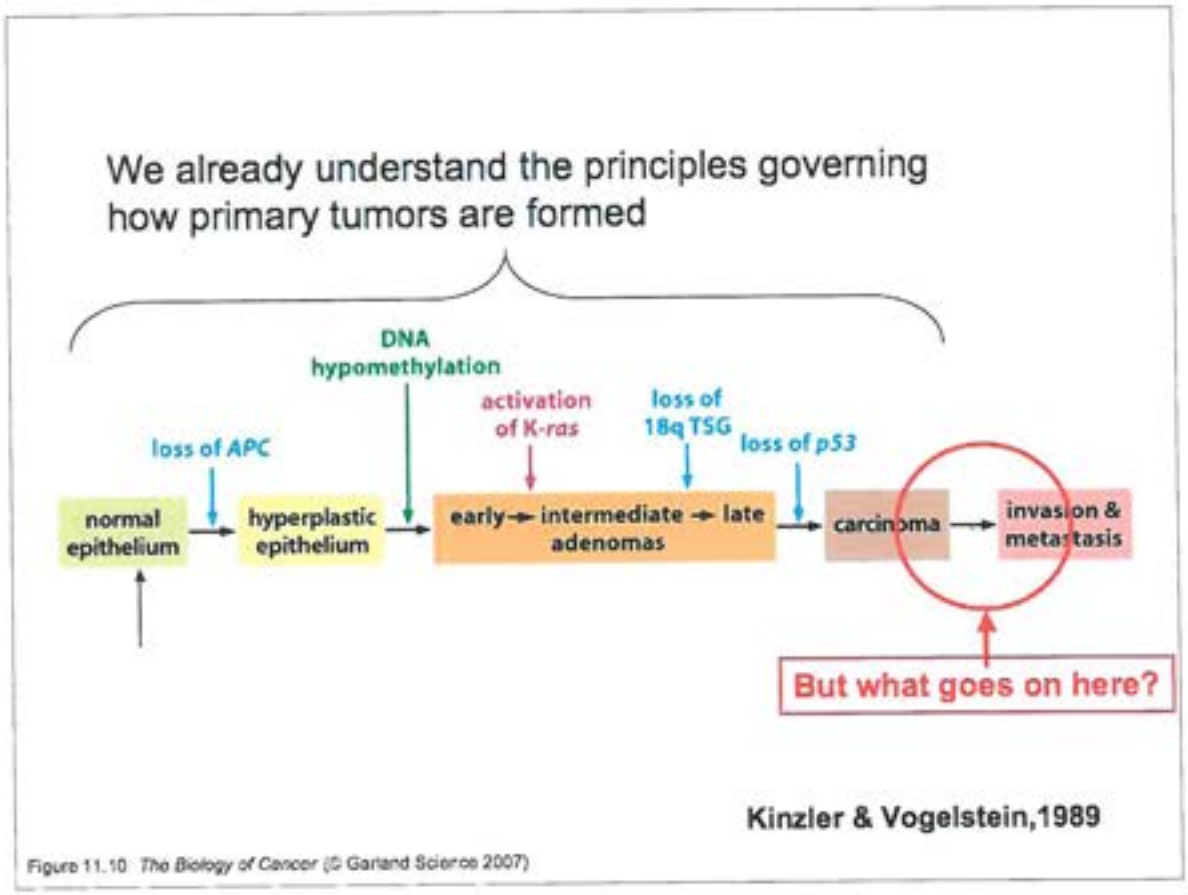


Figure 11.10 *The Biology of Cancer* (© Garland Science 2007)

8.

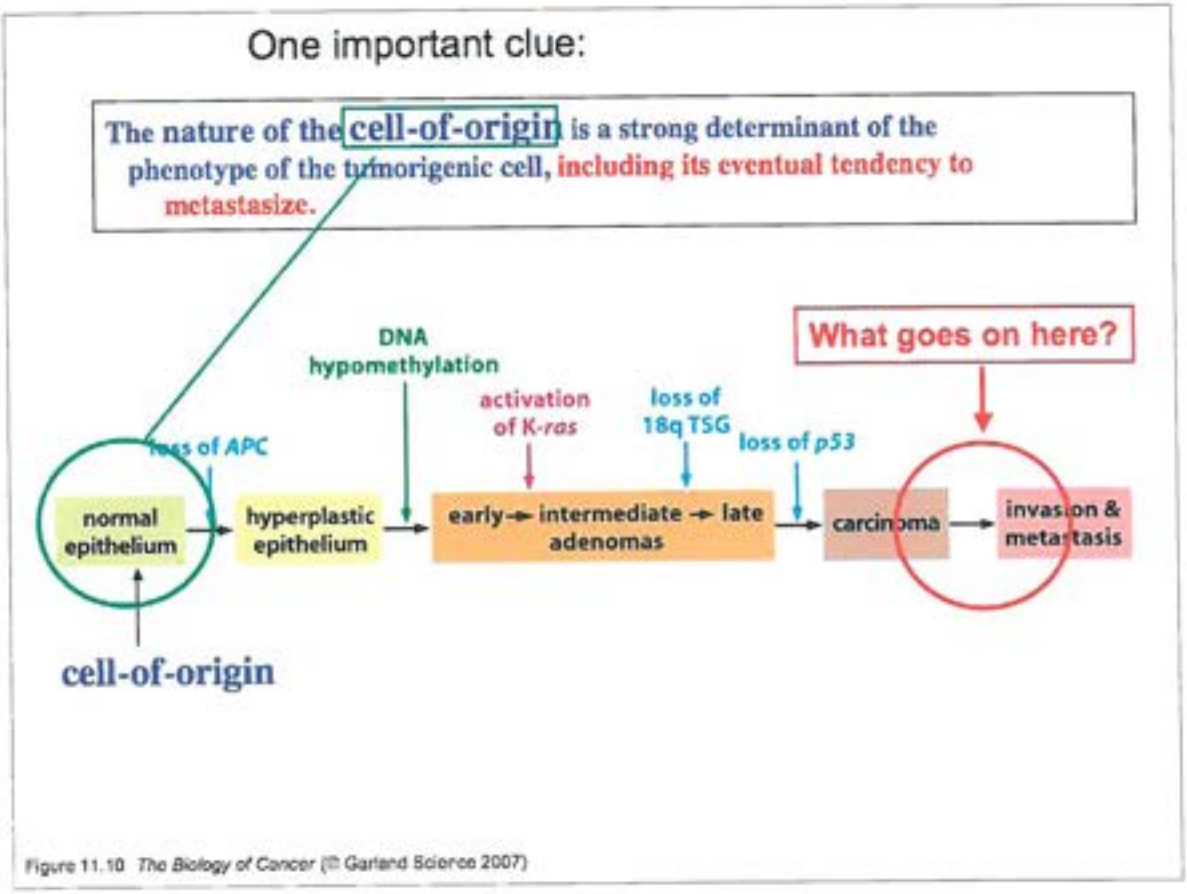


Figure 11.10 *The Biology of Cancer* (© Garland Science 2007)

9.

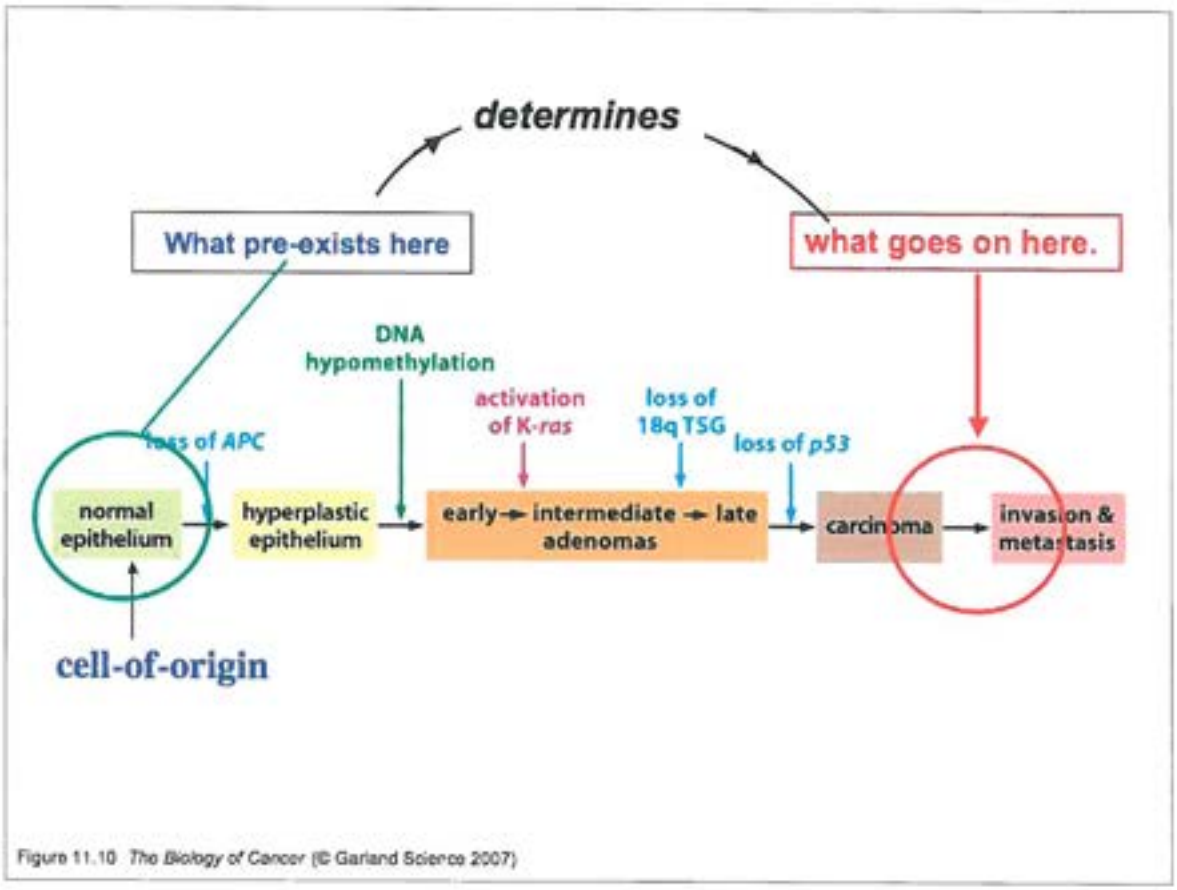


Figure 11.10 *The Biology of Cancer* (© Garland Science 2007)

10.

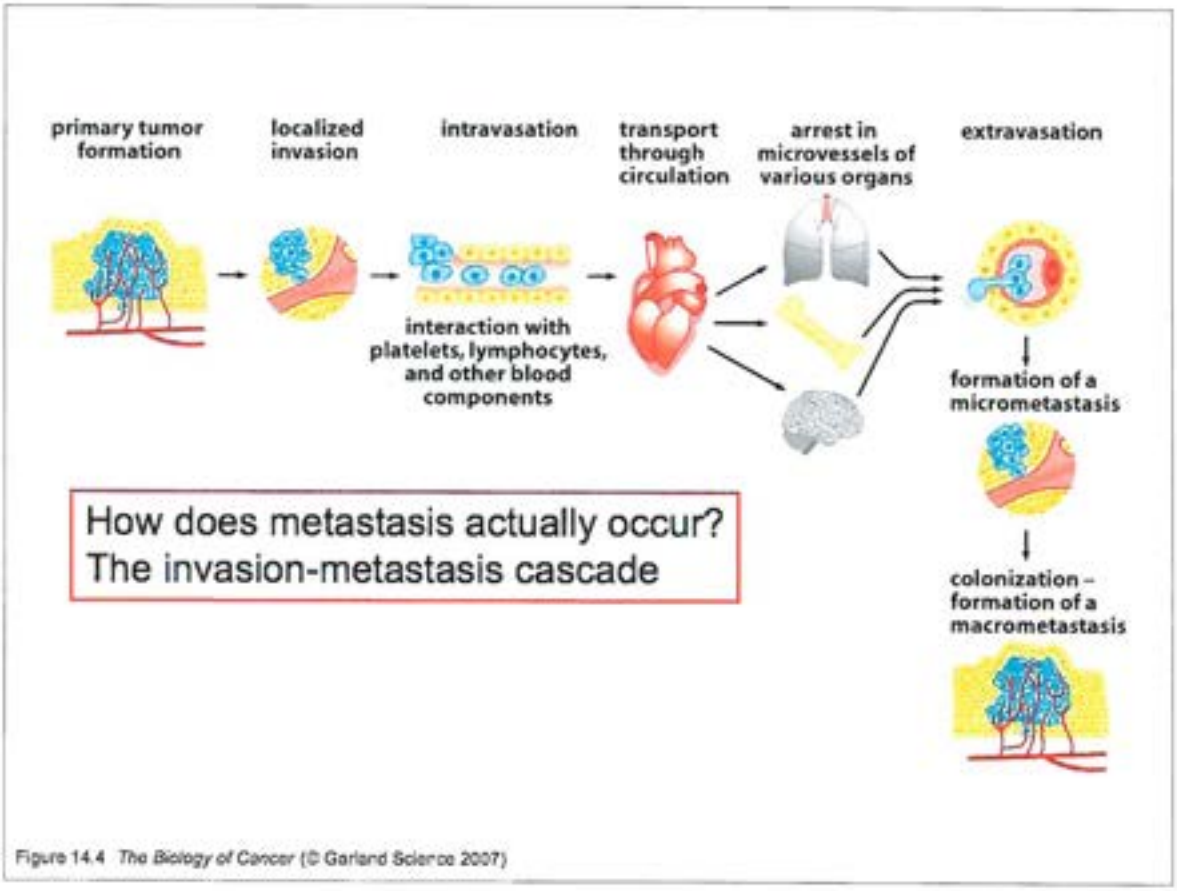


Figure 14.4 *The Biology of Cancer* (© Garland Science 2007)

11.

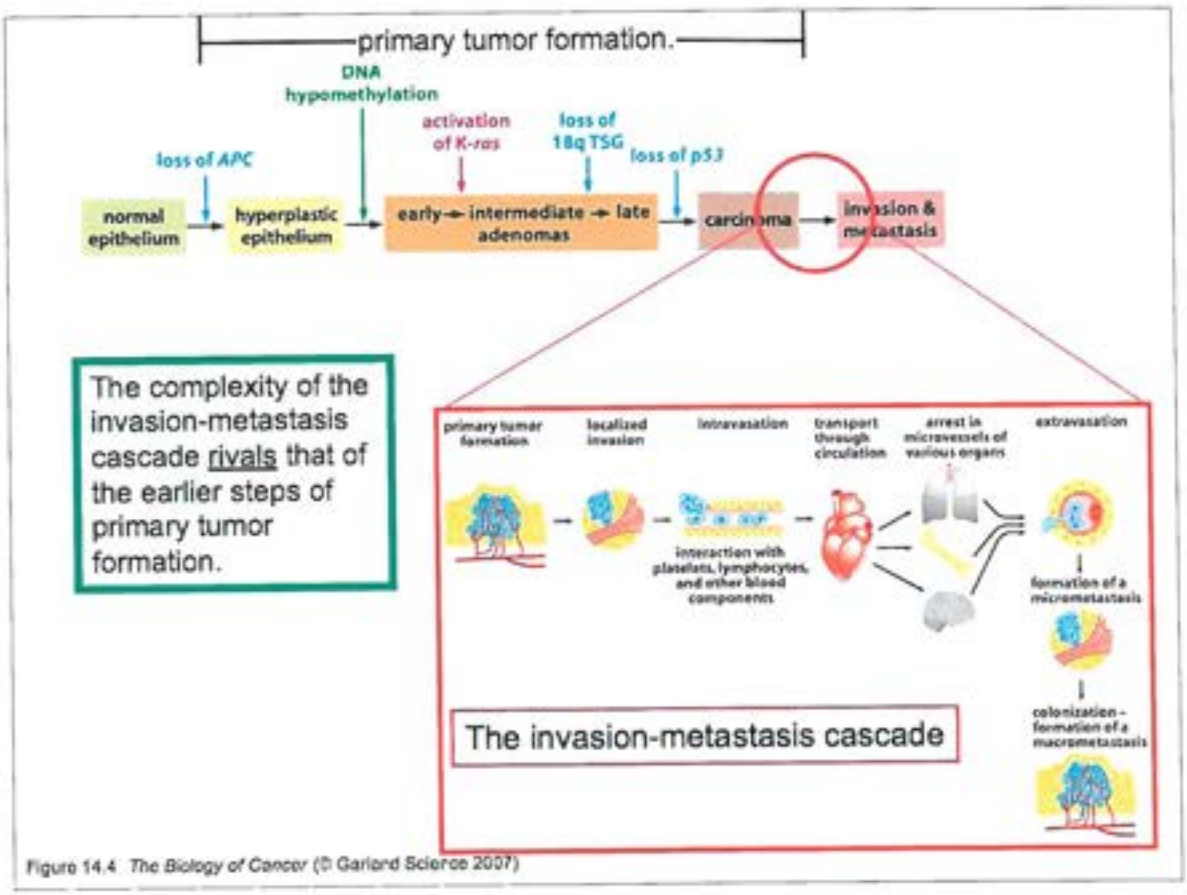


Figure 14.4 *The Biology of Cancer* (© Garland Science 2007)

12.

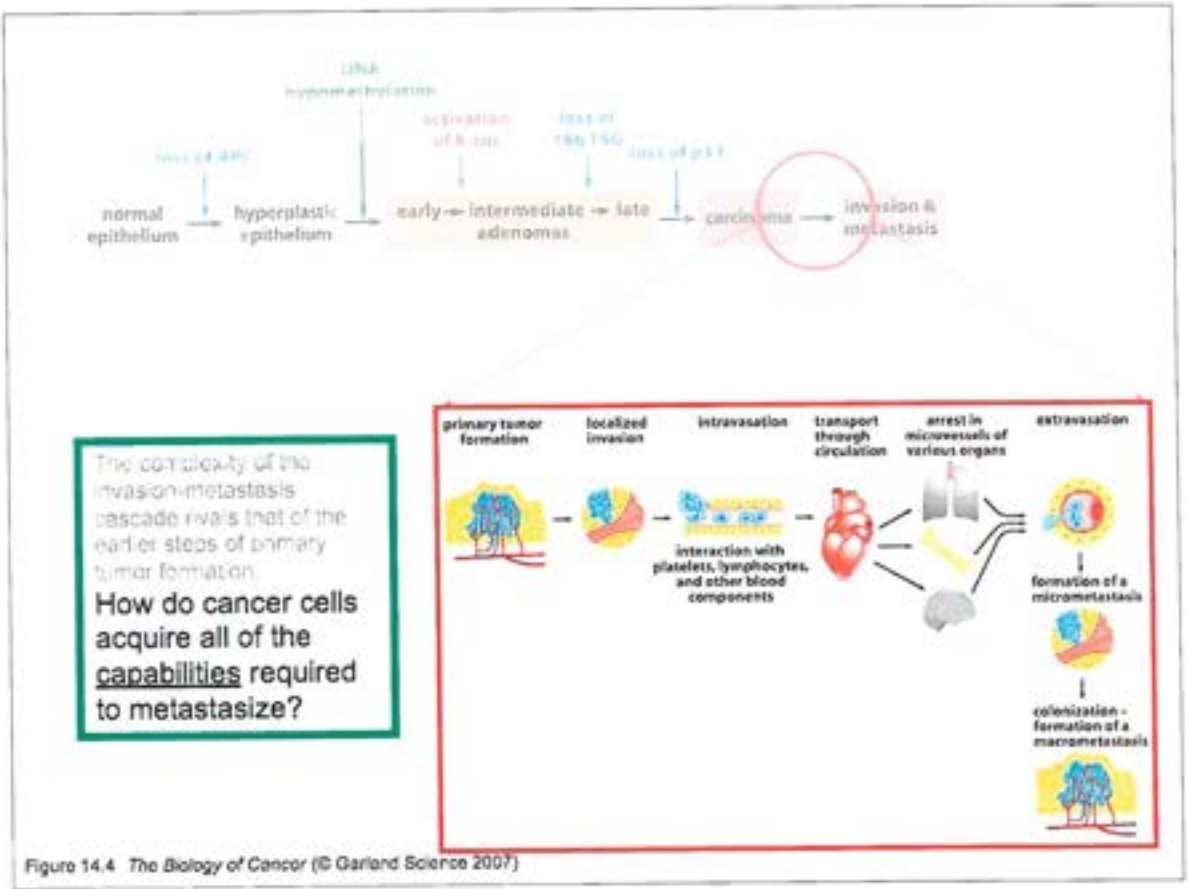
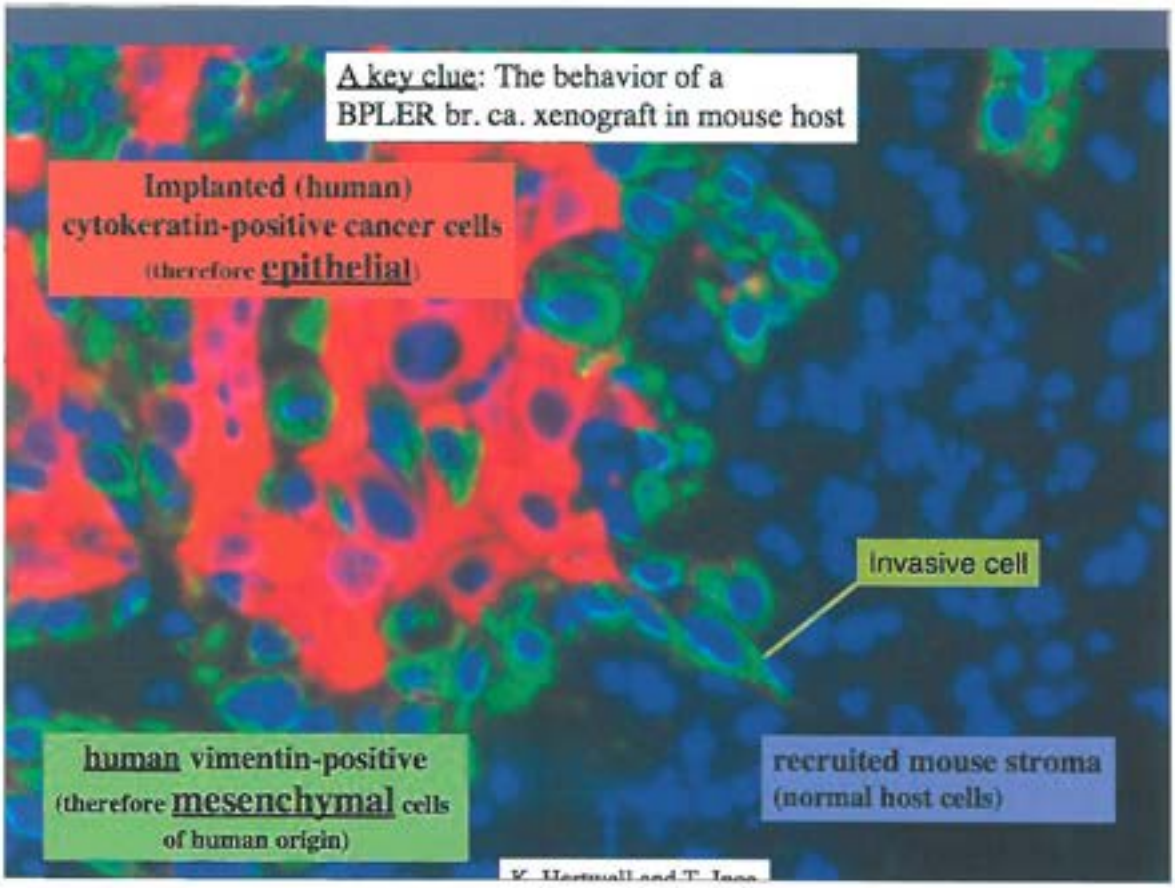
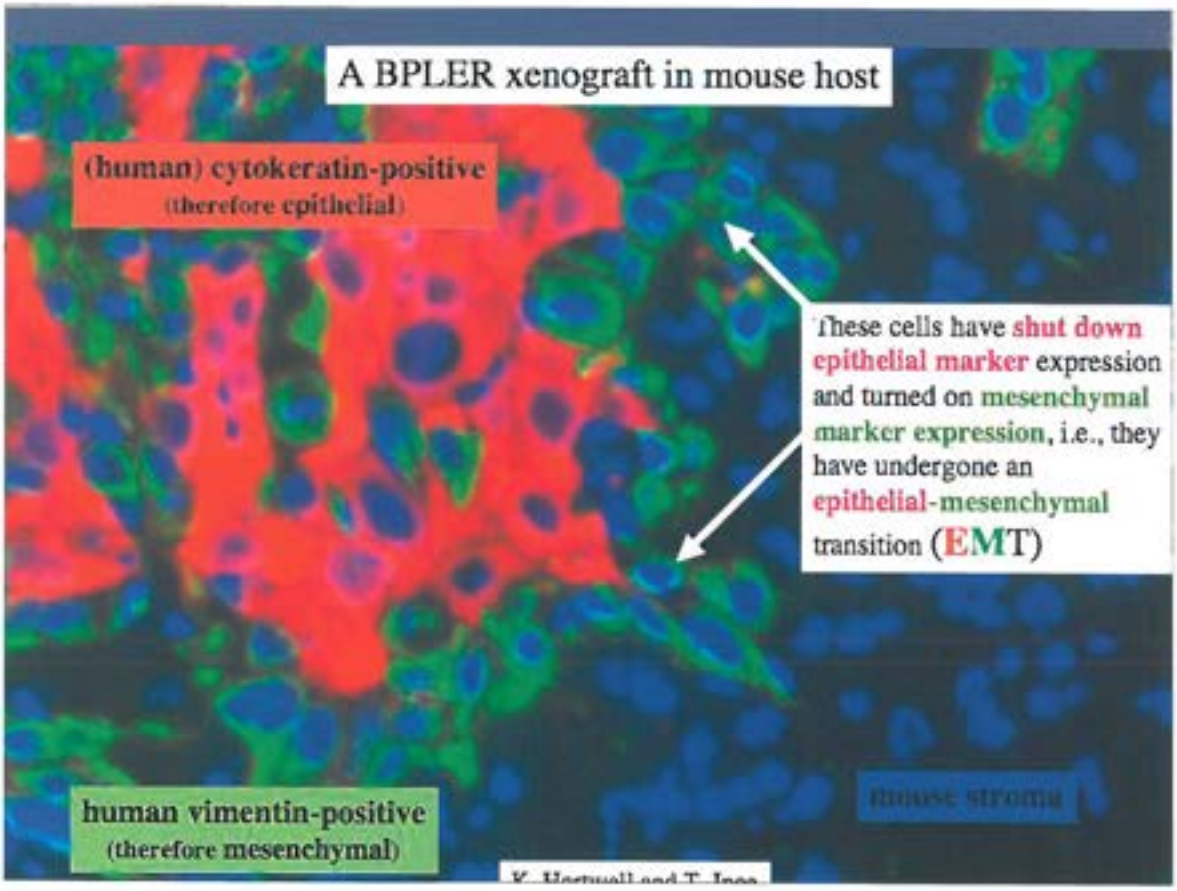


