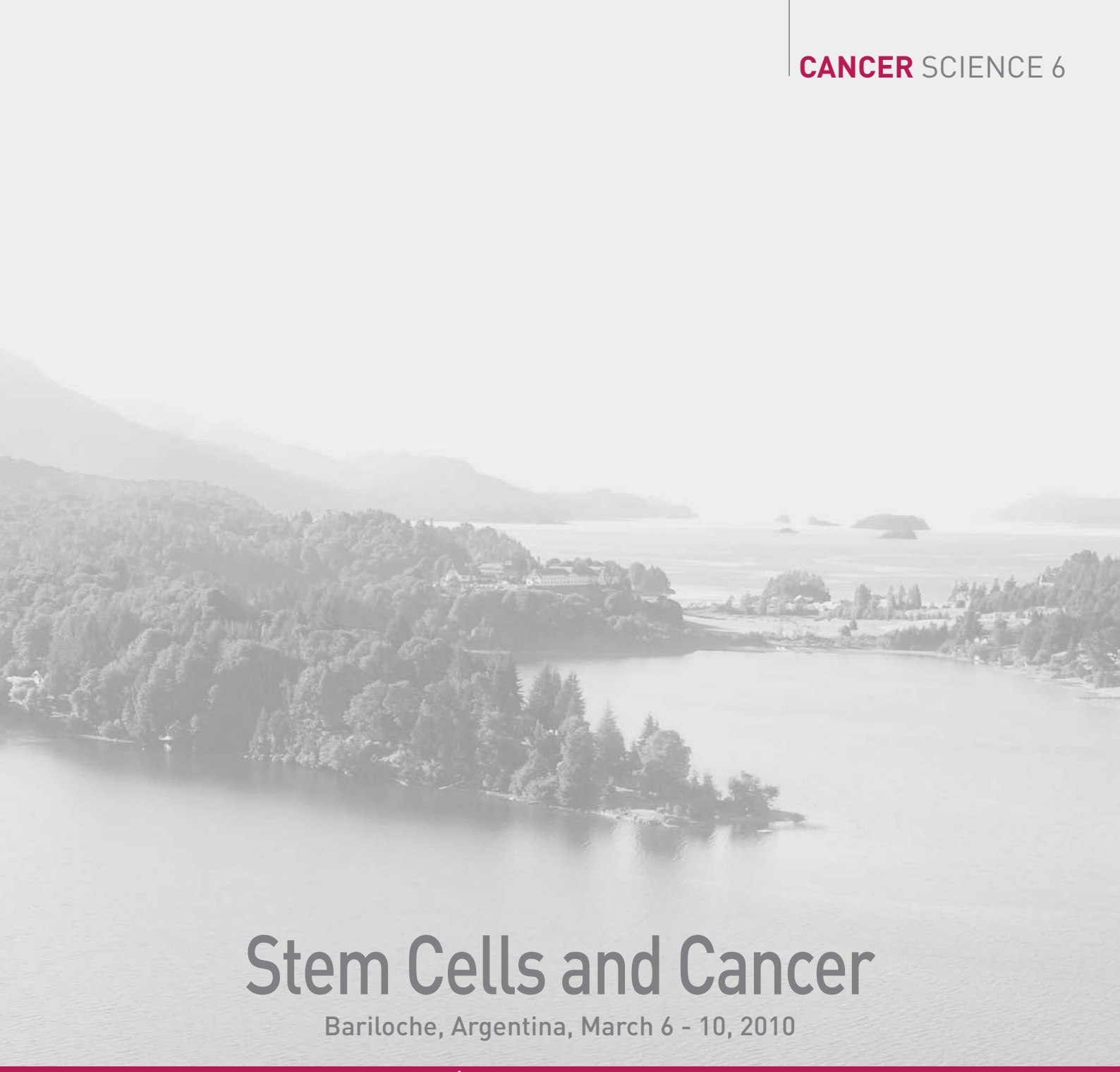

Stem Cells and Cancer

Bariloche, Argentina, March 6 - 10, 2010

COLLOQUES MÉDECINE ET RECHERCHE

FONDATION IPSEN

SCIENTIFIC REPORT BY APOORVA MANDAVILLI

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FOREWORD by INDER M. VERMA

Cancer stem cells (CSC) are cells found within tumors and that possess characteristics associated with normal stem cells, specifically the ability to self-renew and differentiate. CSC have the capacity to both expand the pool and to differentiate into the heterogeneous non-tumorigenic cell types that appear to constitute the bulk of cells within most tumors.