Figure 14.4 *The Biology of Cancer* (© Garland Science 2007)

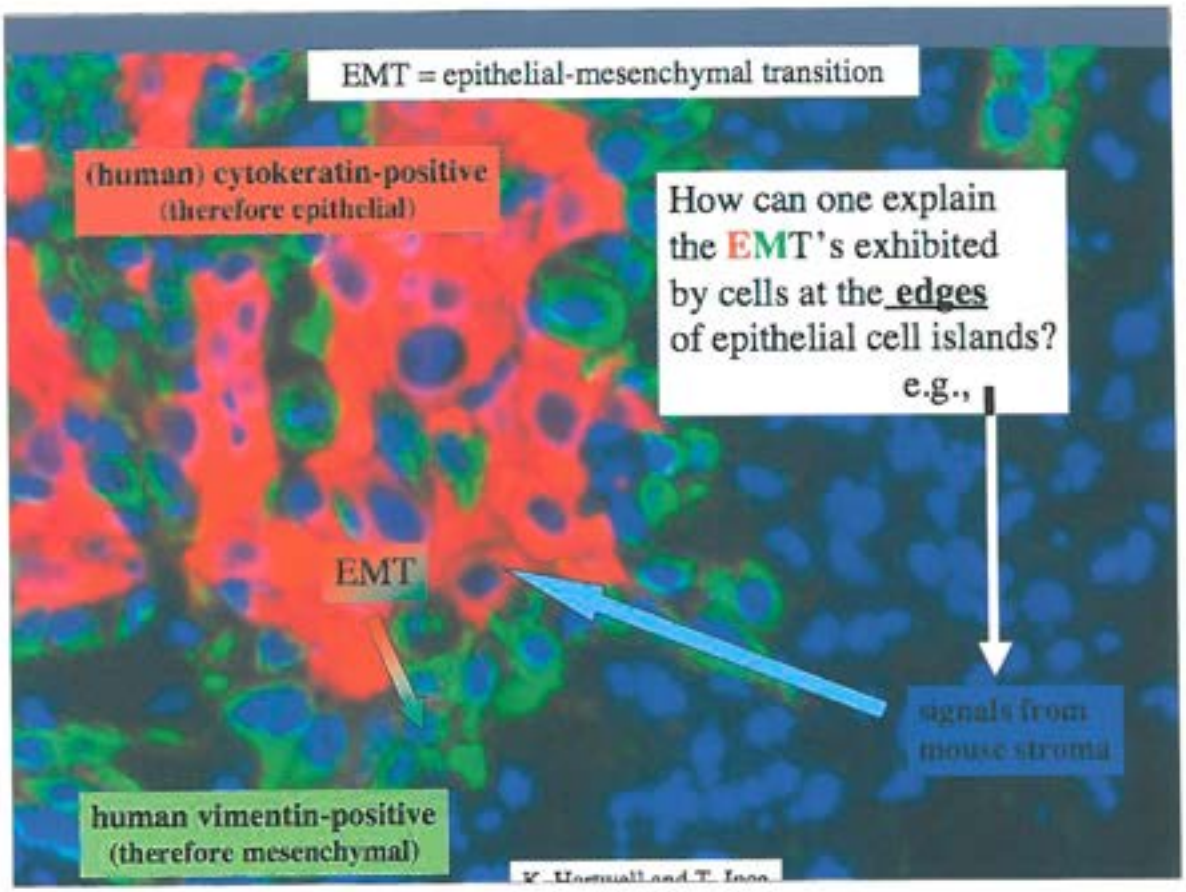
13.


A key clue: The behavior of a BPLER br. ca. xenograft in mouse host
Implanted (human) cytokeratin-positive cancer cells (therefore epithelial)
Invasive cell
human vimentin-positive (therefore mesenchymal cells of human origin)
recruited mouse stroma (normal host cells)

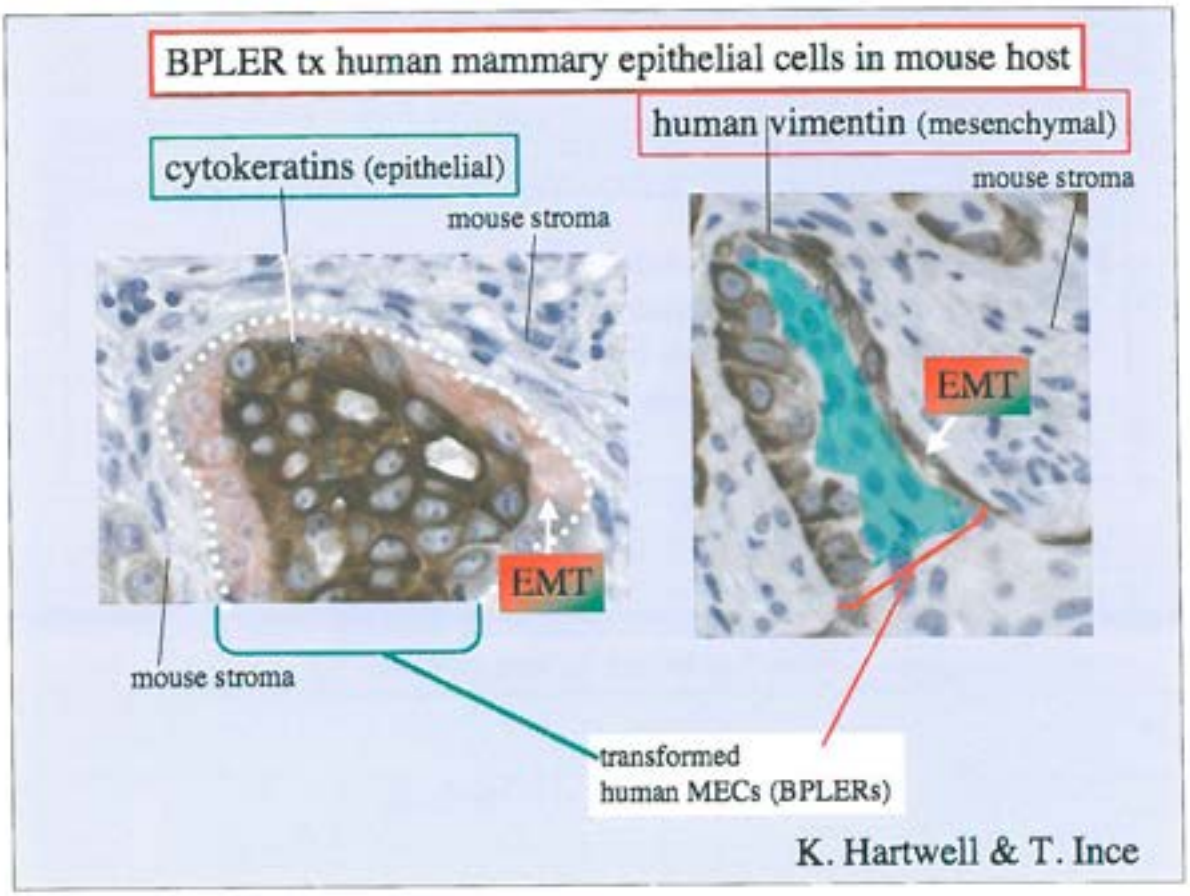
14.


A BPLER xenograft in mouse host
(human) cytokeratin-positive (therefore epithelial)
These cells have shut down epithelial marker expression and turned on mesenchymal marker expression, i.e., they have undergone an epithelial-mesenchymal transition (EMT)
human vimentin-positive (therefore mesenchymal)

15.

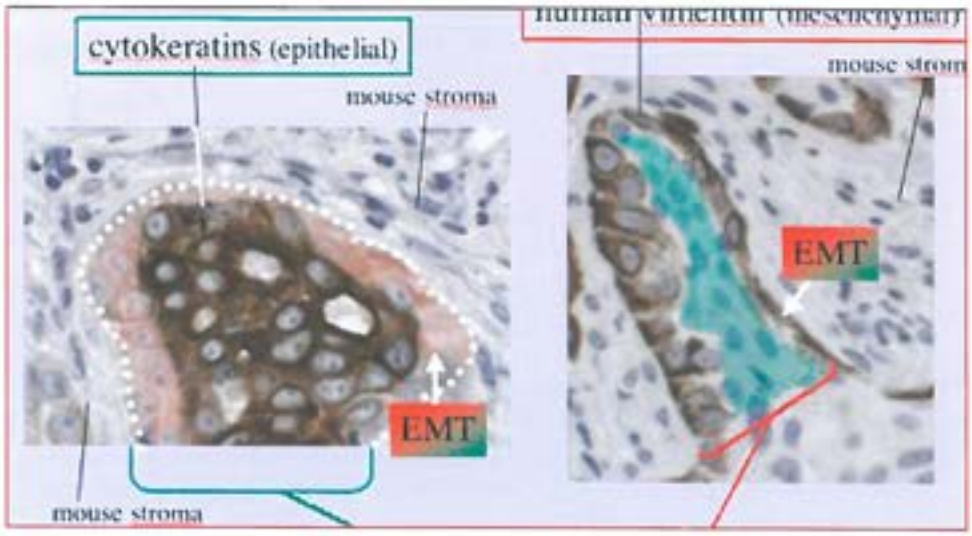

EMT = epithelial-mesenchymal transition
(human) cytokeratin-positive
(therefore epithelial)
How can one explain
the EMT's exhibited
by cells at the edges
of epithelial cell islands?
e.g.,
EMT
signals from
mouse stroma
human vimentin-positive
(therefore mesenchymal)

16.


BPLER tx human mammary epithelial cells in mouse host
human vimentin (mesenchymal)
cytokeratins (epithelial)
mouse stroma
mouse stroma
EMT
EMT
mouse stroma
transformed
human MECs (BPLERs)
K. Hartwell & T. Ince

17.

Close contact between carcinoma cells and surrounding recruited stroma seems to be involved in triggering EMTs .



18.

BPLER tx human mammary epithelial cells in mouse host

human vimentin (mesenchymal)

cytokeratins (epithelial)

mouse stroma

Conclusion:

The microenvironment* of the primary tumor can contribute importantly to the phenotypic conversion occurring during an EMT , which involves a response of carcinoma cells to contextual signals originating in the stroma.

*** created by the stroma**

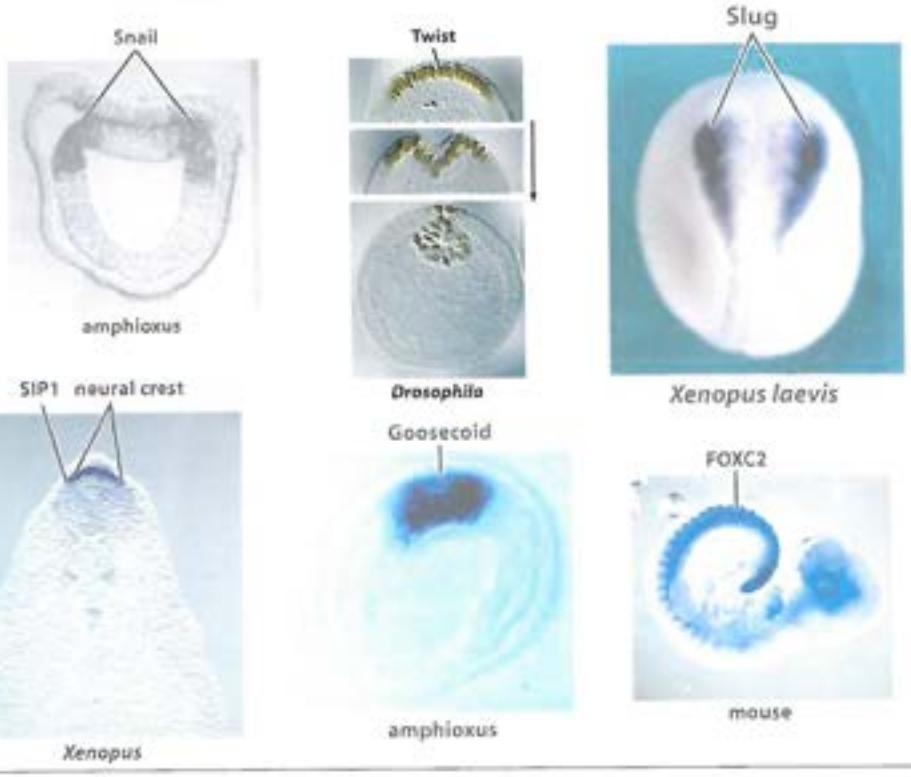
mouse stroma

transformed human MECs (BPLERs)

K. Hartwell & T. Ince

19.

A group of pleiotropically acting **transcription factors** that induce EMTs at various stages of embryogenesis



20.

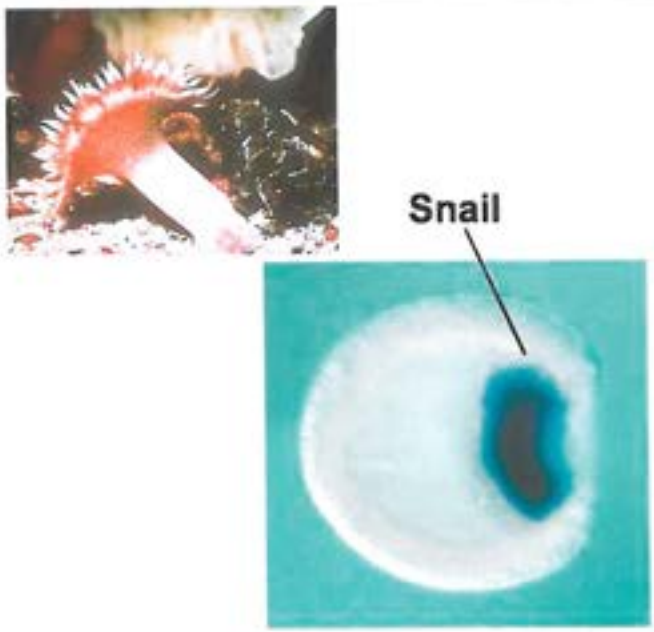
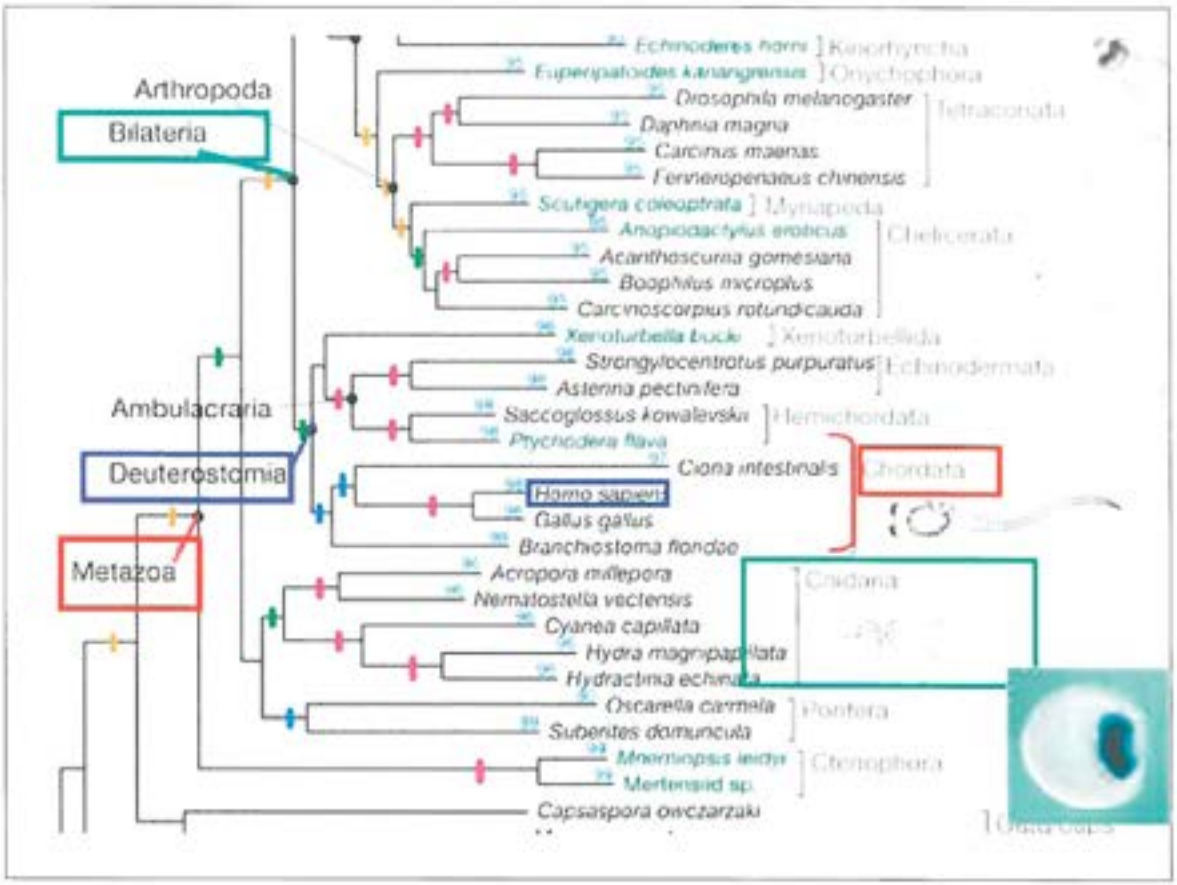


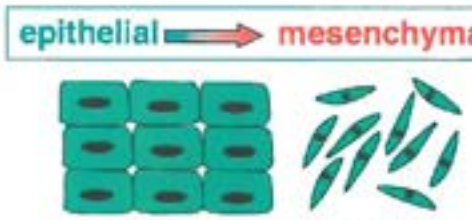
Figure 14.26f *The Biology of Cancer* (© Garland Science 2007)

21.



22.

The epithelial-mesenchymal transition (EMT) (Hay, 1986) is a complex, multi-faceted program involving multiple changes in cell properties.



Cobblestone	Elongated
Non-motile	Motile
Non-invasive	Invasive
Polarised	Non-polarised
Epithelial markers	Mesenchymal markers
• E-cadherin	• N-cadherin
• Desmoplakin	• Vimentin
• Keratin	• Fibronectin

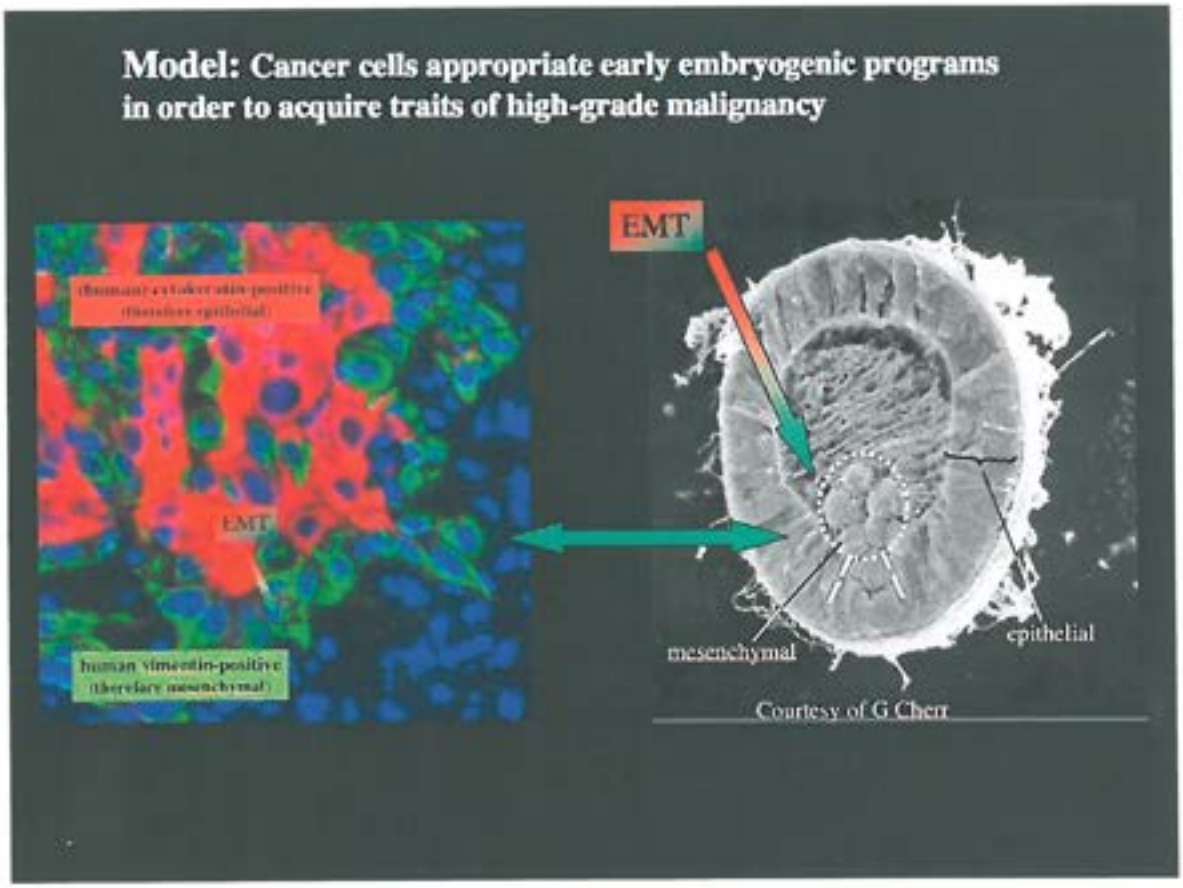
epithelial → mesenchymal

EMT

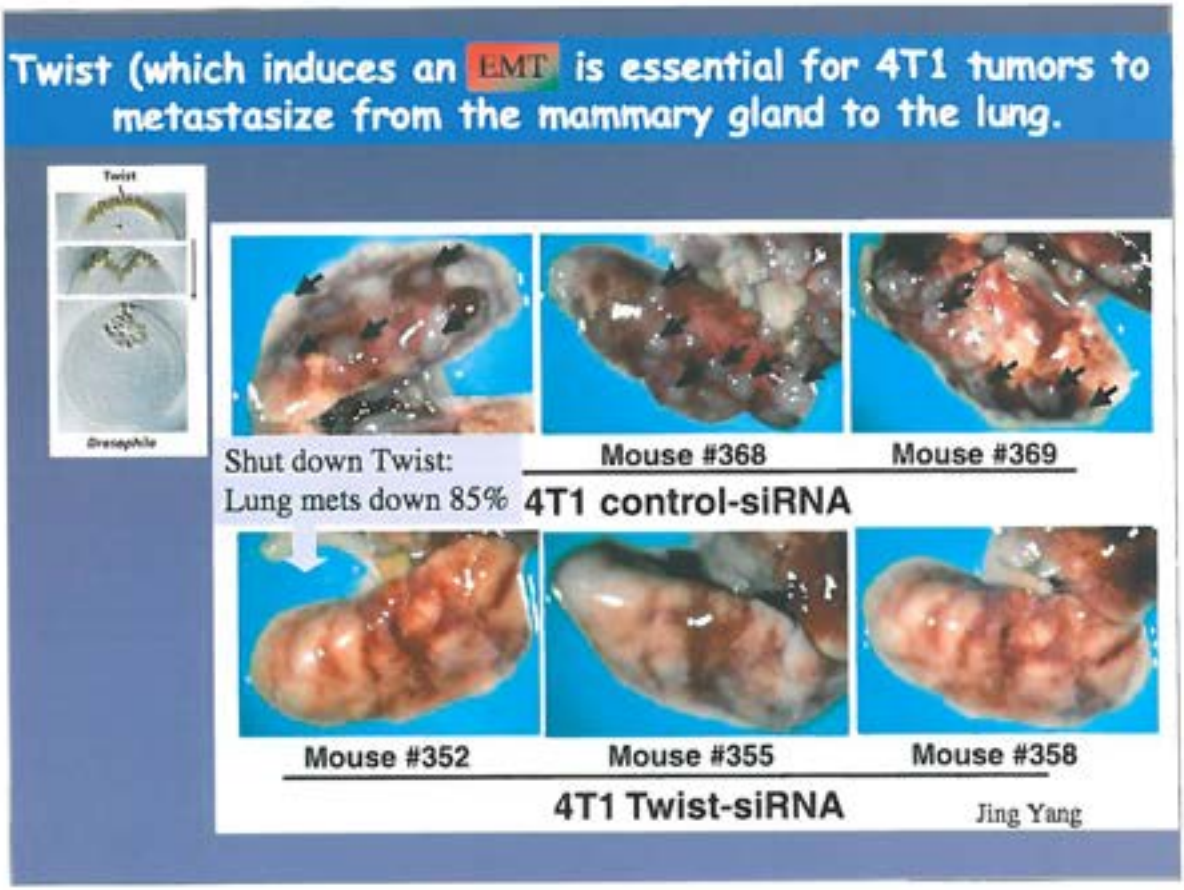
Elizabeth Hay

Growing up, Betty had a deep interest in what makes animals click and, when she took the superb freshman course at Smith in anatomy and physiology, taught by S. Meryl Rose, she was hooked. By sophomore year, she was doing regeneration research with Rose, who became her mentor for the next 6 years and persuaded her to get an MD instead of a PhD degree, because he saw females with PhDs stuck for life teaching in a girls' school like Smith.

23.


Model: Cancer cells appropriate early embryogenic programs in order to acquire traits of high-grade malignancy
EMT
EMT
human vimentin-positive
mesenchymal
epithelial
Courtesy of G Chen

24.


Twist (which induces an EMT is essential for 4T1 tumors to metastasize from the mammary gland to the lung.
Twist
Drosophila
Shut down Twist:
Lung mets down 85%
Mouse #368
Mouse #369
4T1 control-siRNA
Mouse #352
Mouse #355
Mouse #358
4T1 Twist-siRNA
Jing Yang

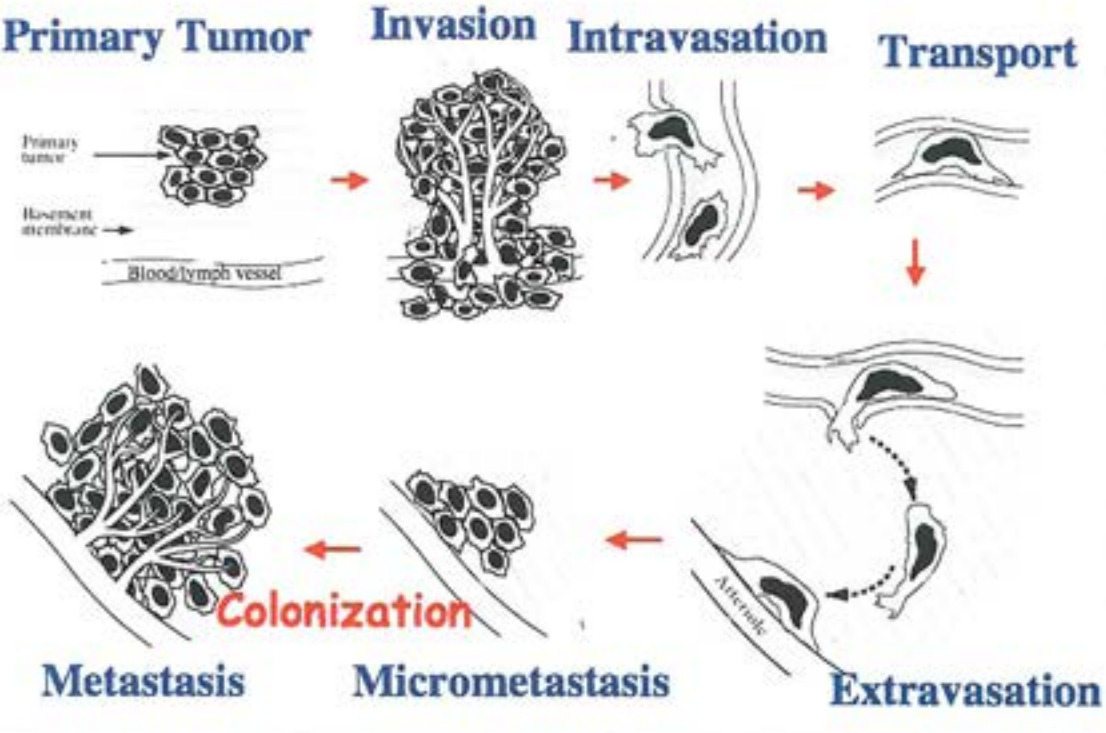
25.

Given the ability of Twist to program an EMT

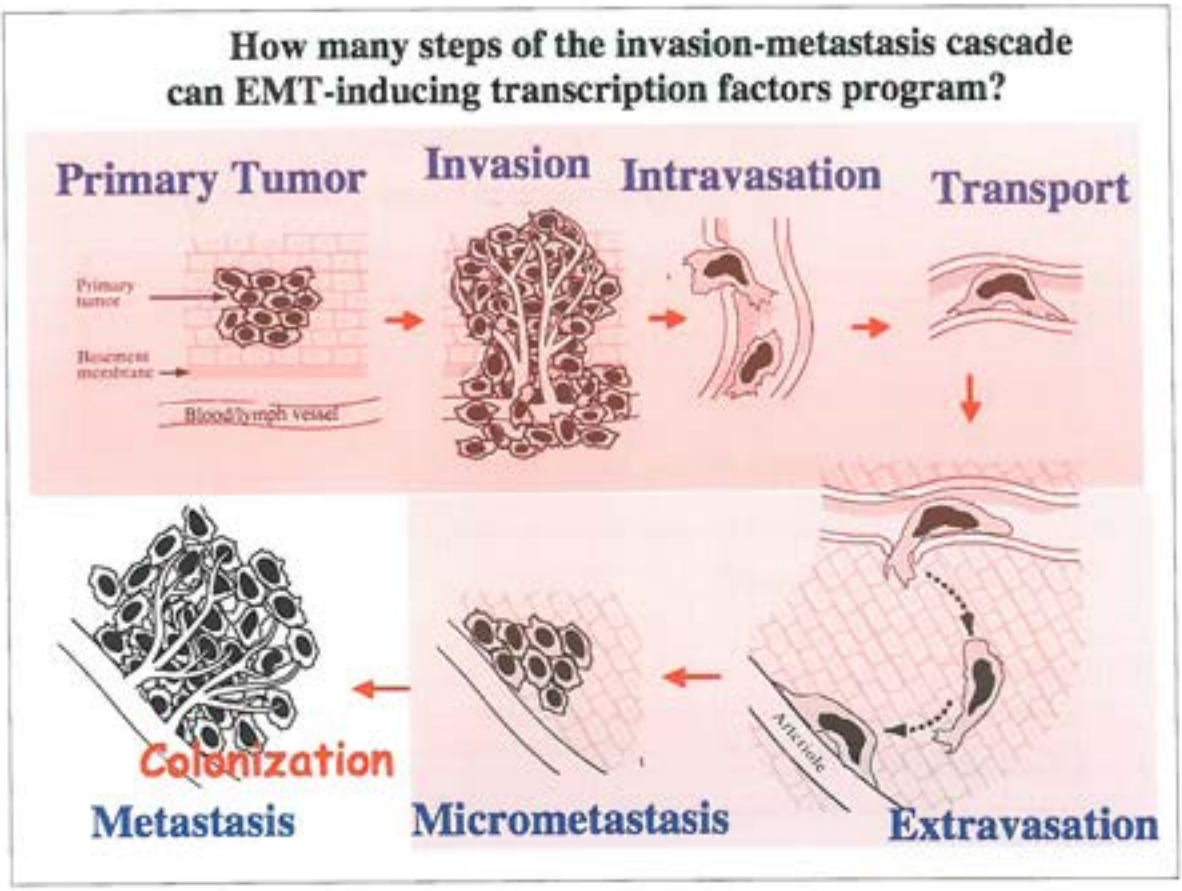
and the role of Twist in metastasis

26.

How many steps of the invasion-metastasis cascade can the EMT Twist enable?



27.



28.

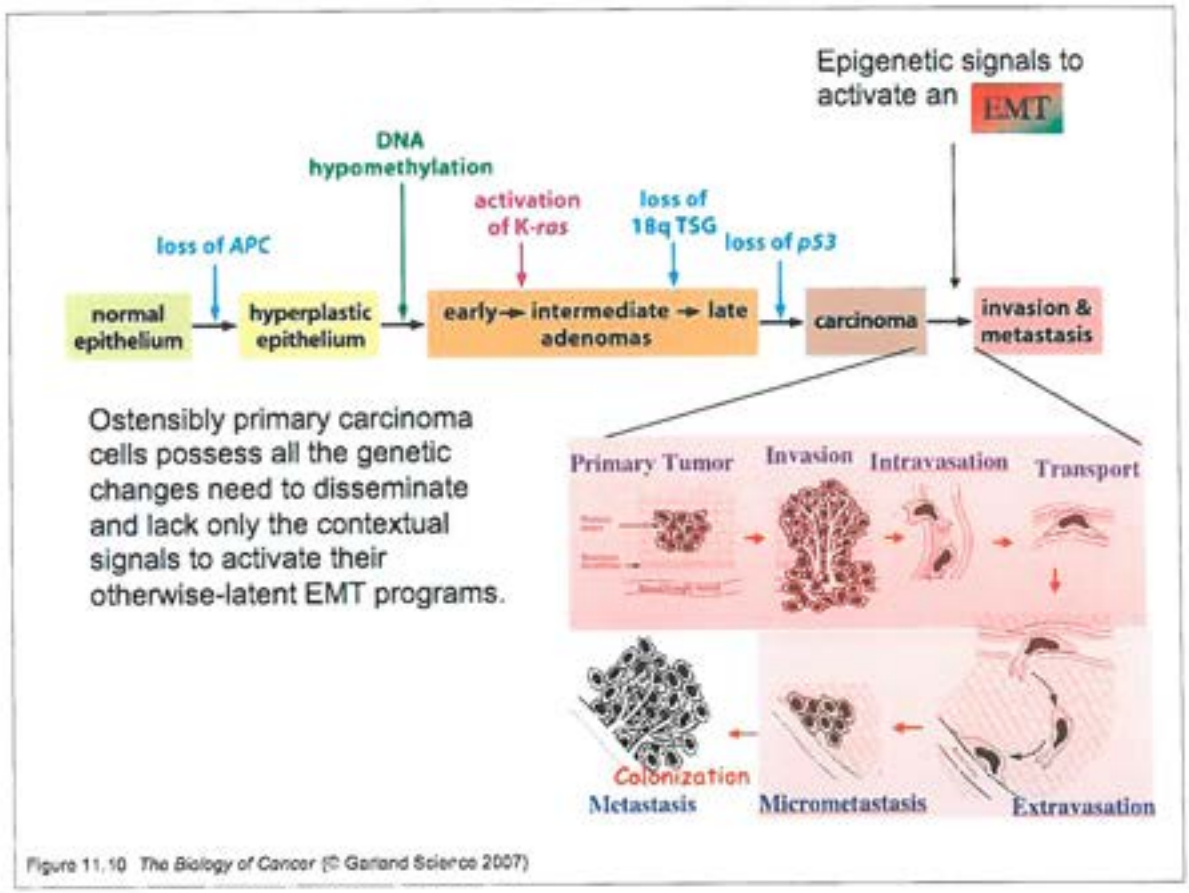


Figure 11.10 *The Biology of Cancer* (© Garland Science 2007)

29.

Switching topics: A distinct attribute of cancer cell populations

Tumor-initiating capability

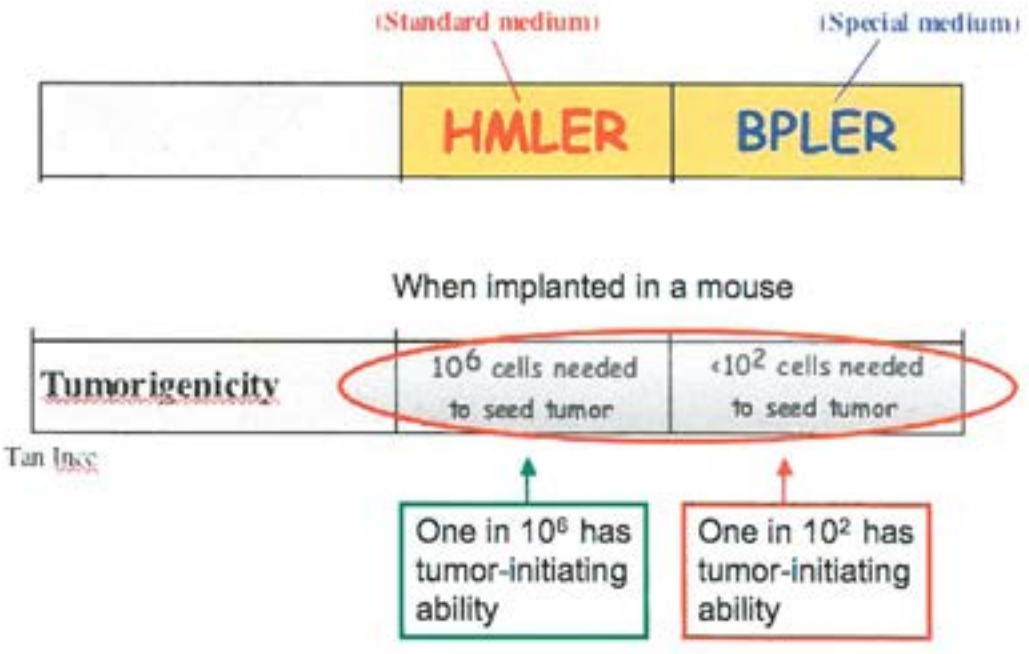
(Two types of breast cancer cells)

	HMLER	BPLER
Ductal differentiation	No	Yes
Invasion	Minimal	Yes
Stromal recruitment	Minimal	Yes
Metastasis	No	Yes
Tumorigenicity	10^6 cells needed to seed tumor	$<10^2$ cells needed to seed tumor

Tan Ince

30.

To return to another notion:



31.

Cell of Origin Determines Tumor Morphology and Behavior

(Standard medium) (Special medium)

Du	UMLER	PPLER
In		
Stromal recruitment	Minimal	Yes
Metastasis	No	Yes
Tumorigenicity	10^6 cells needed to seed tumor	$<10^2$ cells needed to seed tumor

Hence, there must be at least two distinct types of cells in these tumors:
those that can initiate tumors and those that cannot

Tan Ince

32.

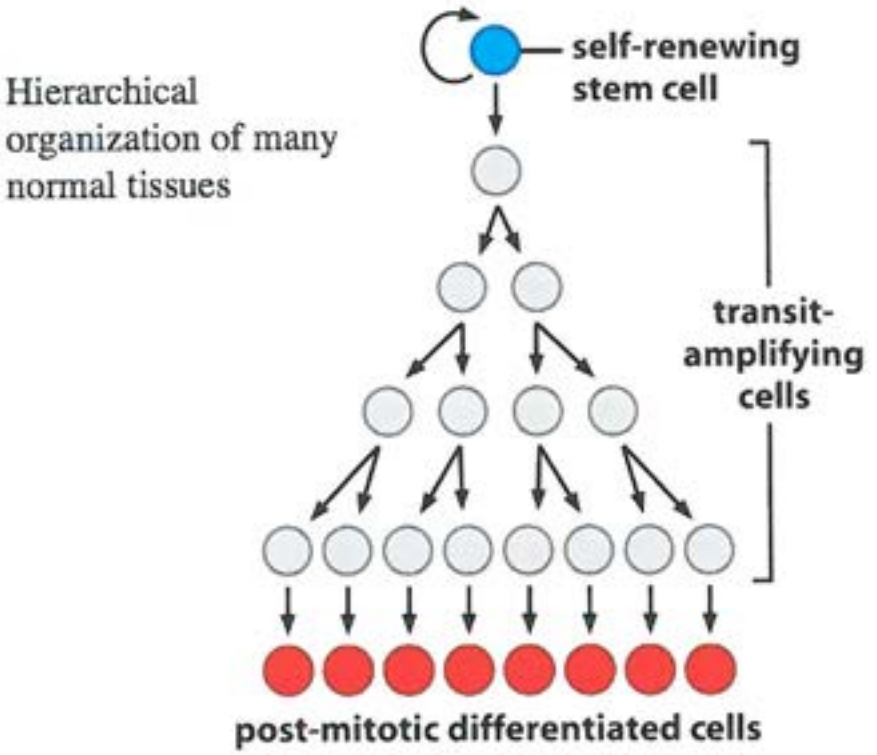


Figure 11.16b *The Biology of Cancer* (© Garland Science 2007)

33.

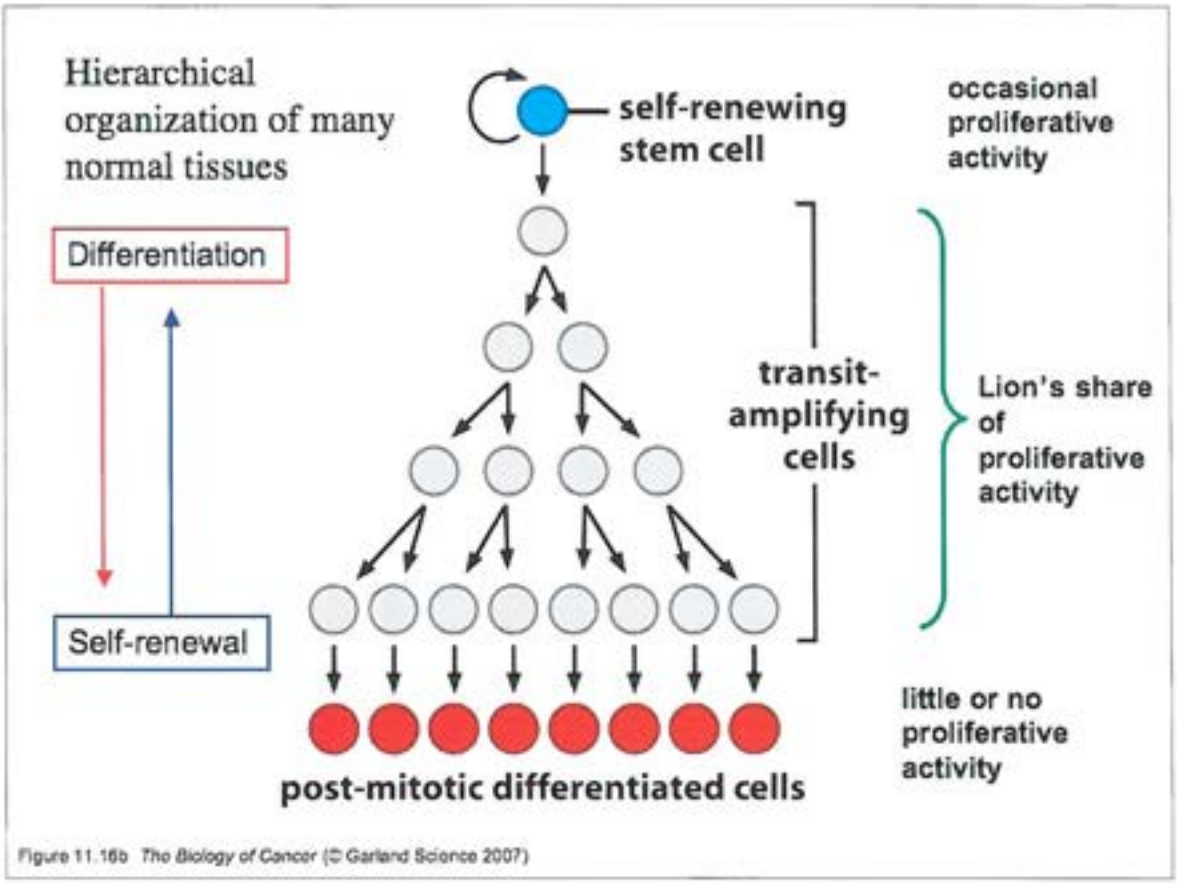


Figure 11.16b *The Biology of Cancer* (© Garland Science 2007)

34.

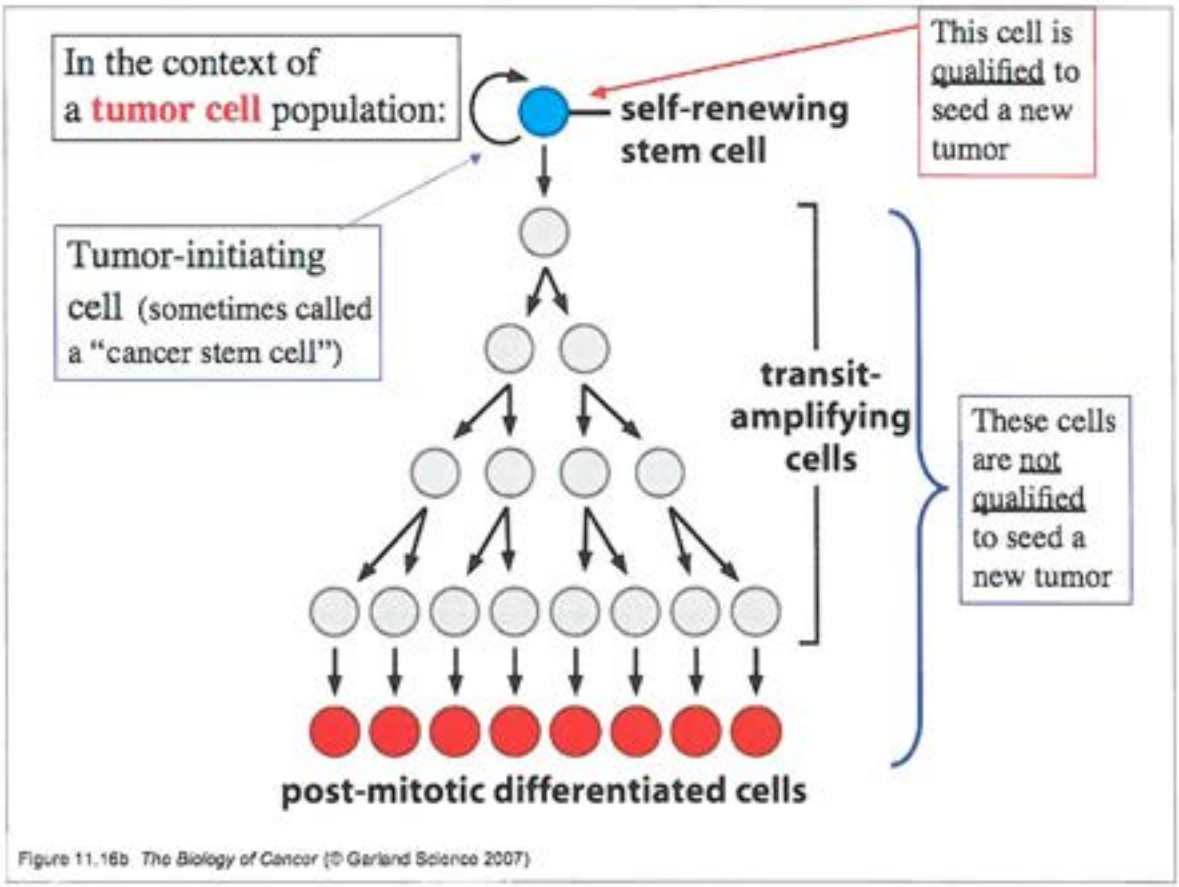


Figure 11.16b *The Biology of Cancer* (© Garland Science 2007)

35.