The existence of CSC has important therapeutic implications. Most therapies target the rapidly dividing cells that comprise the majority of cells within a tumor. If CSC are refractory to these therapies, treatment is unlikely to be curative and relapses are likely. The CSC hypothesis argues for therapies that target the minority stem cell population that fuels tumor growth.

As with many emerging hypotheses, the idea of CSC has not been met with universal acceptance and is a controversial topic in cancer biology. The subject was therefore ripe for the Fondation IPSEN's 6th cancer series meeting.

The meeting opened with an introduction to CSC by **Irving Weissman**, who drew on his experience with hematological cancers. **Hans Clevers** presented LGR5 as a marker for active stem cell populations in multiple adult tissues and cancers. Single sorted LGR5+ cells can initiate crypt-villus organoids that maintain cell hierarchy. Pyloric LGR5+ cells generate entire gastric units within 10 days and maintain multipotent stem cell activity over at least 20 months.

Many research teams are intent on identifying and characterizing these resilient CSC, in order to design therapies that can target them. For example, **John Dick** showed that, based on expression analysis, gene signatures derived from leukemia stem cells and normal hematopoietic stem cells turn out to be the strongest predictor of overall survival compared with signatures obtained from bulk leukemia blasts.

Catriona Jamieson discussed the importance of combining different molecularly targeted therapies to block the abnormal differentiation, self-renewal, survival and homing capacity of CSC.

Existing therapies against many cancers are not based on a full understanding of CSC, which have a close relationship to their microenvironment. **Jeremy Rich** argued that CSC are at the center of a nexus, and must not be studied in isolation.

David Scadden proposed that tumors' dependence on the interaction between CSC and their microenvironment offers the potential for intervention in treatment and prevention strategies.

A major impediment in using embryonic stem cells and induced pluripotent stem cells to study human diseases is the inefficiency of gene targeting. **Rudolf Jaenisch** discussed novel approaches for the genetic manipulation of these cells. I suggested that the use of Cre-loxP controlled lentiviral vectors is a new way to generate mouse models for glioblastoma and other cancers. Glioblastomas may originate by reprogramming of the terminally differentiated glial cells by oncogenes.

Cancer cells invariably express a form of pyruvate kinase, PK-M2, that is normally found in embryonic tissues and in cells that have the capacity for self-renewal. **Lew Cantley** discussed the role of PK-M2 and other metabolic enzymes in maintaining high rates of growth in cancer cells.

Cell lines from different tumors exhibit divergent gene expression signatures and differentiation behavior that correlate with specific neural progenitor subtypes, suggesting distinct CSC phenotypes from different patient tumors.

These human and mouse tumorigenic stem cell lines are valuable tools for defining heterogeneity within the same stem cell pool and between stem cells from different individuals. **Peter Dirks** discussed the possibility that stem cells differentially express neurosignaling pathways, creating a stochastically heterogeneous pool.

Fred Gage suggested that characterization of somatic neuronal diversification will not only be relevant for the understanding of brain complexity and neuronal organization in mammals, but may also shed light on the differences in cognitive abilities.

Although some cancers appear to follow a CSC model, **Sean Morrison** proposed that other cancers, such as melanoma, do not follow this model, and so it will not be possible to cure them by targeting rare CSC.

As with most cancer series meetings, there was lively discussion, carried on during drives to scenic interludes in the beautiful surroundings of Bariloche, the crown jewel of Patagonia, Argentina. As in all the previous meetings, Jacqueline, Yves, Astrid and Sonia made the experience memorable with their organization and hospitality. Apoorva worked diligently, taking copious notes, following the speakers with questions and clarifications and cajoling them to provide high-resolution figures. The outcome is yet another well-summarized and highly readable monograph of the presentations and discussions.