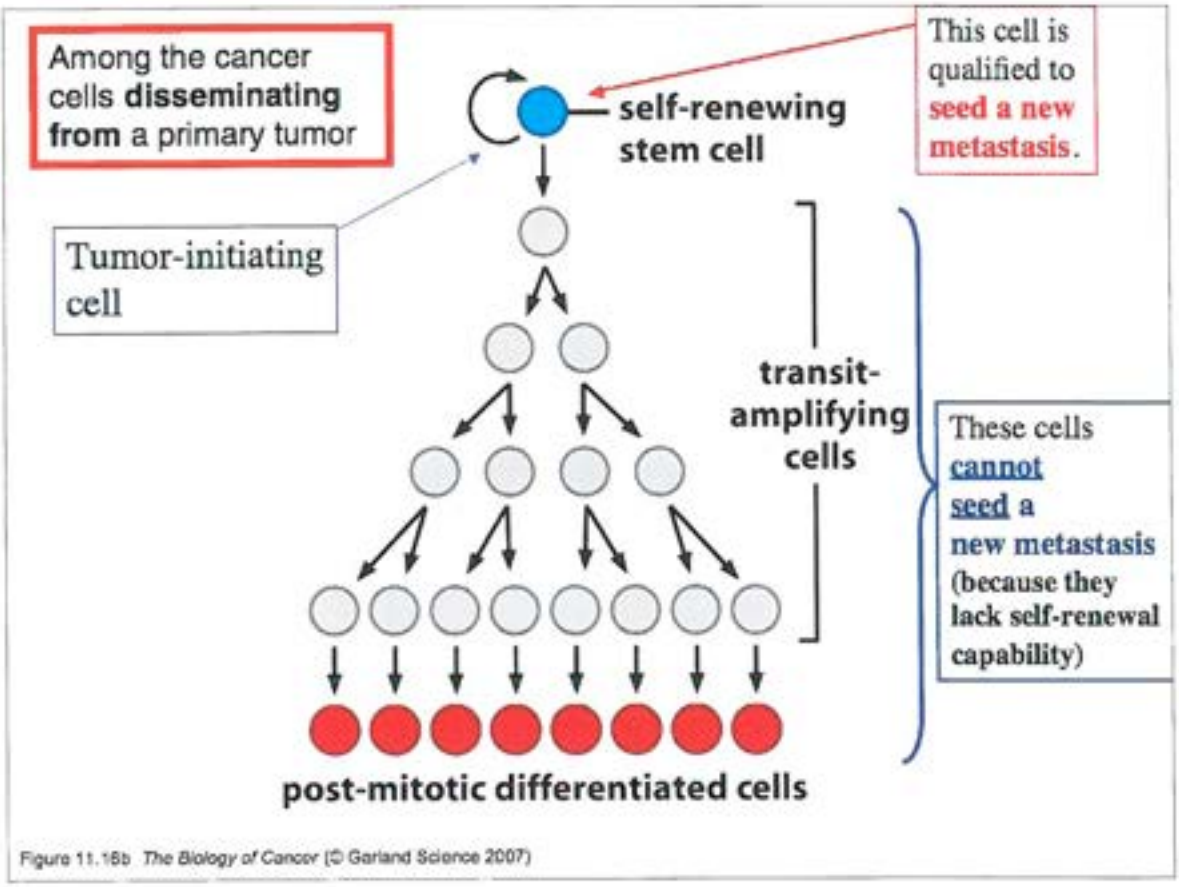


Figure 11.15b *The Biology of Cancer* (© Garland Science 2007)

36.

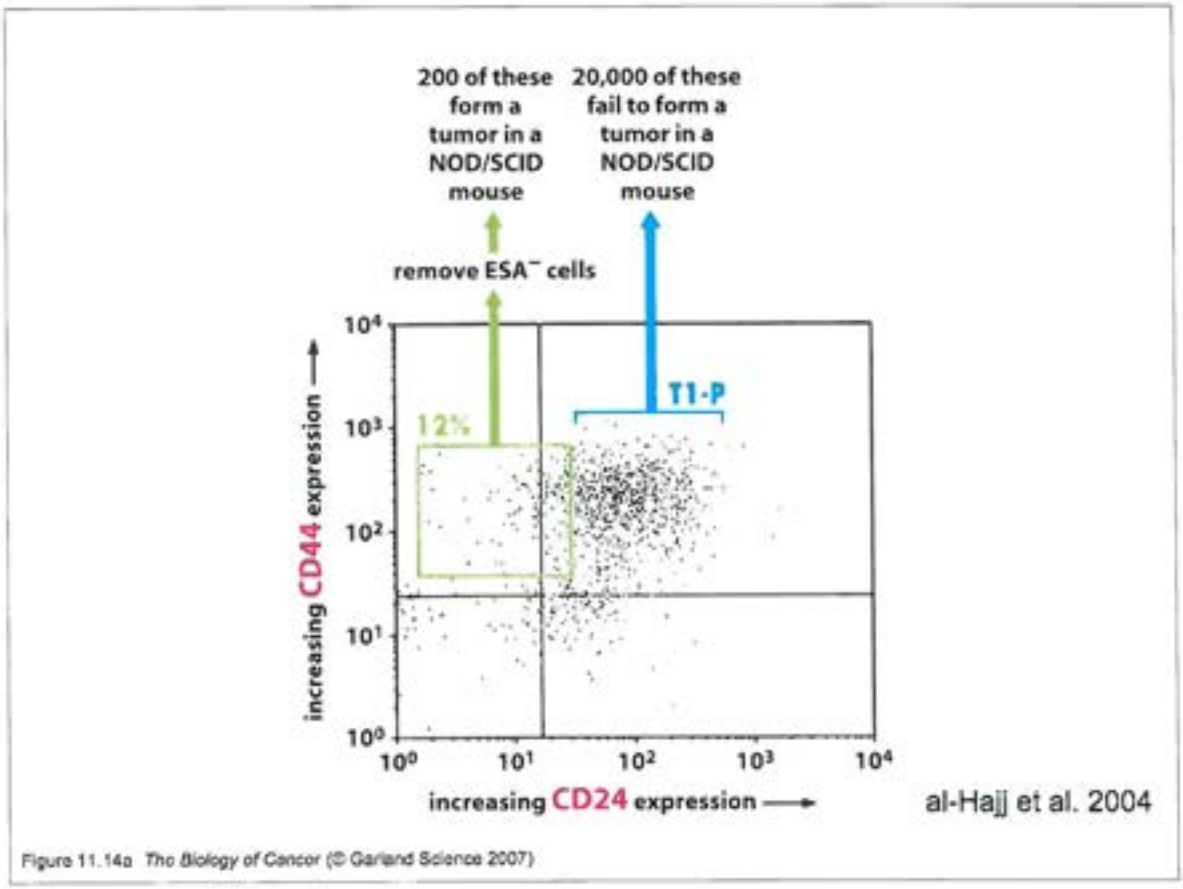
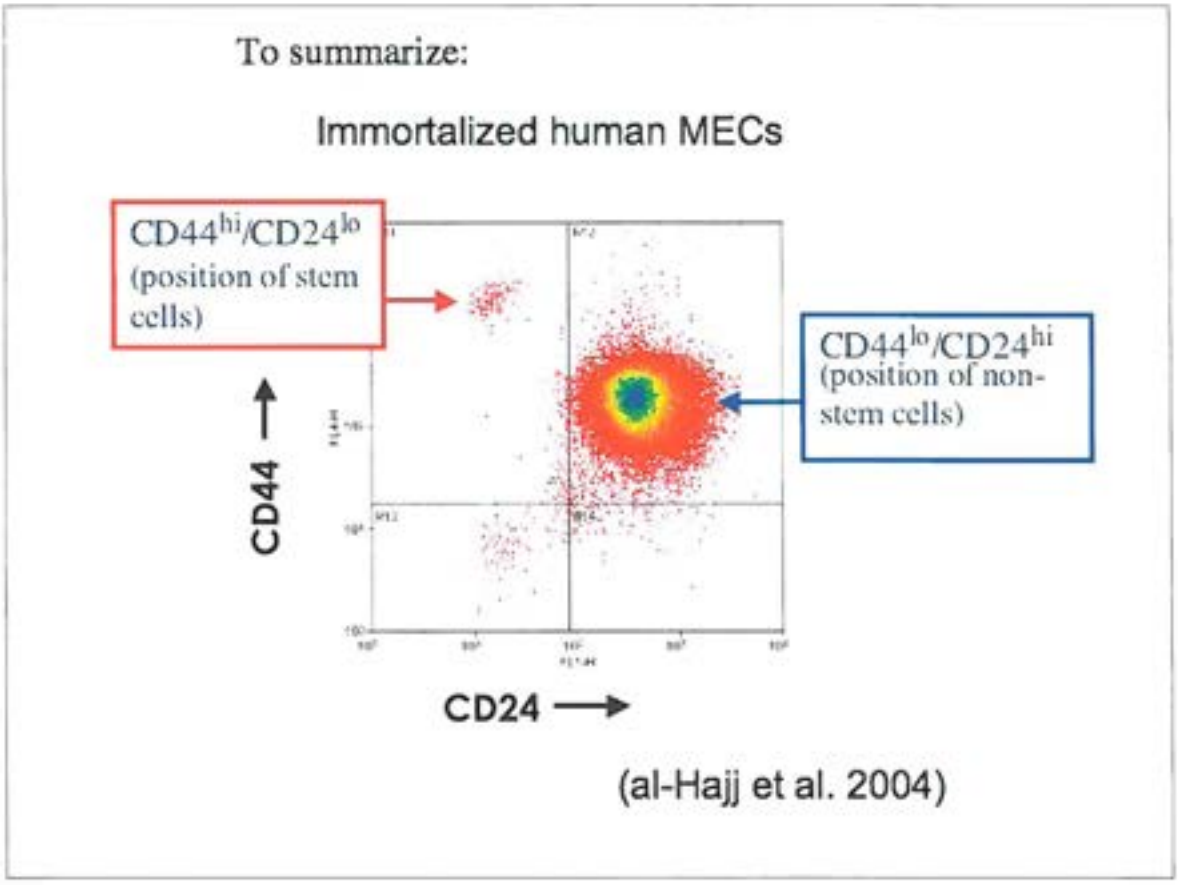
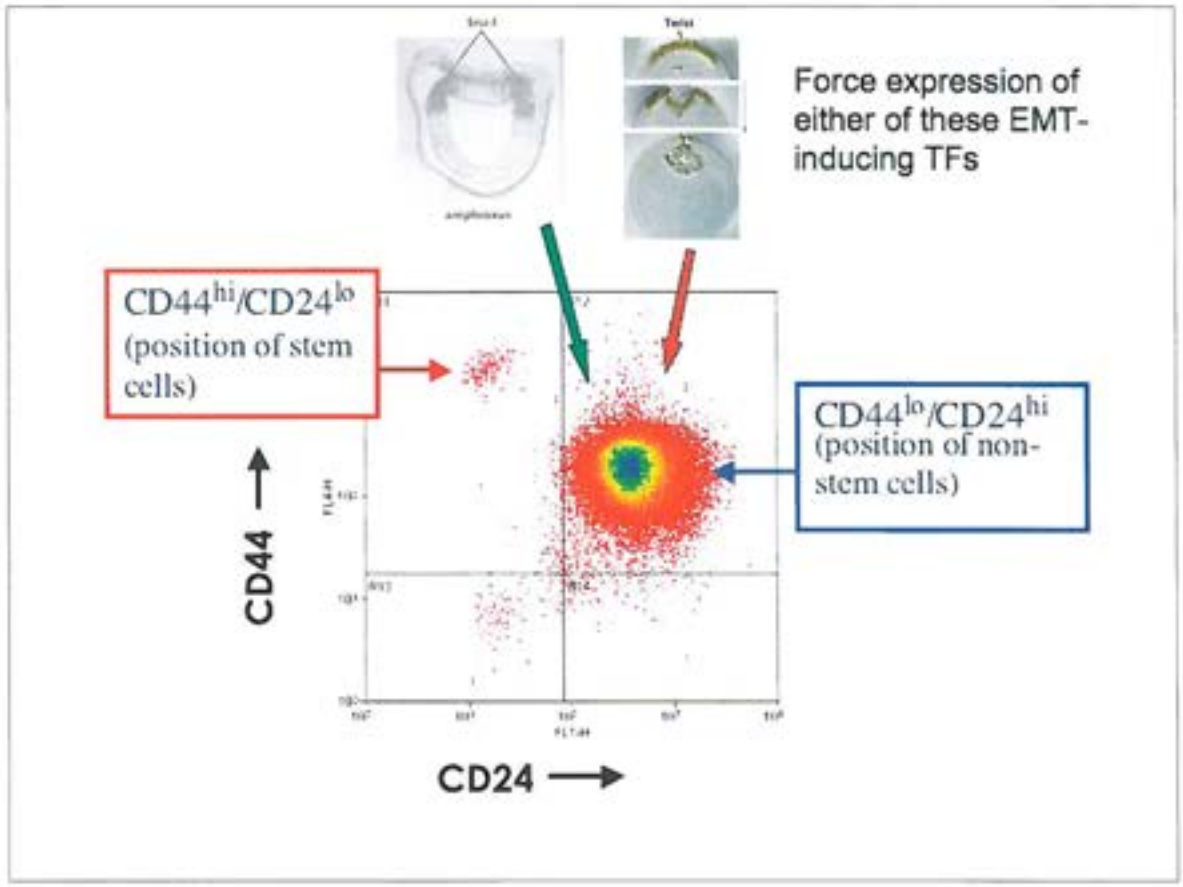


Figure 11.14a *The Biology of Cancer* (© Garland Science 2007)

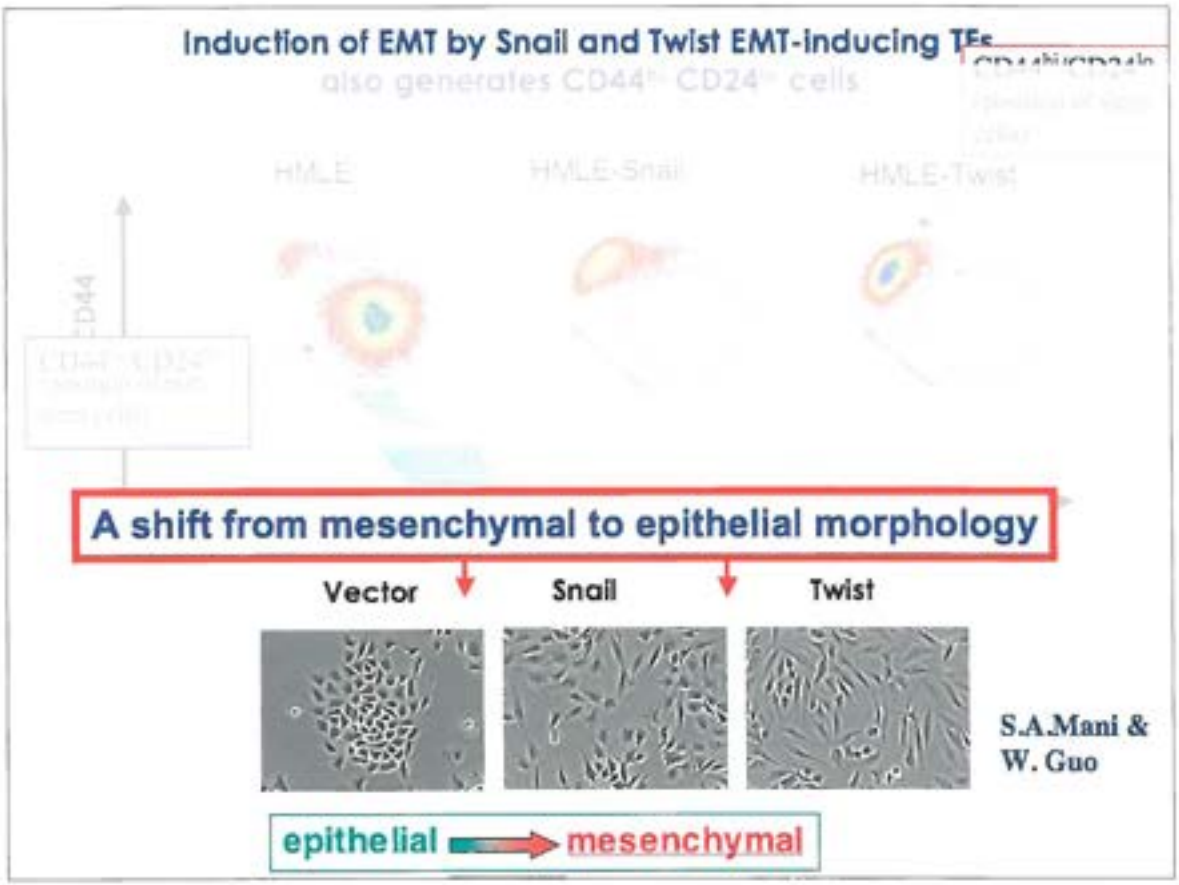
37.


To summarize:
Immortalized human MECs
CD44hi/CD24lo (position of stem cells)
CD44lo/CD24hi (position of non-stem cells)
CD44
CD24
(al-Hajj et al. 2004)

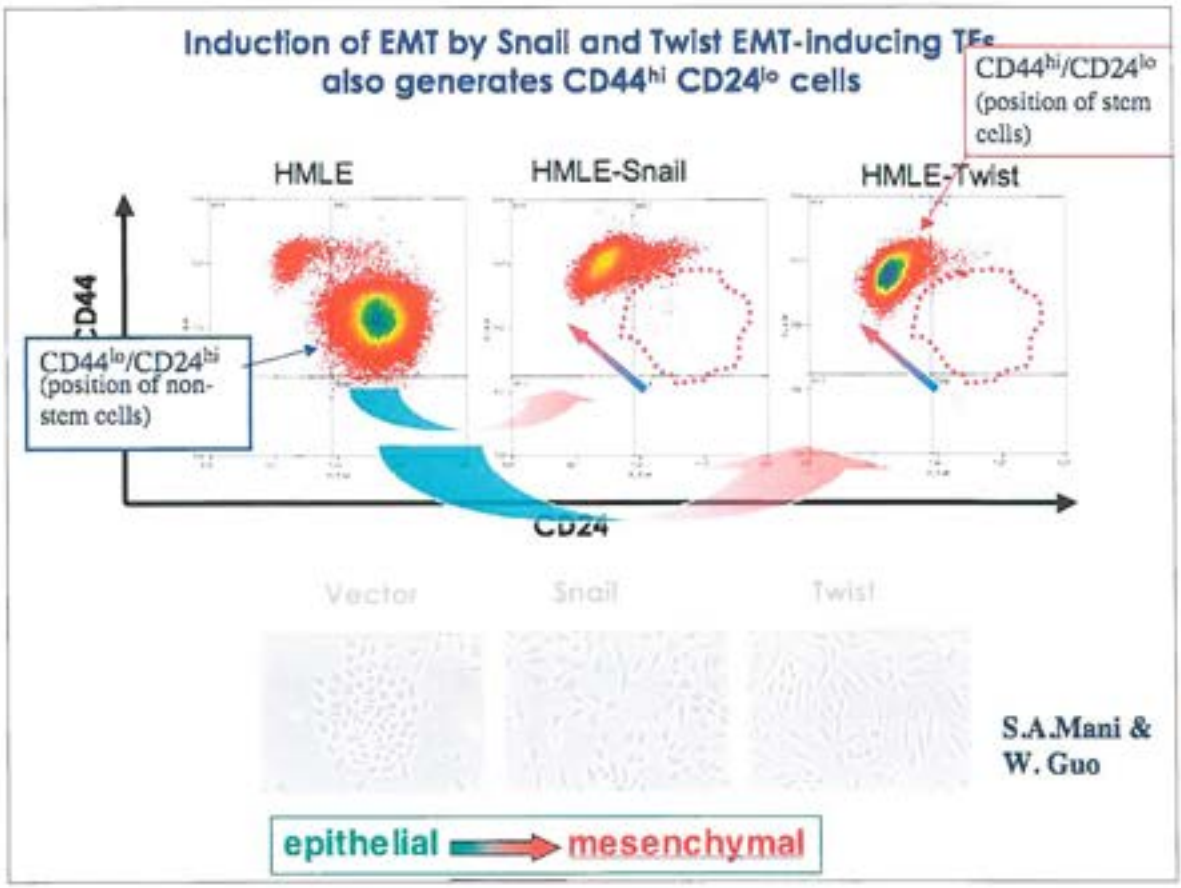
38.


Force expression of either of these EMT-inducing TFs
CD44hi/CD24lo (position of stem cells)
CD44lo/CD24hi (position of non-stem cells)
CD44
CD24

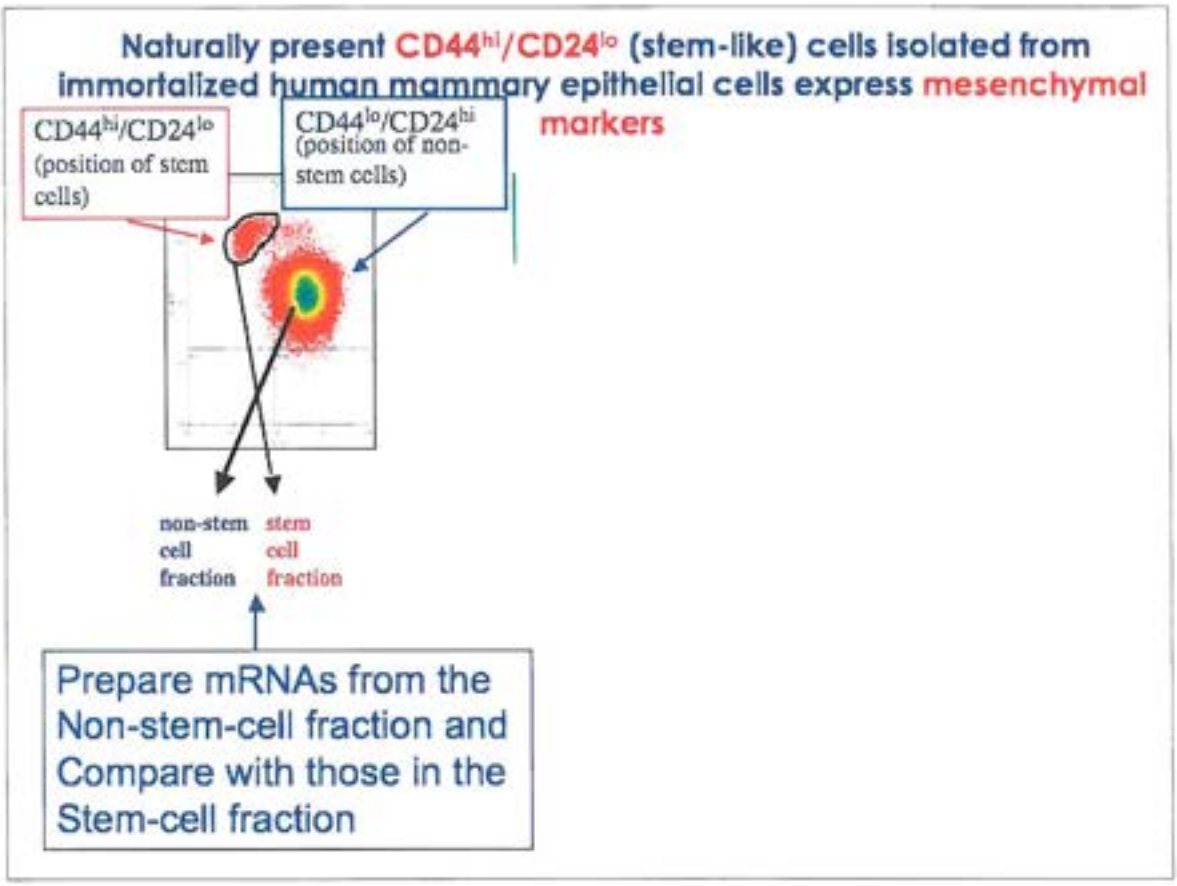
39.


Induction of EMT by Snail and Twist EMT-inducing TFs
A shift from mesenchymal to epithelial morphology
Vector
Snail
Twist
S.A.Mani &
W. Guo
epithelial
mesenchymal

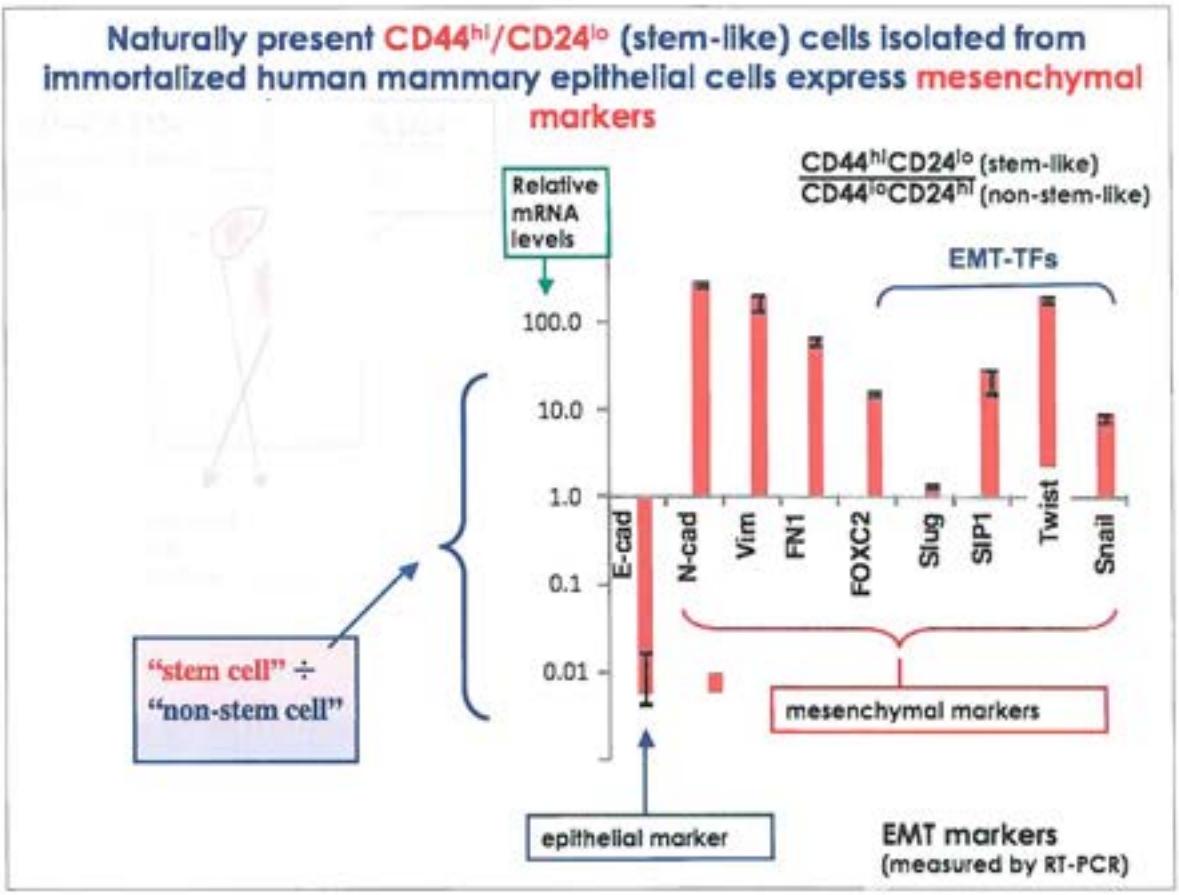
40.


Induction of EMT by Snail and Twist EMT-inducing TFs
also generates CD44hi CD24lo cells
CD44hi/CD24lo
(position of stem
cells)
HMLE
HMLE-Snail
HMLE-Twist
CD44
CD44lo/CD24hi
(position of non-
stem cells)
CD24
Vector
Snail
Twist
S.A.Mani &
W. Guo
epithelial
mesenchymal

41.


Naturally present CD44hi/CD24lo (stem-like) cells isolated from immortalized human mammary epithelial cells express mesenchymal markers
CD44hi/CD24lo (position of stem cells)
CD44lo/CD24hi (position of non-stem cells)
non-stem cell fraction
stem cell fraction
Prepare mRNAs from the Non-stem-cell fraction and Compare with those in the Stem-cell fraction

42.


Naturally present CD44hi/CD24lo (stem-like) cells isolated from immortalized human mammary epithelial cells express mesenchymal markers
CD44hiCD24lo (stem-like) / CD44loCD24hi (non-stem-like)
Relative mRNA levels
EMT-TFs
100.0
10.0
1.0
0.1
0.01
E-cad
N-cad
Vim
FN1
FOXC2
Slug
SIP1
Twist
Snail
"stem cell" ÷ "non-stem cell"
mesenchymal markers
epithelial marker
EMT markers (measured by RT-PCR)

43.

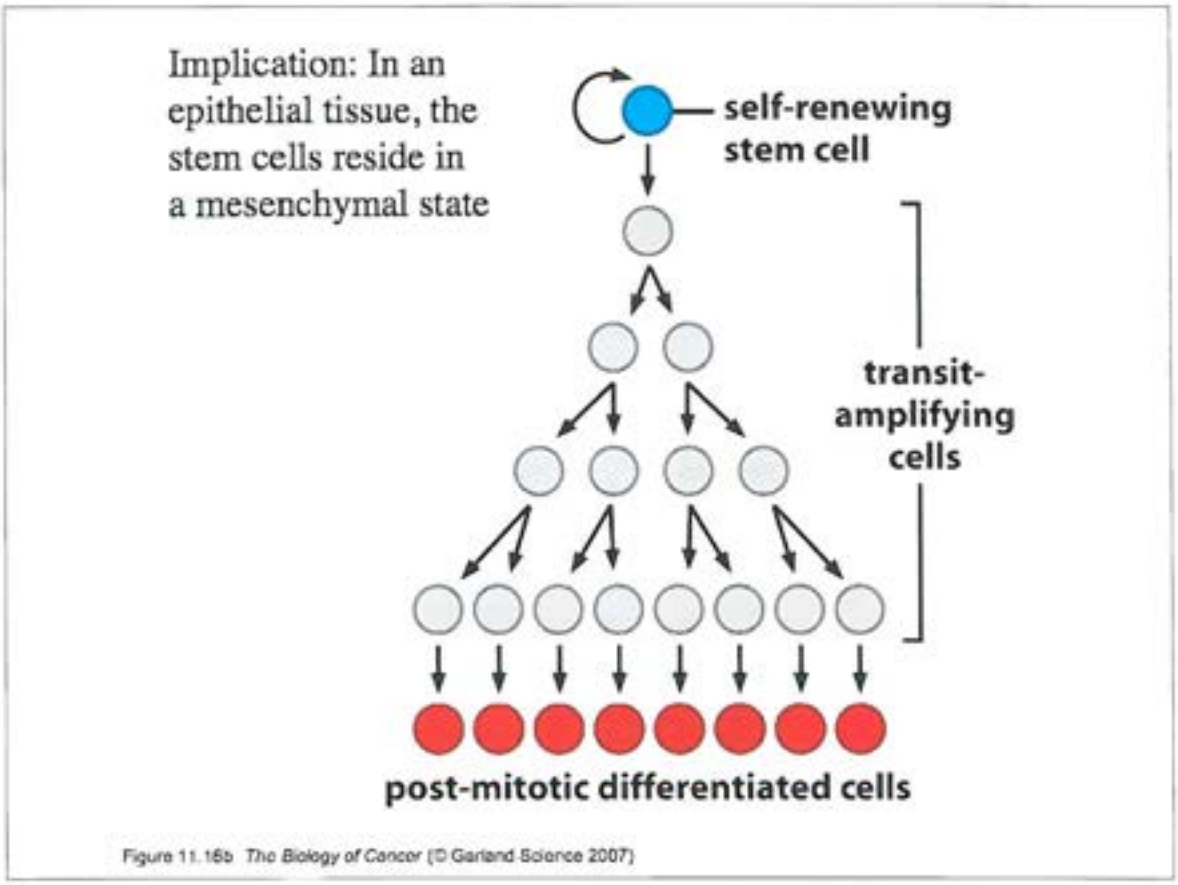
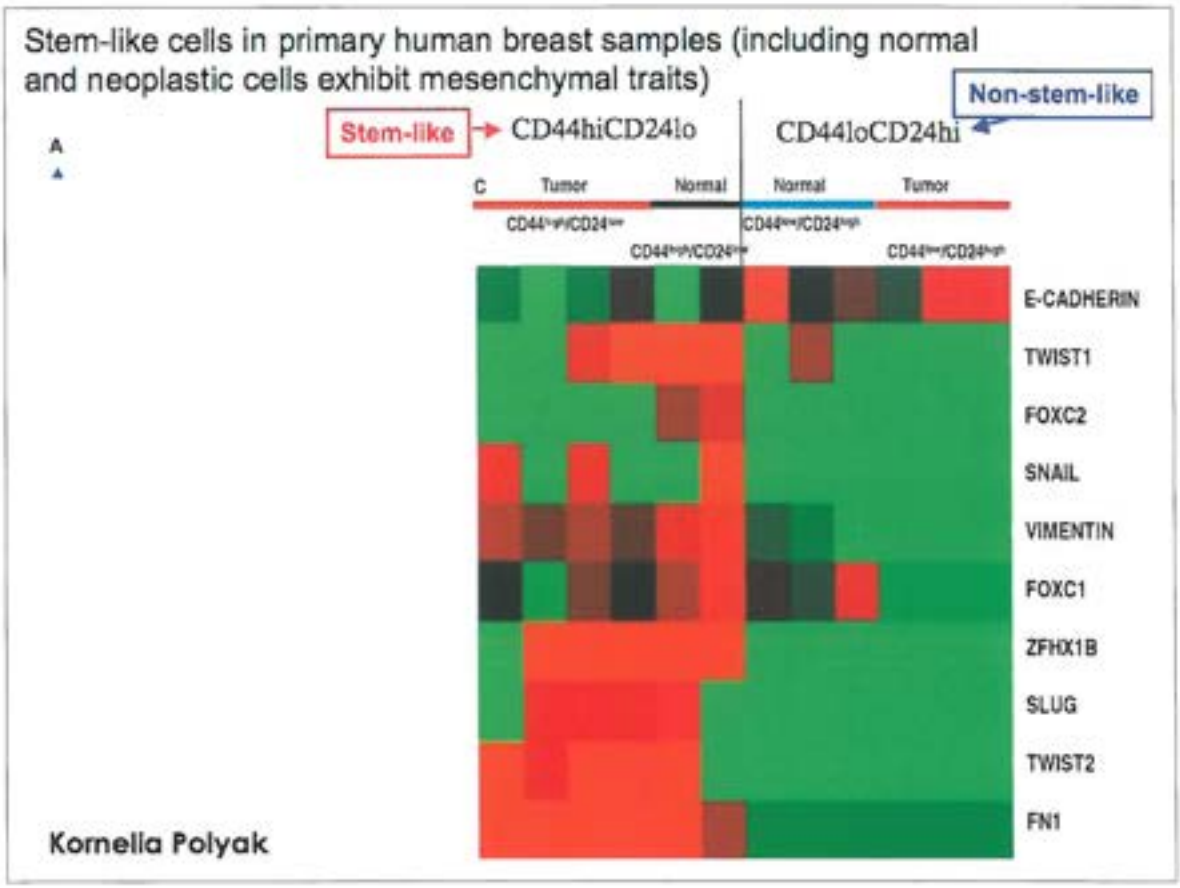


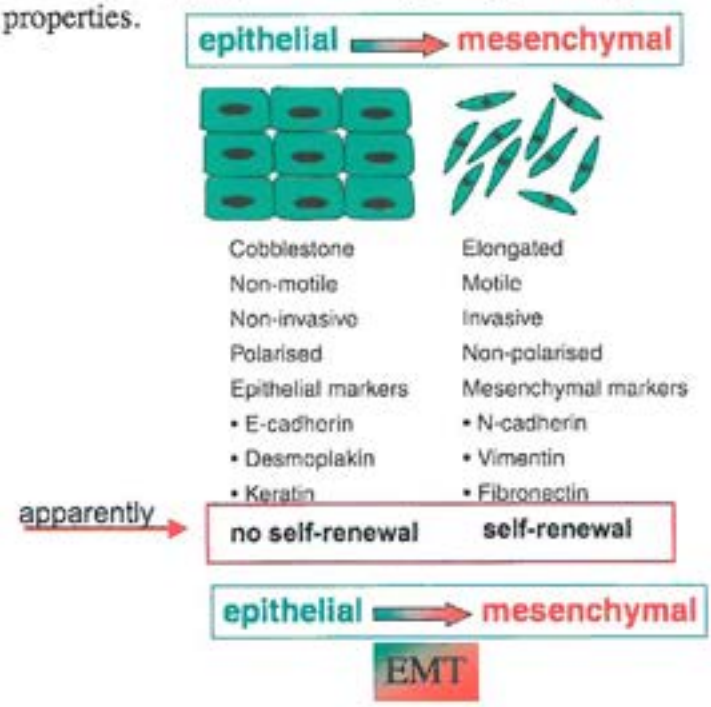
Figure 11.16b *The Biology of Cancer* (© Garland Science 2007)

44.



45.

The epithelial-mesenchymal transition (EMT) is a complex, multi-faceted program involving multiple changes in cell properties.



46.

EMT and cancer progression

1. Normal cells induced to undergo an EMT acquire stem-cell properties.
   a. $CD44^{hi}CD24^{lo}$ antigenic phenotype
   b. Ability to form mammospheres indefinitely
2. Same outcome with cancer cells
3. Epithelial cells in culture that are naturally $CD44^{hi}CD24^{lo}$ show mesenchymal morphology; same is true of cells from reduction mammoplasty
4. Transient exposure to Snail or Twist causes descendants of exposed cells to form mammospheres indefinitely (as gauged by serial passage).

Hence, induction of an EMT should allow cancer cells in a primary tumor
a. To disseminate
b. To become self-renewing

47.

But can one produce more compelling **biological proofs** that EMTs generate epithelial stem cells?

EMT and cancer progression

1. Normal cells induced to undergo an EMT acquire stem-cell properties
   a. $CD44^{high}CD24^{low}$ antigenic phenotype
   b. Ability to form mammospheres indefinitely
2. Same outcome with cancer cells
3. Epithelial cells in culture that are naturally $CD44^{high}CD24^{low}$ show mesenchymal morphology; same is true of cells from reduction mammoplasty
4. Transient exposure to Snail or Twist causes descendants of exposed cells to form mammospheres indefinitely (as gauged by serial passage).

Hence, induction of an EMT allows cancer cells
a. To disseminate
b. To become self-renewing

48.

A robust and stringent *in vivo* mammary stem cell assay

Cleared mammary fat pad transplantation

(Remove endogenous mammary epithelial cells from adolescent mammary fat pad)

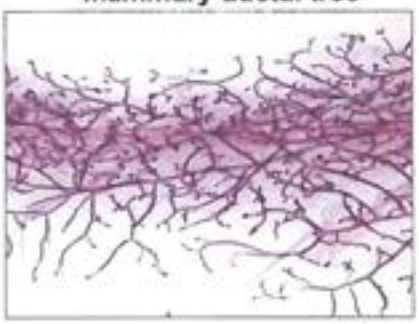
Ability to regenerate entire mammary ductal tree evidence of mammary epithelial stem cells

remove mammary epithelial rudiment

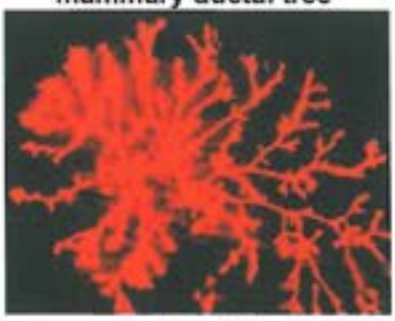
transplant mammary epithelial cells

regeneration of mammary ductal tree

endogenous mammary ductal tree

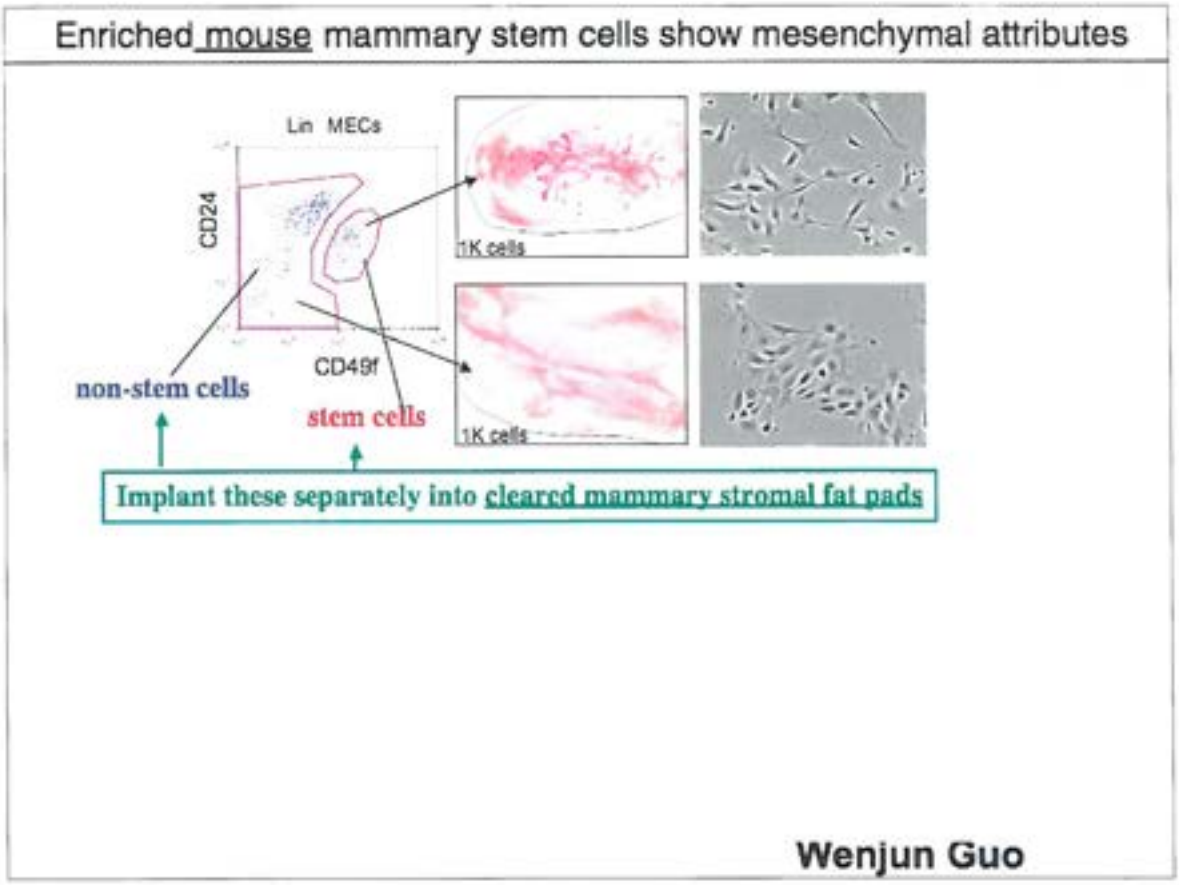


reconstituted mammary ductal tree

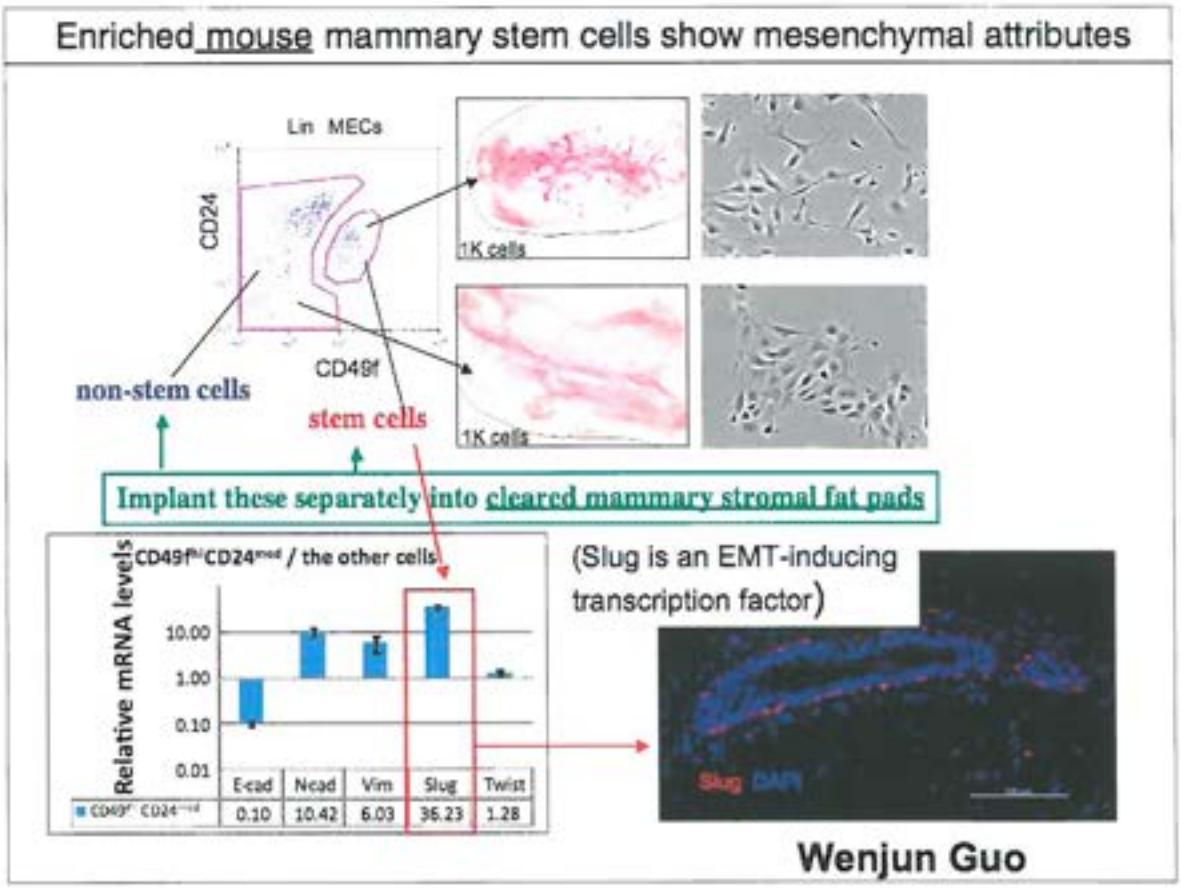


Wenjun Guo

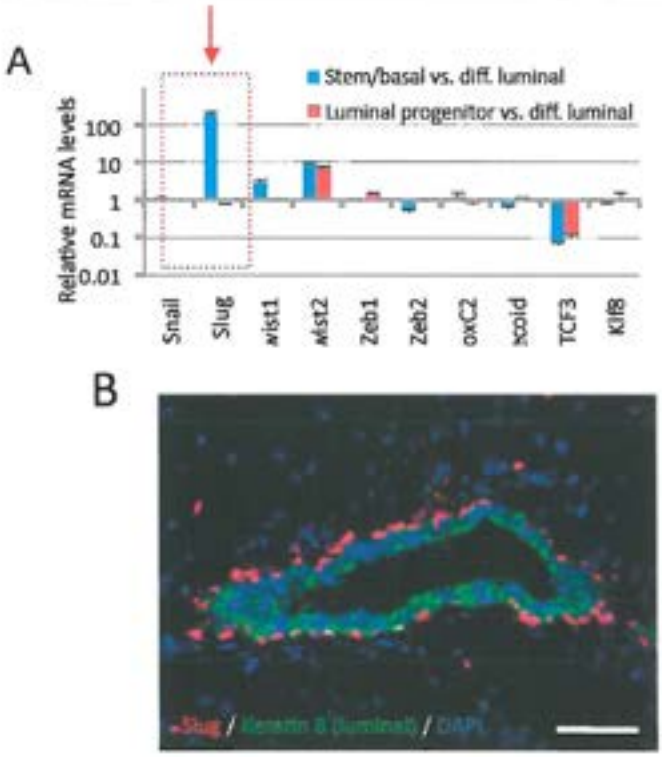
49.


Enriched mouse mammary stem cells show mesenchymal attributes
Lin MECs
CD24
CD49f
non-stem cells
stem cells
1K cells
1K cells
Implant these separately into cleared mammary stromal fat pads
Wenjun Guo

50.