Inder Verma

PART I: The identity of stem cells

LGR5 stem cells in self-renewal and cancer

A report on a lecture by
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The intestinal epithelium is the most rapidly self-renewing tissue in adult mammals. Current models state that crypt stem cells reside at the +4 position immediately above the Paneth cells in the small intestine. Using an inducible Cre knock-in allele and the Rosa26-LacZ reporter strain, lineage tracing experiments have shown that LGR5+ crypt base columnar cells represent the stem cell of the small intestine and colon. The expression pattern of LGR5 suggests that it marks stem cells in multiple adult tissues and cancers. Single sorted LGR+ cells can initiate crypt-villus organoids that maintain cell hierarchy. Intestinal cancer is initiated by Wnt pathway-activating mutations in genes such as APC. Deleting APC in in LGR5+ stem cells leads to their transformation within days and causes the growth of adenomas within 6 weeks. In the stomach, LGR5 expression is confined to a small population of cells at the gland base. Pyloric LGR5+ cells generate entire gastric units within 10 days and maintain multipotent stem cell activity over at least 20 months. ***Hans Clevers*** *presented LGR5 as a marker for active stem cell populations in several different organ systems.*

In the small intestine of mammals, the presence of villi enormously extends the surface epithelium. Cells in the villi are born in crypts, and live for only a short time. There are about 200 rapidly proliferating cells, called the transit amplifying cells, with a cell cycle of about 12 hours.

The assumption in the field has long been that, in contrast to these rapidly proliferating cells, stem cells of the small intestine cycle slowly and may even be quiescent. The leading candidates for these stem cells were cells located at the +4 position[1]. However, experiments have since shown that, in fact, the little-studied crypt-based columnar (CBC) cells are the workhorse stem cells of this epithelium.

LGR5, a gene that encodes a G-protein coupled receptor, seems to mark these CBC cells[2]. The ligand for LGR5 is as yet unidentified, but LGR5 is closely related to well-studied receptors for luteinizing hormone, follicle stimulating hormone and thyroid hormone. LGR4, LGR5 and LGR6 are other members of this class, and LGR6 also seems to mark stem cells in a large number of tissues.

LGR5 was originally discovered as a Wnt target in colon cancer cells. One of about 300 such genes, it was carefully mapped by expression analysis, and then picked up in the rare small cells at the base of the crypt. The stem cells can be visualized in a knock-in mouse with LGR5 driving the expression of green fluorescent protein (GFP) and the CreERT2 recombinase protein for lineage tracing purposes.

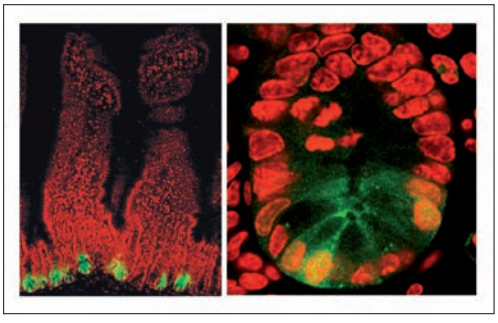

Figure 1
LGR5-driven expression of GFP in crypt base columnar cells.

Using this method, there are between 6 and 10 CBC cells with high levels of GFP, and a few daughter cells — indicating lineage tracing — that carry over some GFP. Post-mitotic Paneth cells appear as dark areas, and are closely associated with the GFP^{hi} stem cells.

By electron microscopy, more than 80% of the volume of these small, flat cells is taken up by the nucleus, which is not surprising. The cells have a few mitochondria, but

metabolic analysis shows that they seem to burn glutamate and lactate for energy, and don't rely on their mitochondria much.

Contrary to stem cell dogma, however, BrdU labeling suggests that these cells cycle every 24 hours, and are not quiescent. Cells isolated from mouse crypts can be sorted into three generations of stem cells: the $LGR5^{hi}/GFP^{hi}$ stem cells, a second generation of cells, and then the bulk of the cells. All three groups of cells are undifferentiated, and other so-called stem cell markers, such as CD133 or CD44, stain all these cells in a gradient. In contrast, LGR5 is restricted to CBC cells.

Expression analysis

When the different groups of cells are compared by gene expression profiling, very satisfyingly, the most differential gene between the real stem cells and the daughter cells is LGR5 itself. Even though GFP carries over into the daughter cells, LGR5 doesn't, and is an extremely strict marker for CBC cells. Overall, about 60 genes that are functionally interesting are highly enriched in these stem cells.