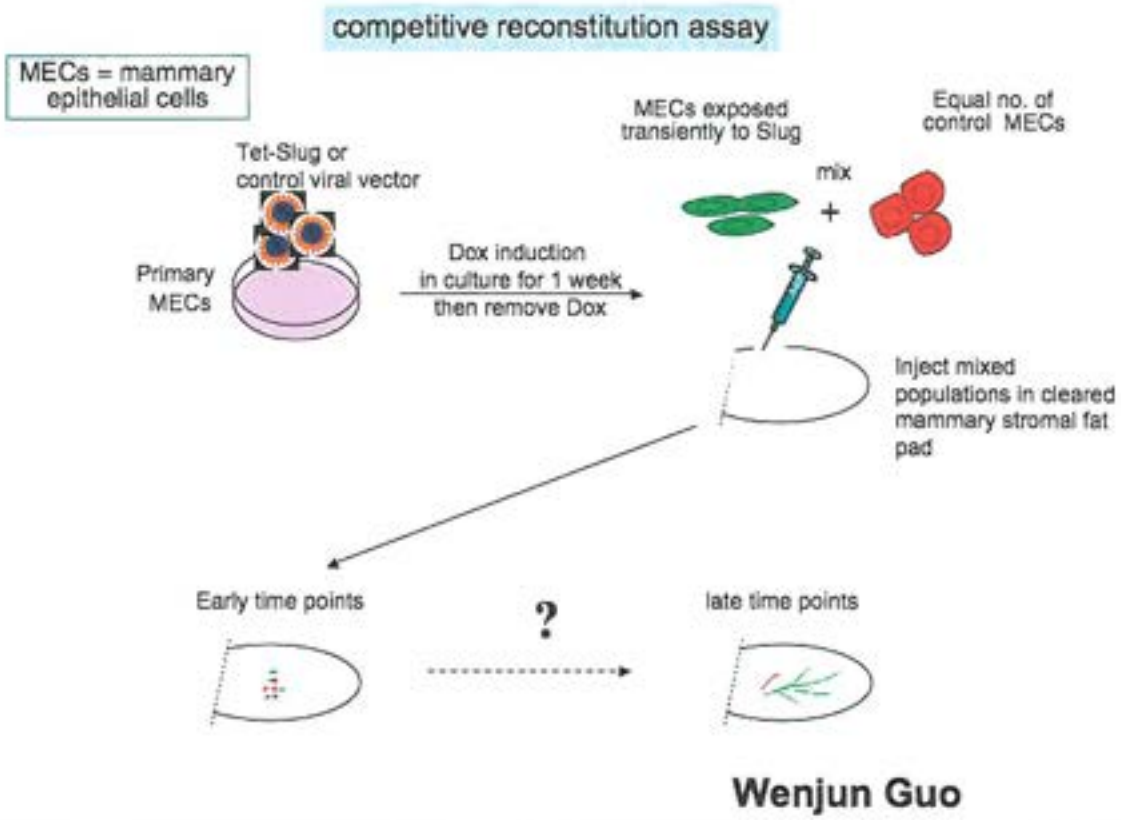

Enriched mouse mammary stem cells show mesenchymal attributes
Lin MECs
CD24
CD49f
non-stem cells
stem cells
1K cells
1K cells
Implant these separately into cleared mammary stromal fat pads
CD49f+CD24mod / the other cells
Relative mRNA levels
10.00
1.00
0.10
0.01
E-cad
Ncad
Vim
Slug
Twist
0.10
10.42
6.03
36.23
1.28
(Slug is an EMT-inducing transcription factor)
Slug DAPI
Wenjun Guo

51.



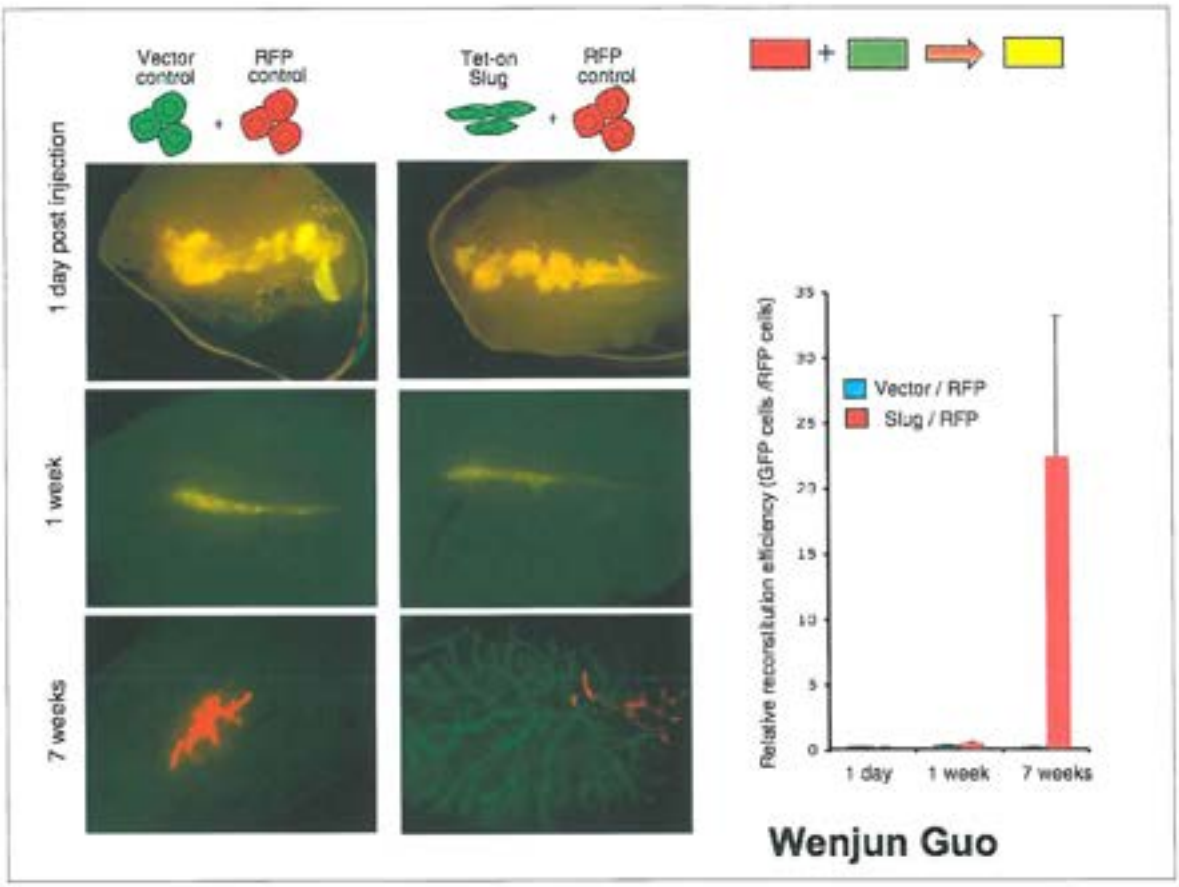
Wenjun Guo

52.

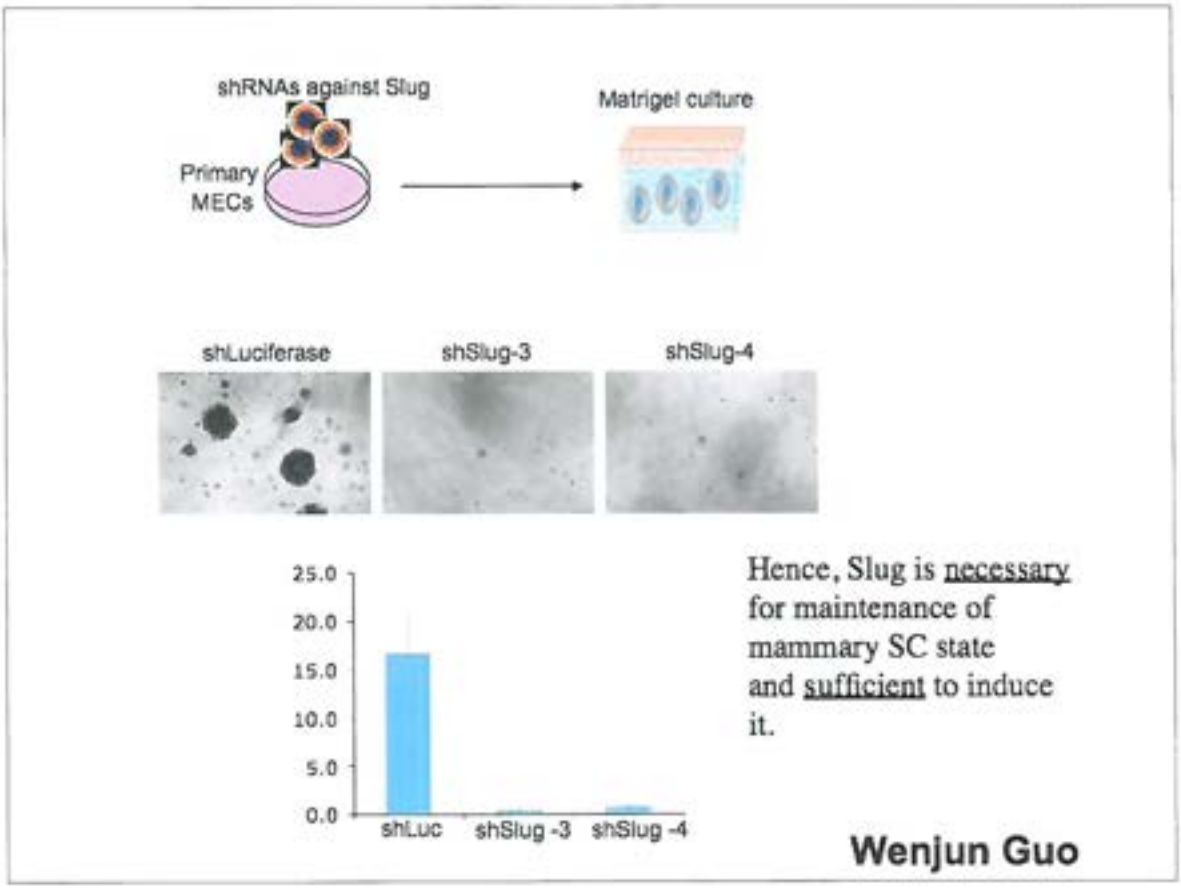


Wenjun Guo

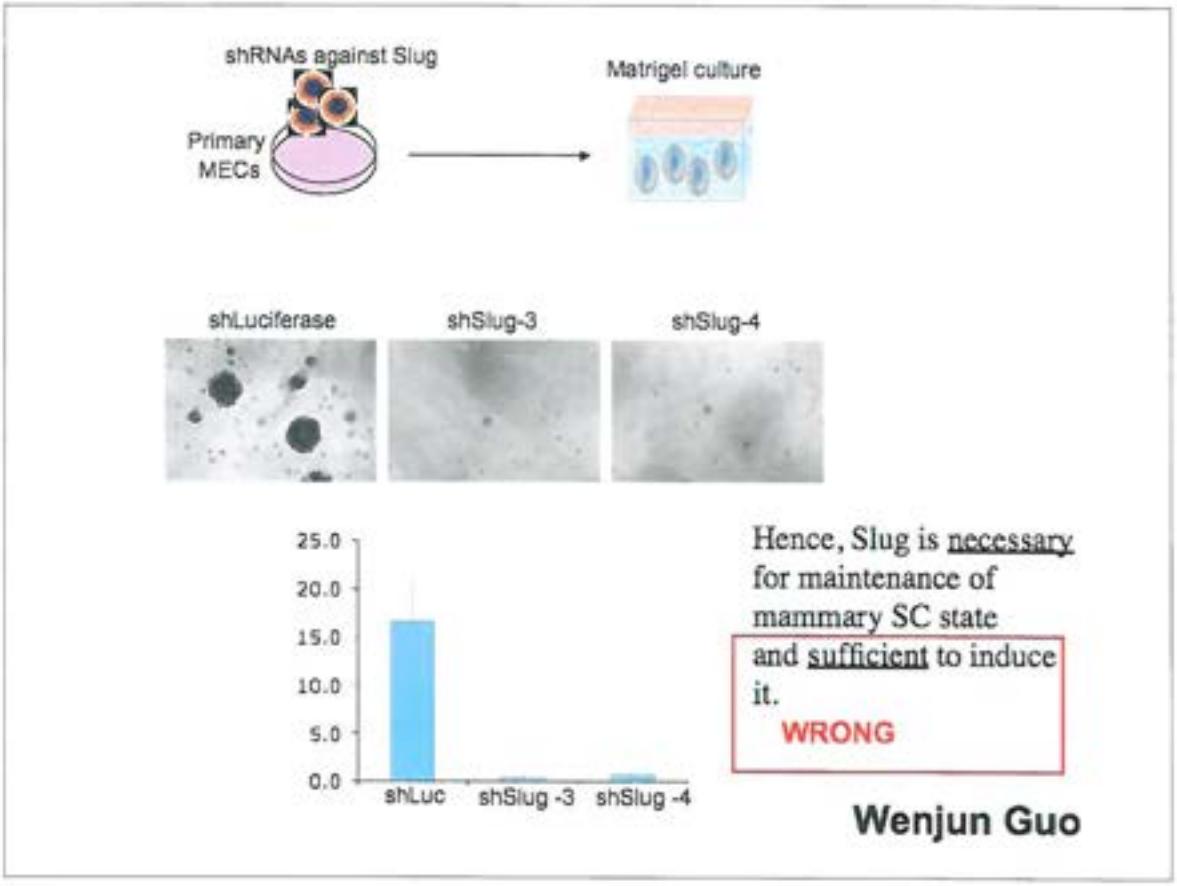
53.


Vector control
RFP control
Tet-on Slug
RFP control
1 day post injection
1 week
7 weeks
Vector / RFP
Slug / RFP
Relative reconstitution efficiency (GFP cells /RFP cells)
1 day
1 week
7 weeks
Wenjun Guo

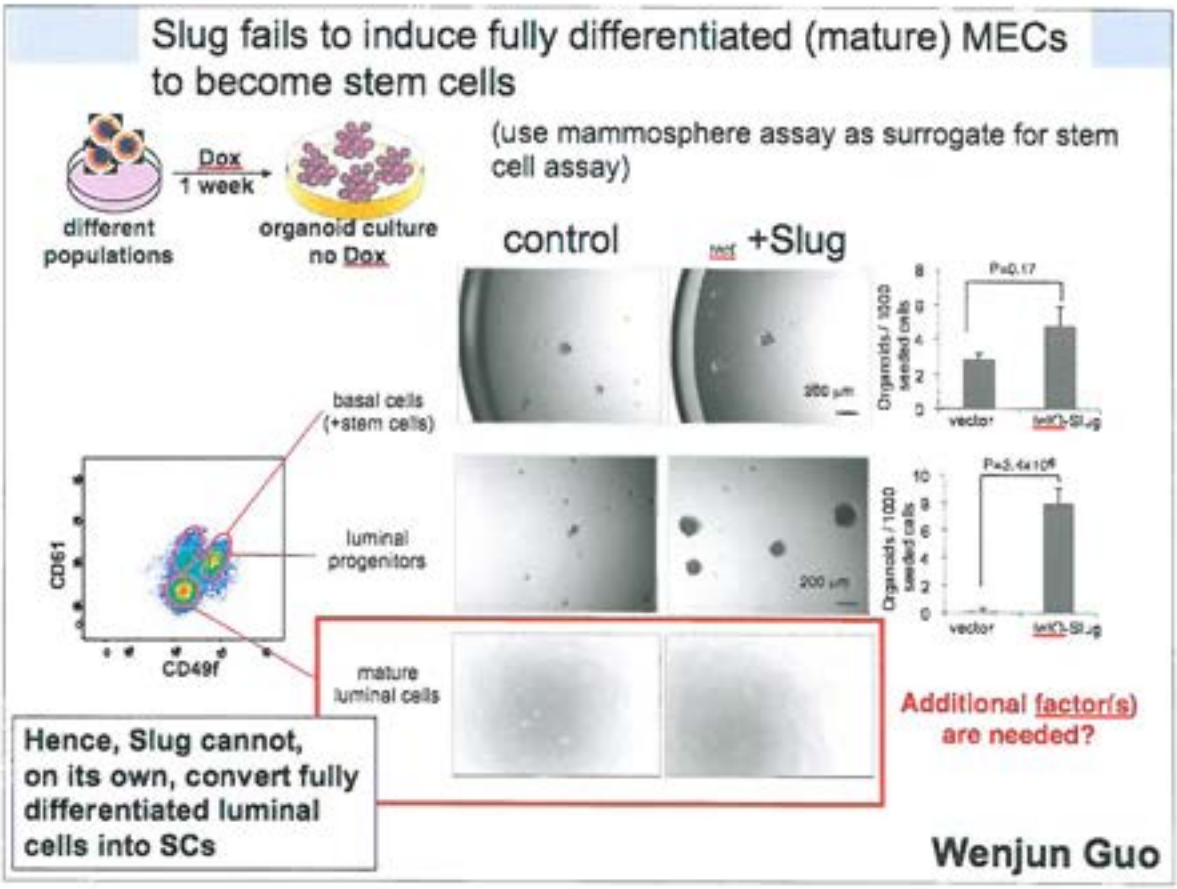
54.


shRNAs against Slug
Primary MECs
Matrigel culture
shLuciferase
shSlug-3
shSlug-4
25.0
20.0
15.0
10.0
5.0
0.0
shLuc
shSlug -3
shSlug -4
Hence, Slug is necessary for maintenance of mammary SC state and sufficient to induce it.
Wenjun Guo

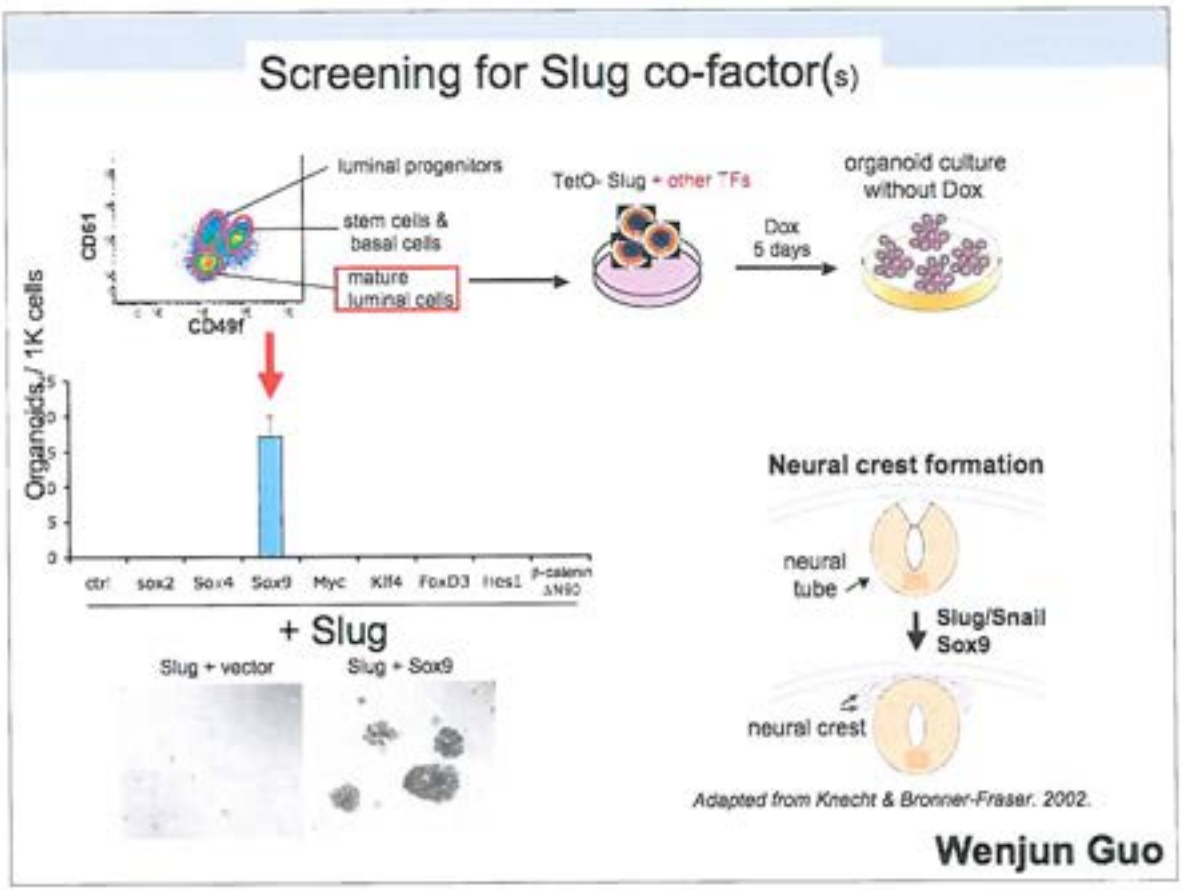
55.


shRNAs against Slug
Primary MECs
Matrigel culture
shLuciferase
shSlug-3
shSlug-4
25.0
20.0
15.0
10.0
5.0
0.0
shLuc
shSlug -3
shSlug -4
Hence, Slug is necessary for maintenance of mammary SC state and sufficient to induce it.
WRONG
Wenjun Guo

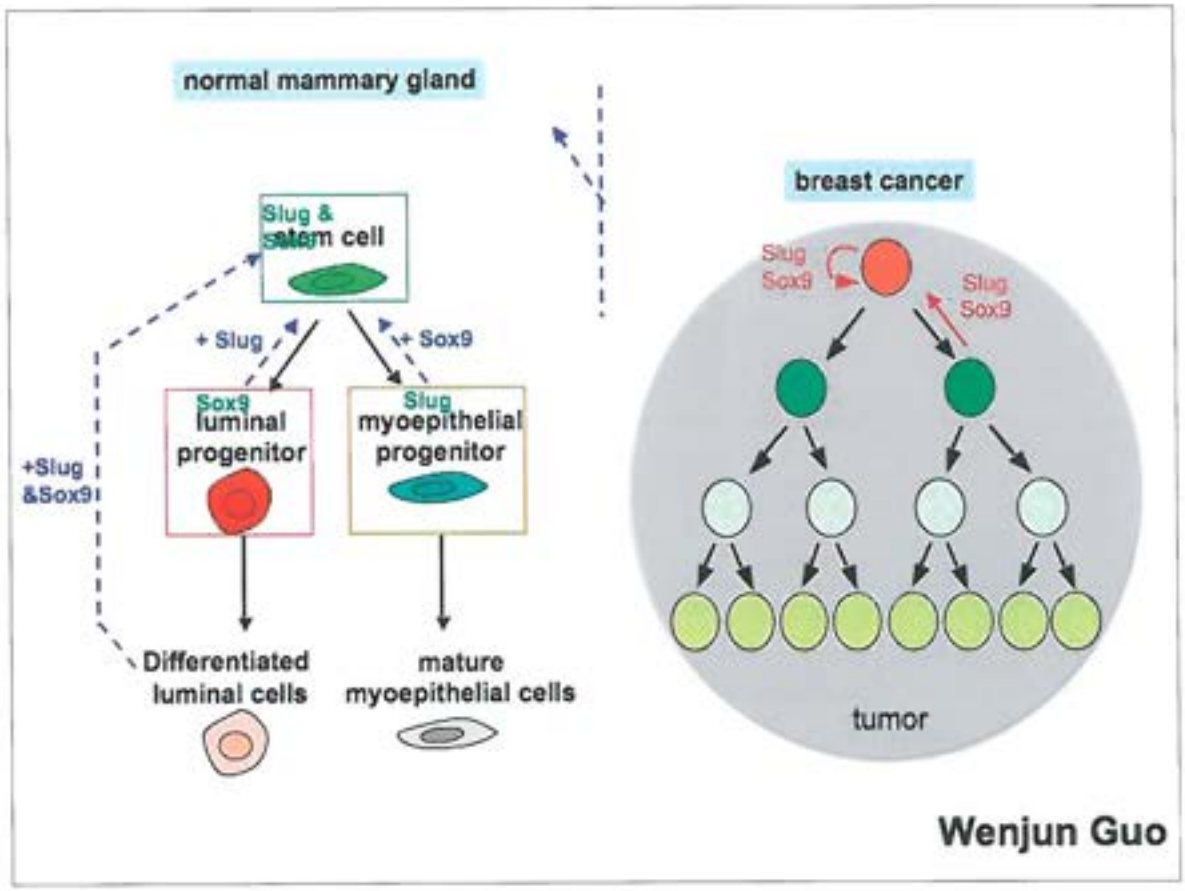
56.


Slug fails to induce fully differentiated (mature) MECs to become stem cells
Dox
1 week
different populations
organoid culture no Dox
(use mammosphere assay as surrogate for stem cell assay)
control
+Slug
basal cells (+stem cells)
luminal progenitors
mature luminal cells
CD61
CD49f
Hence, Slug cannot, on its own, convert fully differentiated luminal cells into SCs
Additional factor(s) are needed?
Wenjun Guo

57.

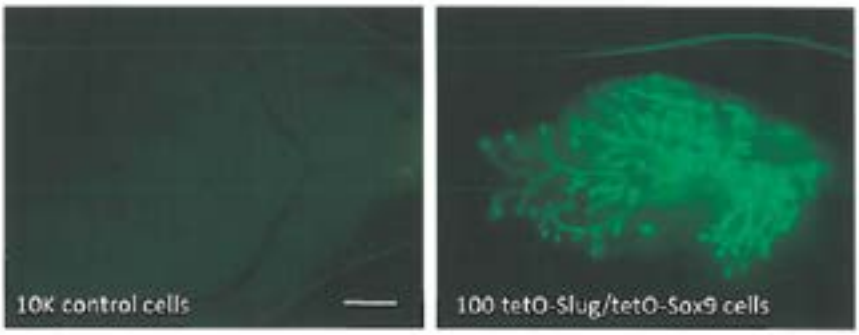

Screening for Slug co-factor(s)
luminal progenitors
stem cells & basal cells
mature luminal cells
CD61
CD49f
TetO- Slug + other TFs
Dox 5 days
organoid culture without Dox
Organoids / 1K cells
ctrl
sox2
Sox4
Sox9
Myc
Klf4
FoxD3
Hes1
β-catenin ΔN90
+ Slug
Slug + vector
Slug + Sox9
Neural crest formation
neural tube
Slug/Snail Sox9
neural crest
Adapted from Knecht & Bronner-Fraser. 2002.
Wenjun Guo

58.


normal mammary gland
Slug & stem cell
+ Slug
+ Sox9
Sox9 luminal progenitor
Slug myoepithelial progenitor
+Slug &Sox9
Differentiated luminal cells
mature myoepithelial cells
breast cancer
Slug Sox9
Slug Sox9
tumor
Wenjun Guo

59.

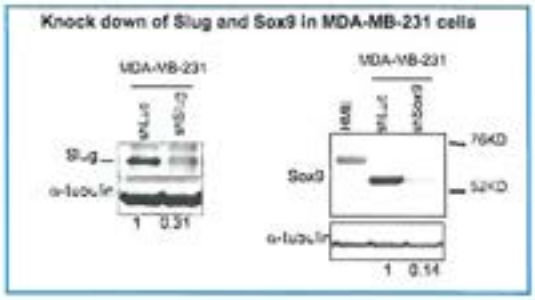
D



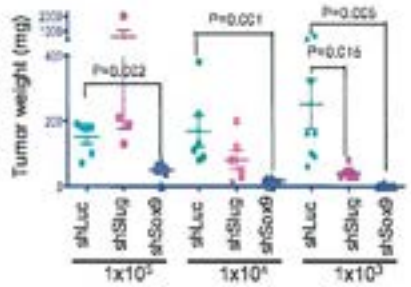
Wenjun Guo

60.

Knock down of Slug and Sox9 inhibits tumorigenicity of MDA-MB-231 cells



Tumor growth



Tumor incidence

# of cells injected	shLuc	shSlug	shSox9
1x10⁵	5/5	4/4	5/5
1x10⁴	5/5	5/5	5/5
1x10³	5/5	5/5	1/5

Cell lines injected	Frequency of tumor initiating cells (95% confidence interval)
shLuc	1/1 (1/1 – 1/952)
shSlug	1/1 (1/1 – 1/952)
shSox9	1/2387 (1/827 – 1/6903)

Wenjun Guo

61.

Questions

Are the transcription factors(TFs) that cause passage through an EMT the key regulators of entrance into the epithelial SC state? Or, are there alternative routes to enter into the epithelial SC state that are independent of the EMT program?

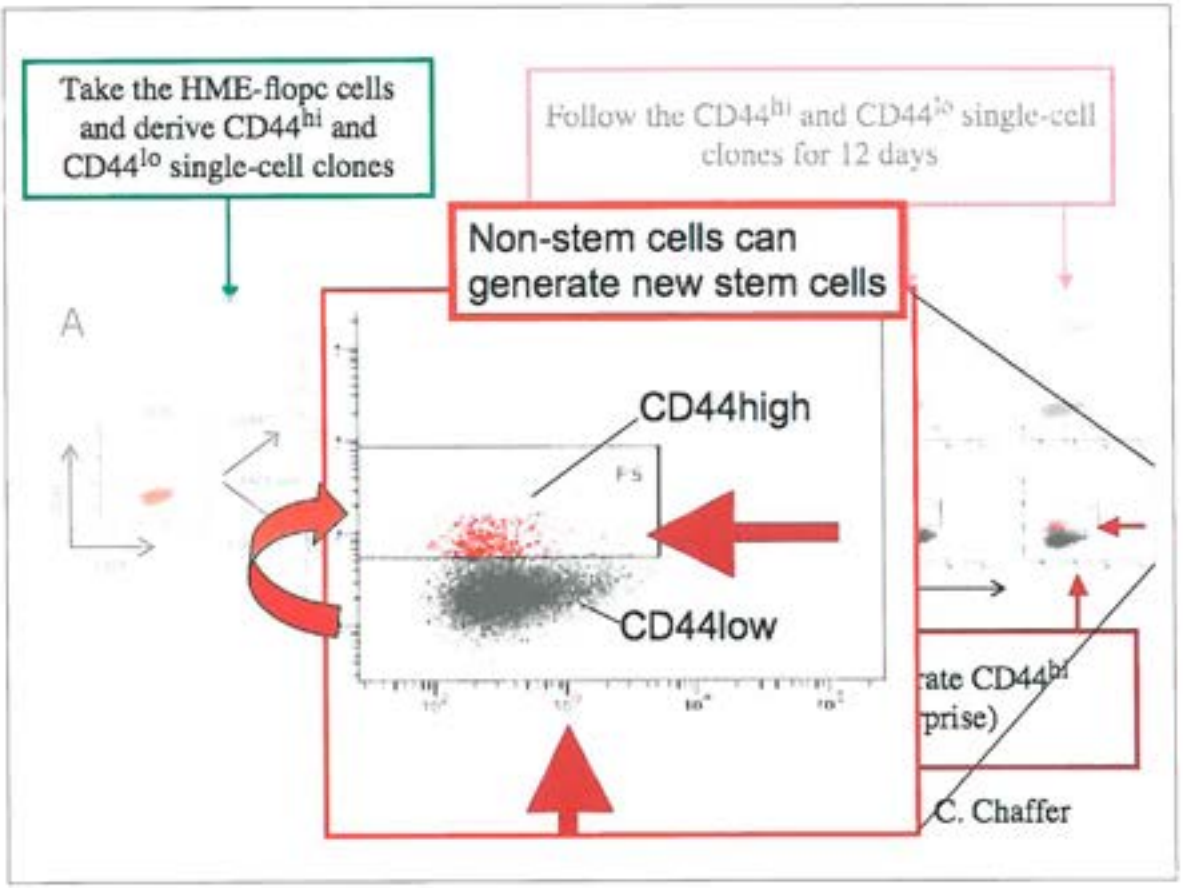
Do these lessons, learned from studying human MECs, extend as well to non-mammary epithelial cells? I.e., are they generalizable?

Does the EMT program and its TFs shed light on the behavior of non-epithelial cell types?

Are the contextual signals that induce passage through an EMT identical with those that induce entrance into the epithelial SC state?

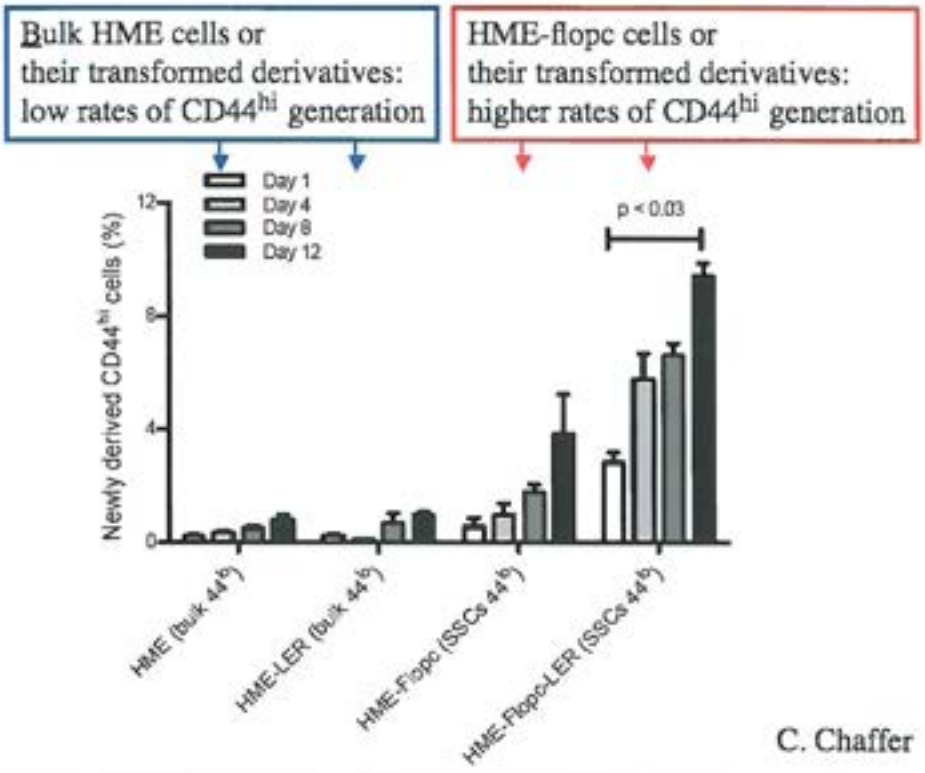
Do the neoplastic cells in human carcinomas pass entirely through an EMT or, more commonly, enter only part way?

62.



63.

Spontaneous appearance of $CD44^{hi}$ (SCs) cells from $CD44^{lo}$ cells (non-SCs)



C. Chaffer

64.

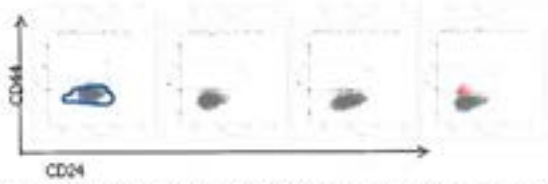
Conversion rate per cell generation
$CD44^{lo} \rightarrow CD44^{hi}$
(non stem cells $\rightarrow$ stem cells)

HME-Flopc: 0.0049 per cell generation
HMLE-Flopc: 0.0082 per cell generation
HMLER-Flopc: 0.017 per cell generation

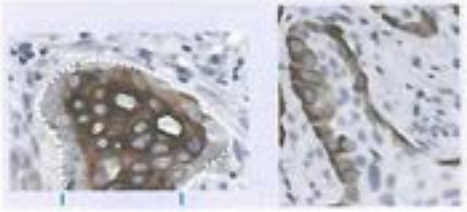
Christine Chaffer and Paul Wiggins

65.

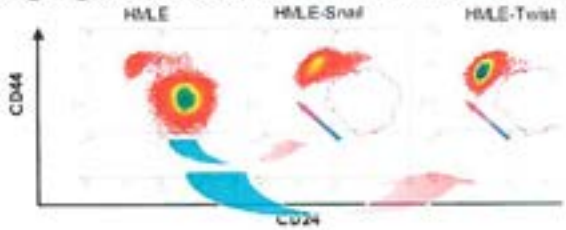
If immortalized mammary epithelial cells (MECs) can generate CD44 hi cells



And if immortalized contextual signals can induce tumorigenic MECs to undergo an EMT

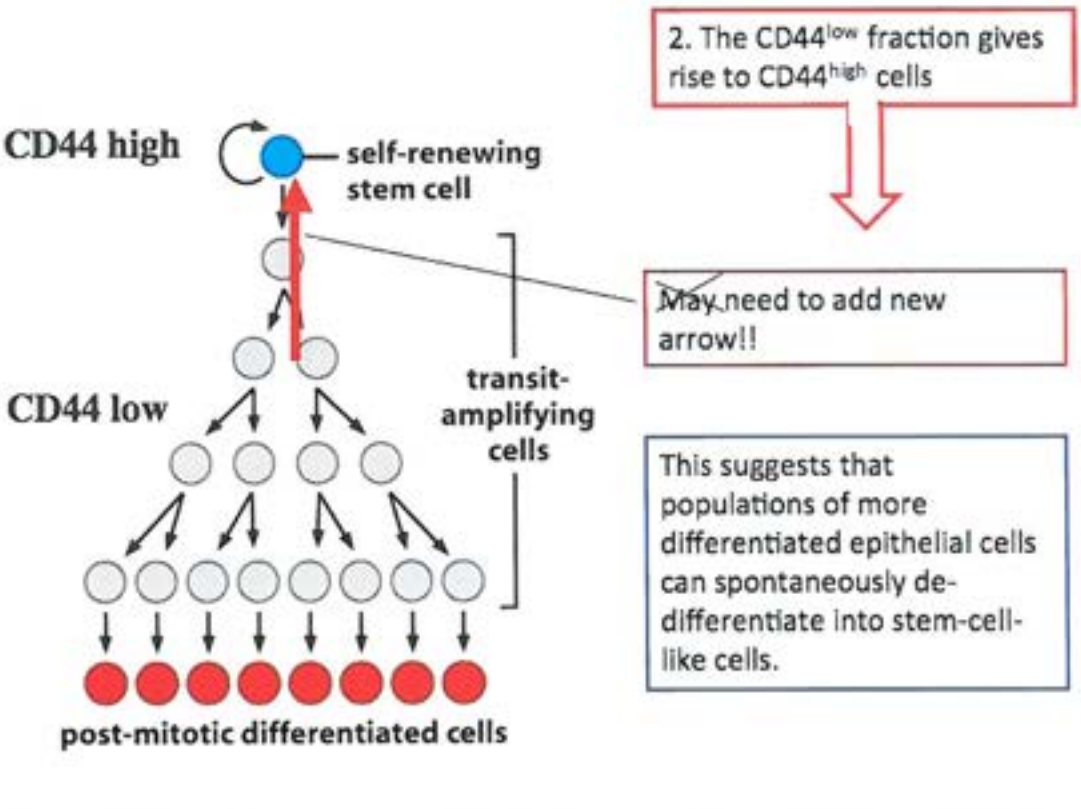


And if EMT-inducing regulators create CD44 hi immortalized MECs



It follows that in a variety of non-stem-cell MECs can be converted into stem cells

66.



67.

2. The CD44^{low} fraction gives rise to CD44^{high} cells

CD44 high — self-renewing stem cell

May need to add new arrow!!

Hence, epithelial cell populations exhibit great plasticity in vitro.

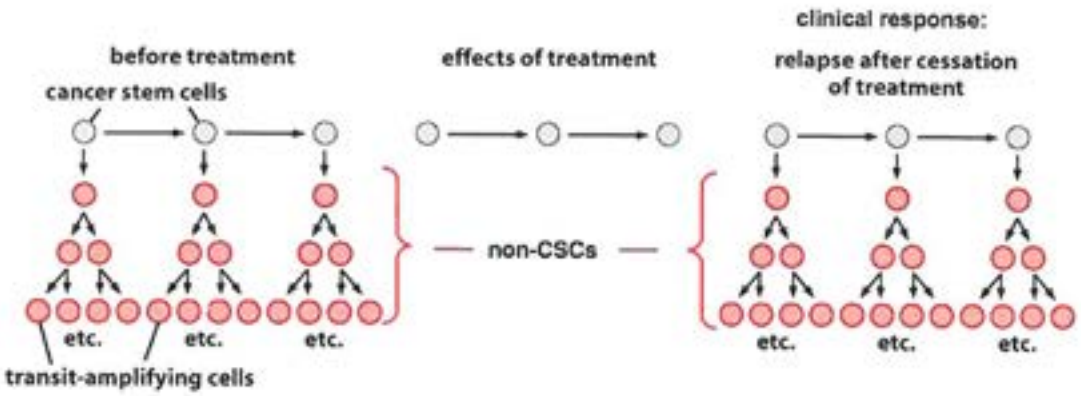
(In vivo, a population of transformed HMLER cells from which CD44 hi cells have been eliminated following implantation in mouse host generates tumors with >20% CD44hi cells within several weeks.)

And certain cells are intrinsically highly susceptible to induction of an EMT.

68.

Most currently used chemotherapeutics kill non-CSCs preferentially.

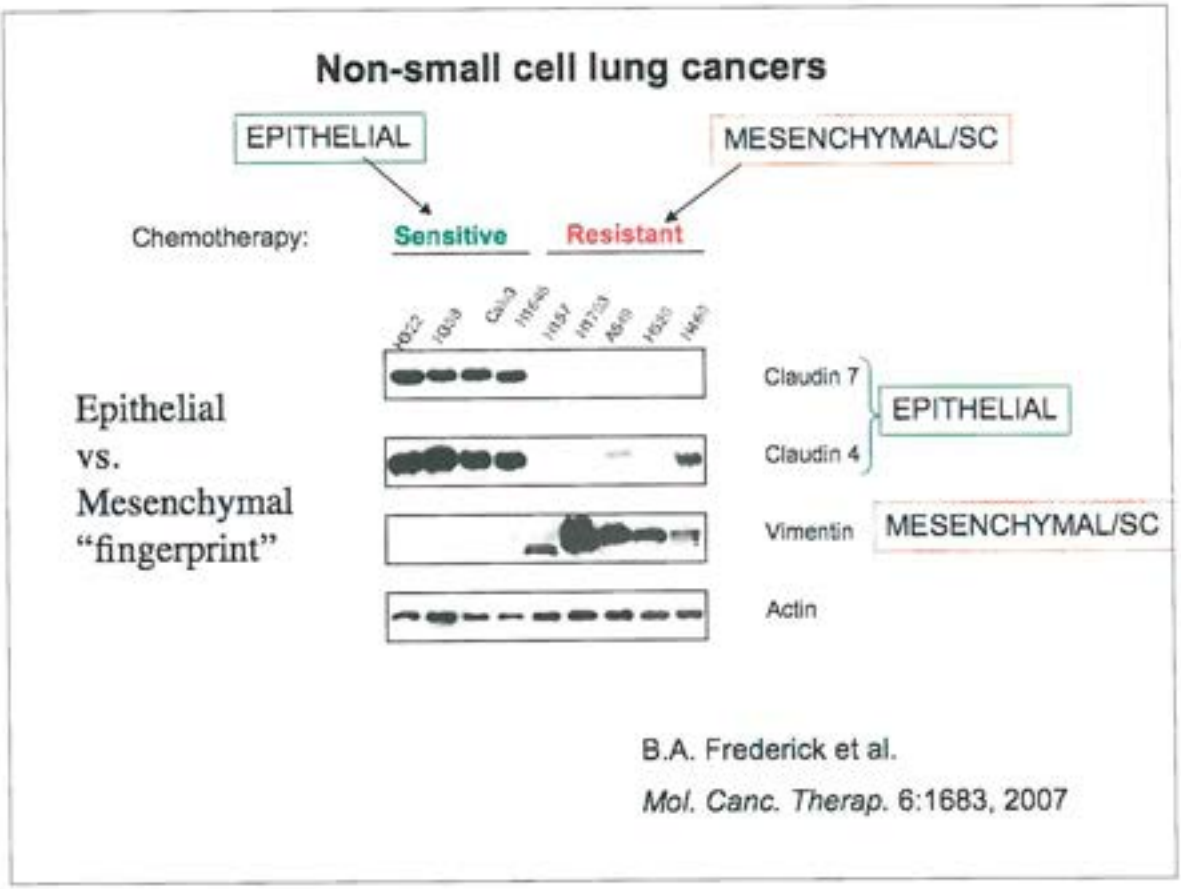
Putative effects of eliminating the non-CSCs therapeutically



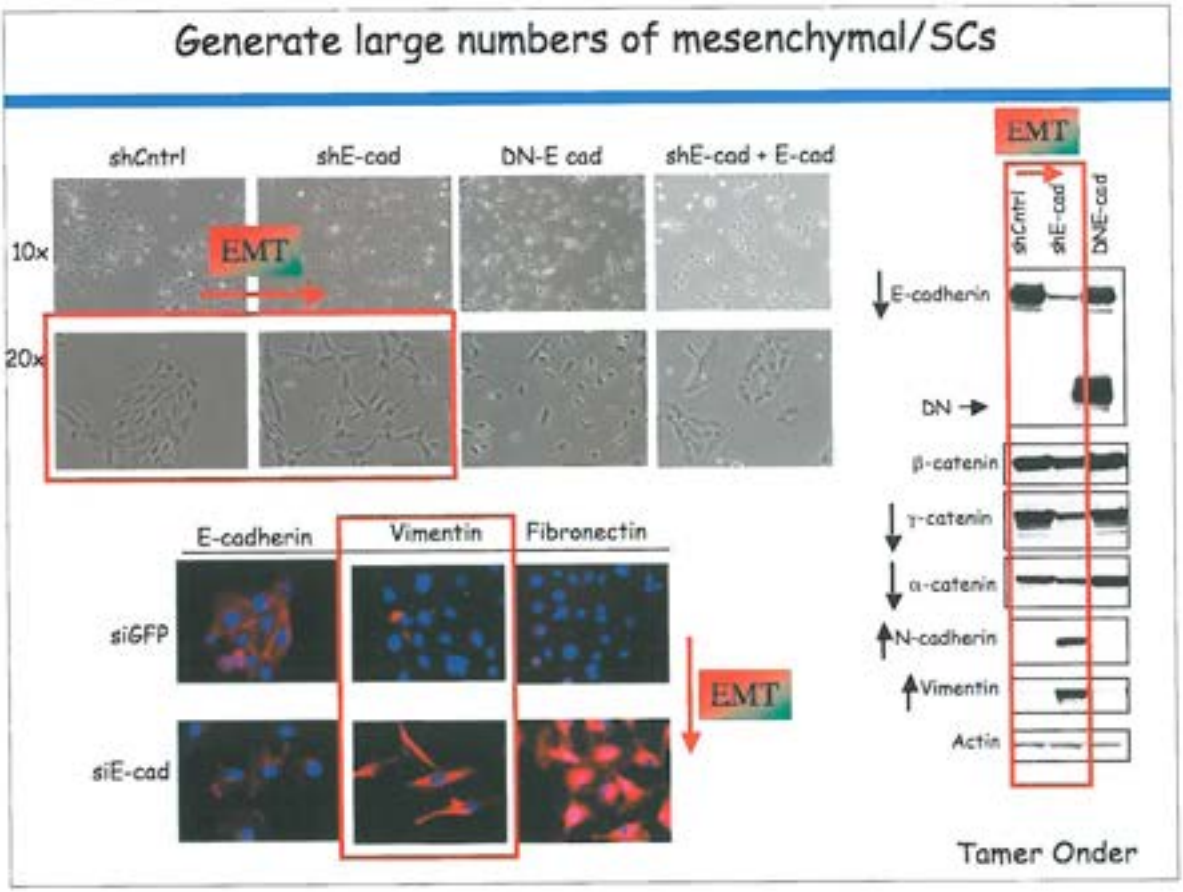
CSC = cancer stem cell

Figure 16.30 *The Biology of Cancer* (© Garland Science 2007)

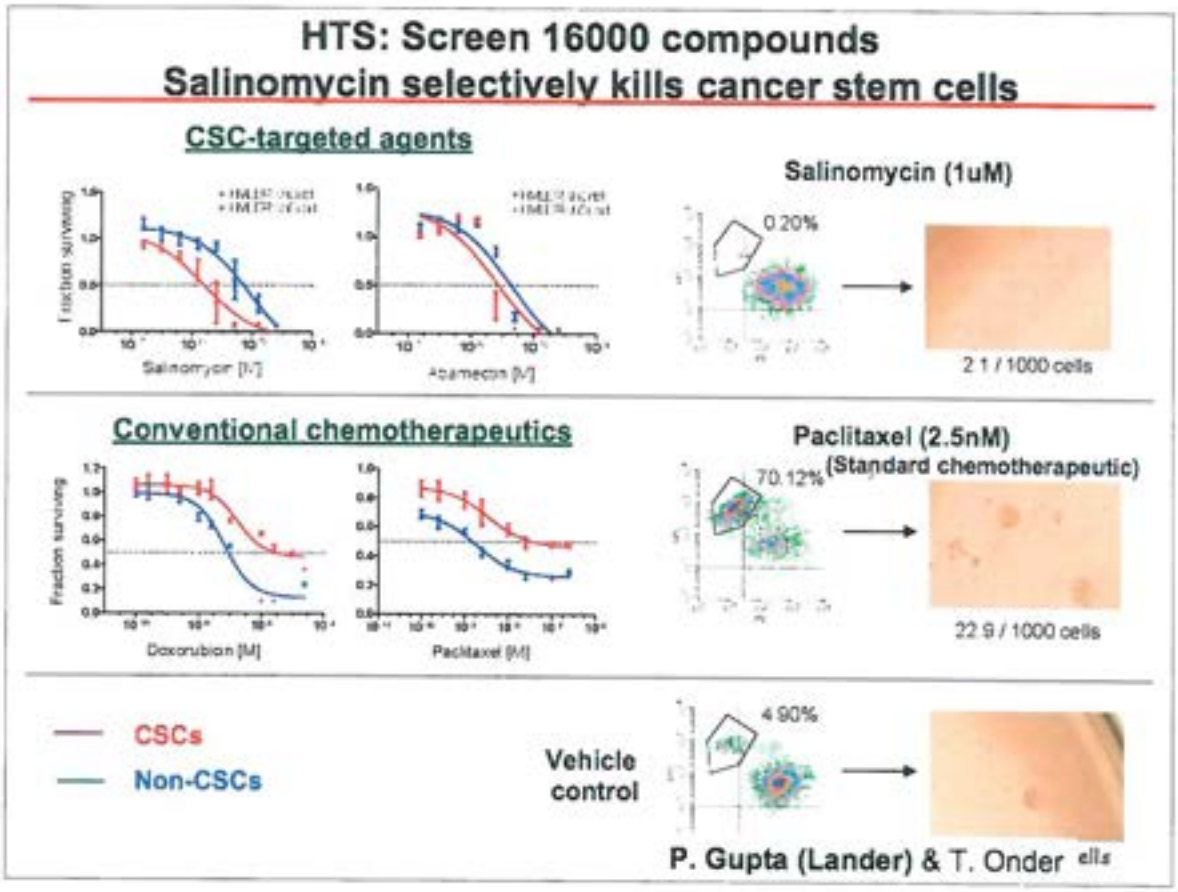
69.


Non-small cell lung cancers
EPITHELIAL
MESENCHYMAL/SC
Chemotherapy:
Sensitive
Resistant
Epithelial
vs.
Mesenchymal
"fingerprint"
Claudin 7
Claudin 4
EPITHELIAL
Vimentin
MESENCHYMAL/SC
Actin
B.A. Frederick et al.
Mol. Canc. Therap. 6:1683, 2007

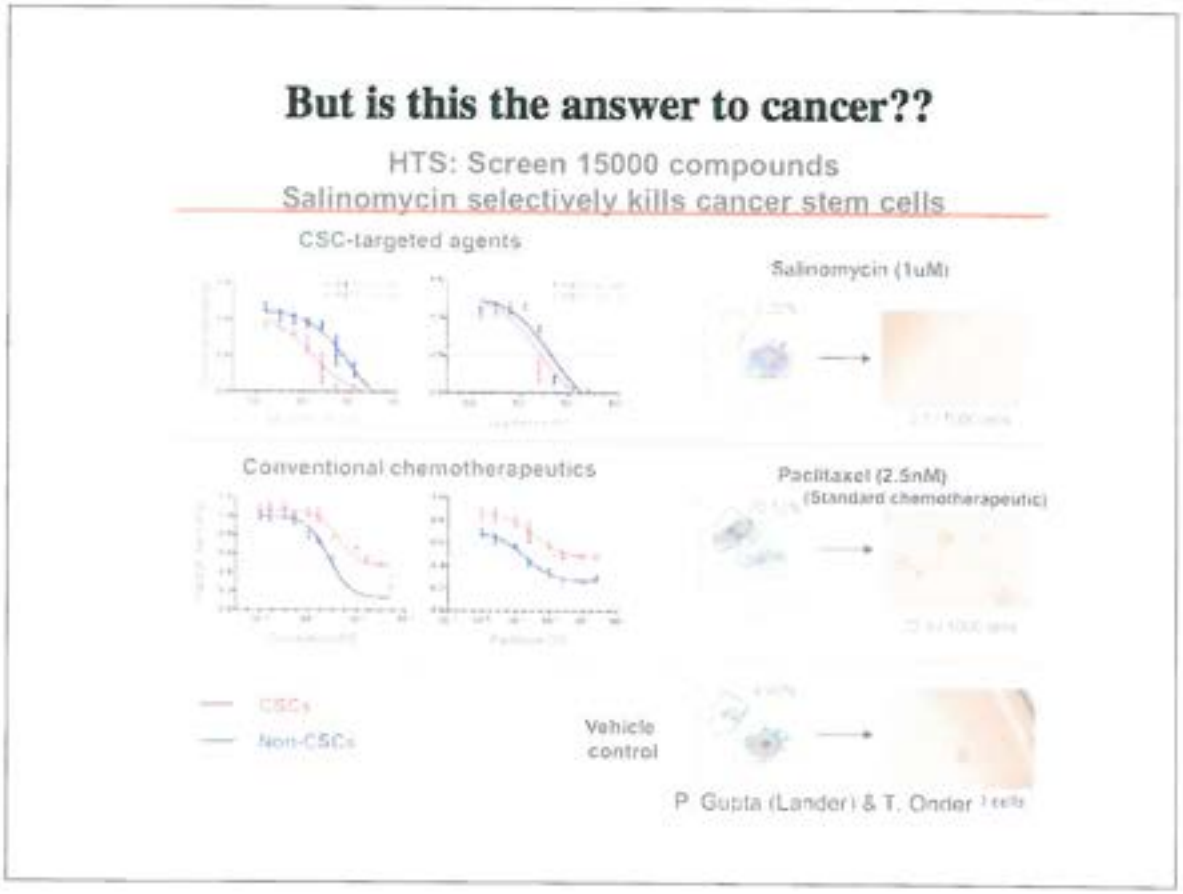
70.


Generate large numbers of mesenchymal/SCs
shCntrl
shE-cad
DN-E cad
shE-cad + E-cad
10×
20×
EMT
E-cadherin
Vimentin
Fibronectin
siGFP
siE-cad
EMT
EMT
shCntrl
shE-cad
DNE-cad
E-cadherin
DN
β-catenin
γ-catenin
α-catenin
N-cadherin
Vimentin
Actin
Tamer Onder

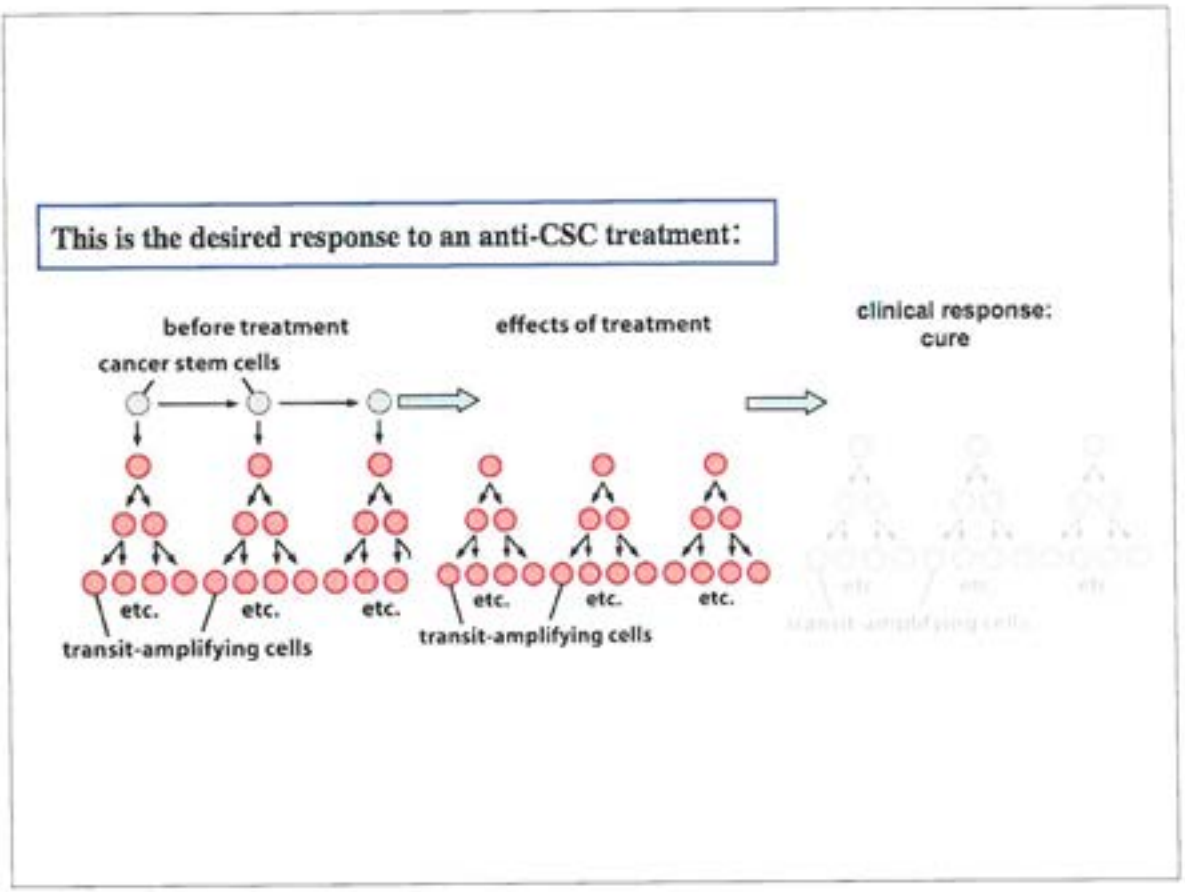
71.


HTS: Screen 16000 compounds
Salinomycin selectively kills cancer stem cells
CSC-targeted agents
Salinomycin (1uM)
0.20%
2.1 / 1000 cells
Conventional chemotherapeutics
Paclitaxel (2.5nM)
(Standard chemotherapeutic)
70.12%
22.9 / 1000 cells
CSCs
Non-CSCs
Vehicle control
4.90%
P. Gupta (Lander) & T. Onder

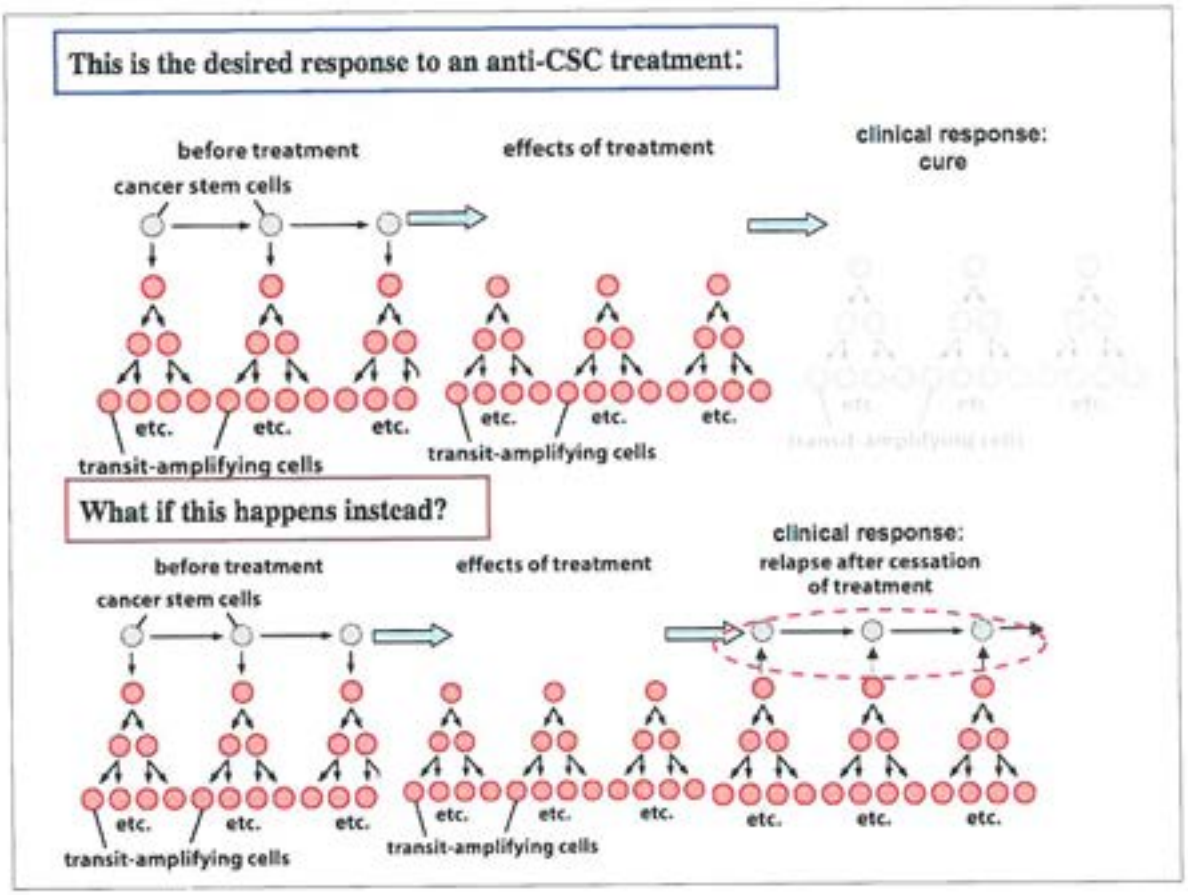
72.


But is this the answer to cancer??
HTS: Screen 15000 compounds
Salinomycin selectively kills cancer stem cells
CSC-targeted agents
Salinomycin (1uM)
Conventional chemotherapeutics
Paclitaxel (2.5nM)
(Standard chemotherapeutic)
CSCs
Non-CSCs
Vehicle control
P. Gupta (Lander) & T. Onder

73.


This is the desired response to an anti-CSC treatment:
before treatment
cancer stem cells
effects of treatment
clinical response:
cure
etc.
etc.
etc.
etc.
etc.
etc.
transit-amplifying cells
transit-amplifying cells

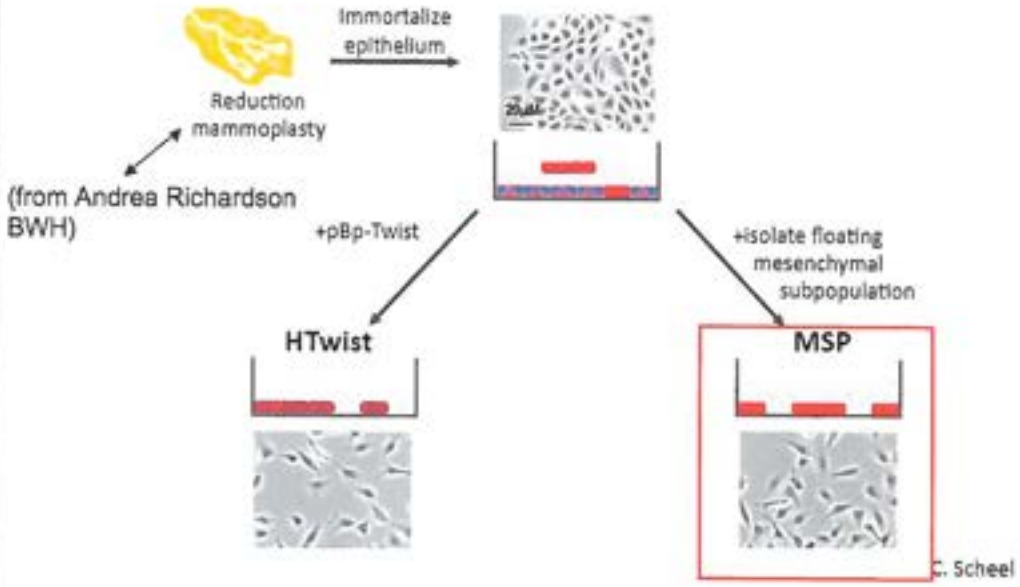
74.


This is the desired response to an anti-CSC treatment:
before treatment
cancer stem cells
effects of treatment
clinical response:
cure
etc.
etc.
etc.
etc.
etc.
etc.
transit-amplifying cells
transit-amplifying cells
What if this happens instead?
before treatment
cancer stem cells
effects of treatment
clinical response:
relapse after cessation
of treatment
etc.
etc.
etc.
etc.
etc.
etc.
etc.
etc.
etc.
transit-amplifying cells
transit-amplifying cells

75.

What extrinsic signals induce an epithelial cell to activate its EMT program?

A Mesenchymal Subpopulation (MSP) of Immortalized Human Mammary Epithelial Cells (HMLE)



76.

How do the MSP & Twist cells **maintain their residence** in the mesenchymal/SC state?

77.

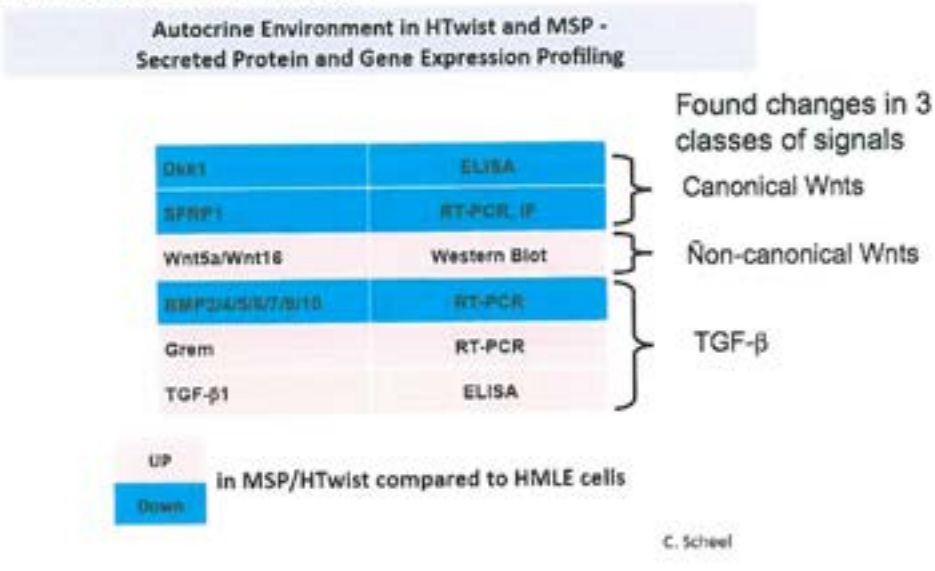
How do the MSP & Twist cells maintain their residence in the mesenchymal/SC state?

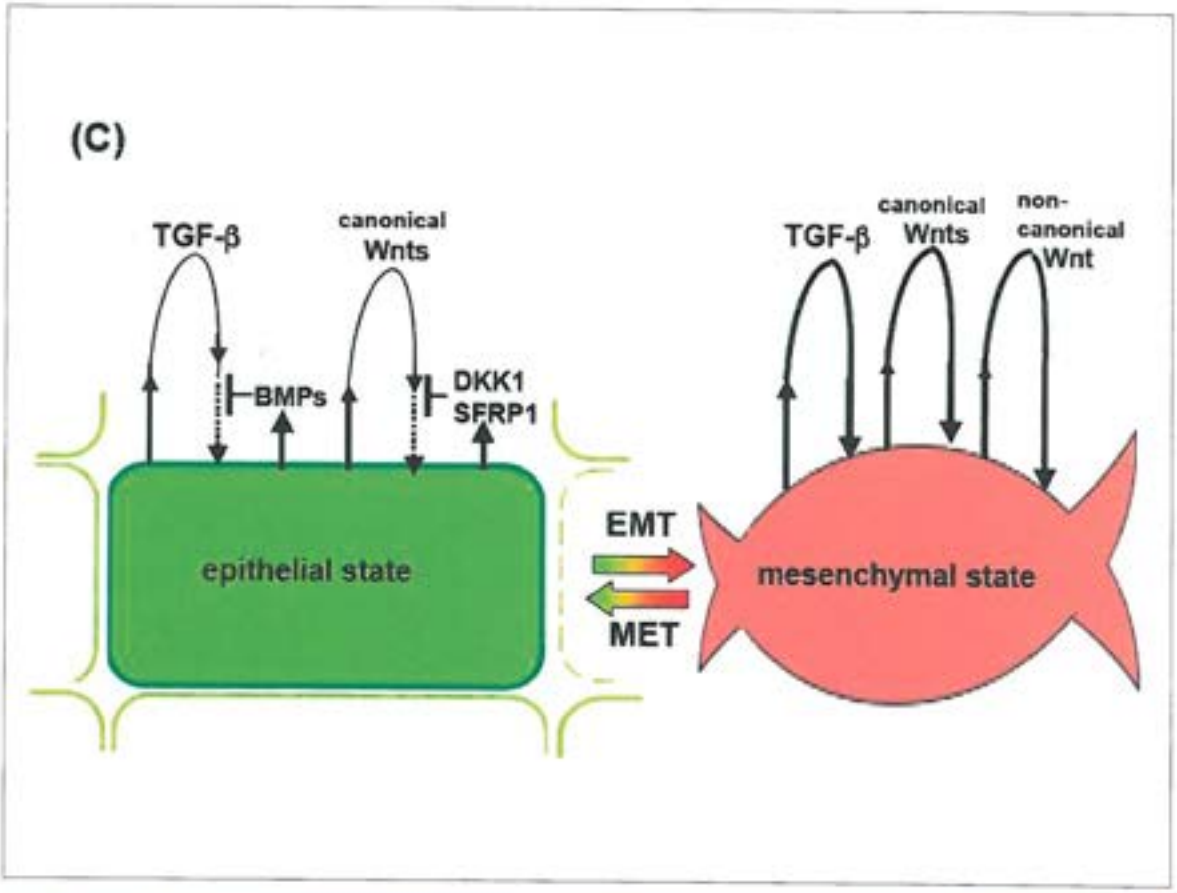
(In the case of Twist cells,
The answer may be simple: constitutive expression of introduced Twist)
But what about the MSP cells that became mesenchymal spontaneously?

Hypothesis: **Autocrine signaling**
Therefore measure the extracellular signaling environment of MSP and Twist cells.
(What factors do these cells release into their surrounding environment?)

78.

How do the MSP & Twist cells maintain their residence in the mesenchymal/SC state?
Hypothesis: Autocrine signaling
Therefore measure the extracellular environment of MSP and Twist cells.




(C)
TGF-β
canonical Wnts
BMPs
DKK1
SFRP1
epithelial state
EMT
MET
TGF-β
canonical Wnts
non-canonical Wnt
mesenchymal state

79.


Sendurai Mani
Piyush Gupta
Samuel Godar
Antoine Kamoun
Christine Chaffer
Tamer Onder
Wenjun Guo
Mai-Jing Liao

80.

2013 Pezcoller Symposium

Metabolism and Tumorigenesis
June 20-22, 2013
Trento, Italy

The Symposium will be held in Trento from June 20 to 22, 2013.

The program has been formulated by

Dr. Bill Kaelin
(Dana Farber Cancer Institute and Harvard Medical School, Boston)
Dr. David Livingston
(Dana Farber Cancer Institute, Boston)
Dr. Massimo Loda
(Dana Farber Cancer Institute and Harvard Medical School, Boston)
Dr. Karen Vousden
(Beatson Institute, Glasgow)
Dr. Enrico Mihich
(Dana Farber Cancer Institute, Boston)

"Tumor cells often reveal complex sets of metabolic abnormalities. Certain metabolic abnormalities in tumors are grounded in the operations of mutant or dysfunctional genes, underscoring the value of these perturbations in the tumorigenesis process. This Symposium will explore key aspects of cancer cell metabolism with an emphasis on understanding the mechanisms that give rise to it, on defining how it serves the survival needs of tumors, on identifying abnormal tumor metabolic phenotypes and on assessing the potential clinical effects of interfering with these abnormalities".

For posters and scientific matters contact
dr. Enrico Mihich:
enrico_mihich@dfci.harvard.edu

For The local organizational matters contact
The Pezcoller Foundation:
pezcoller@pezcoller.it

Two year Pezcoller Foundation Grants

1. The "Fondazione Pezcoller - Ferruccio ed Elena Bernardi fellowship" in collaboration with the Società Italiana di Cancerologia (SIC). Among many qualified candidates has been selected Dr. Samantha Solito, of the University of Padova, Department of Surgery, Oncology and Gastroenterology - Oncology and Immunology Section- and her project "*Characterization of molecular mechanisms involved in MDSC mediated tolerance: paving the way to overcome tumor attack*".

2. The "Fondazione Pezcoller - Fondazione Cassa di Risparmio di Trento e Rovereto fellowship" has been given to Dr. Ivan Raimondi and his project "*Post-transcriptional control mechanisms of CDKN2A/p16INK4a gene expression and impact of 5'UTR VariantsPredisposing To Melanoma*", which will be conducted at the CIBIO (Centre for Integrative Biology), University of Trento.

Other two grants, the "Pezcoller Foundation - Prof. Vittorio Erspamer" and the "Pezcoller Foundation - Dr. Marcello Marchi" will be given later in 2013.

Save the date!

25th Pezcoller Symposium

June 20-22, 2013
Trento, Italy

Metabolism and Tumorigenesis

The Pezcoller Foundation

Journal

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