When the stem cells divide, the first differentiating cells appear by day 2 or 3, and enterocytes, goblet cells and several other rare cell types in this epithelium move up in bands. By day 5 or 6, the first cells start dying at the tip of the villi. The ribbons of cells persist for the lifetime of the mouse.

To study stem cells independently in the same structure, a cassette with LGR5 and 4 fluorescent proteins separated by loxP was created. This technique has been used in mice to study individual neurons with color labels. In this case, activating Cre with different variations of this cassette stochastically creates stem cells of individual colors. As a result, parallel bands of different-colored cells emanate from the crypt. The method reveals that there are between 5 and 10 active individual stem cells fueling self-renewal in the crypt.

These stem cells look and behave identically. There are two models for stem cell behavior. In the deterministic model, the cells go through asymmetric divisions, so that each time a stem cell divides, it generates one daughter cell and a stem cell. In the stochastic model, by contrast, the 10 stem cells double every day and 10 of the 20 stem cells that exist are taken out randomly.

When a single LGR5+ stem cell is labeled stochastically, it generates a chaotic distribution of stem cells, clone size and daughters within one clone, and the pattern follows the stochastic rather than deterministic asymmetric model of cell division.

Similarly, when a Cre that activates extremely well in all the cells of the epithelium is used, a few days after labeling, every cell has taken up a color in a completely random pattern, rather than in ribbons. After a month or two, the picture resolves into contributions from only the stem cells, and the crypt essentially becomes clonal.

There are also mixed crypts, as predicted by the stochastic or "competing neighbors" model, in which neighboring stem cells compete with each other. This model predicts a neutral drift to clonality over time for individual crypts. Based on these results, LGR5+ cells are intestinal stem cells, even though they are not quiescent and don't divide asymmetrically.

Triggering cancer

Colon cancer is unique in that a single molecular event, the loss of APC, appears to kick start the transformation process. There are other ways to activate the Wnt pathway, but more than 90% of sporadic colon cancer cases have mutations or losses in both APC alleles. In mice, deleting APC with Cre causes adenomas, indicating that loss of APC alone is enough to transform the epithelium.

There are two different Cre regimens. The first is cytochrome P450 promoter-driven Cre, which can be activated by a low dose of β-naphthoflavone. When it's given orally, this hits the transit amplifying compartment

and many of the differentiated cells, but not the stem cells.

This method deletes APC everywhere except in the stem cells, and does generate adenomas. However, the mice live several months, much longer than in the APC^{min} mouse. Although the mice have lesions, the tumors stop growing at the 50-100 cell stage.

The other approach is to use the LGR5-CreERT2 mouse, and delete APC in stem cells. It takes about 3 days to see the first stem cell that over-expresses β-catenin. By day 5, more than one stem cell expresses high levels of β-catenin, and several cells in the clone do not express LGR5, suggesting that there is already a hierarchy in the clone.

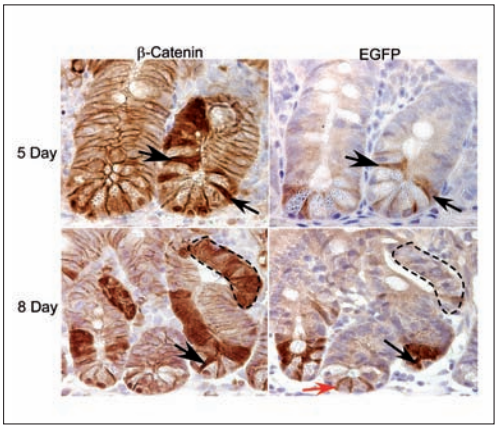

Figure 2
LGR5-GFP in early adenomas: immediate maintenance of a hierarchy.

By 3 or 4 weeks, the mice start dying because of massive adenomas. These tumors are not malignant, but kill the mouse by their sheer size. By fluorescence-activated cell sorting, $LGR5^{hi}$ cells comprise about 3% of these adenomas, conserving the normal proportion seen in the crypts[3].

The results suggest that stem cells in this system are the most likely targets of APC deletion to kick start an adenoma, and rapidly outcompete the neighboring wild type stem cells. The daughter cells behave very much like normal daughter cells until they reach the crypt-villus junction, where they can't differentiate because they can't switch off the Wnt pathway. The cells keep proliferating, move sideways and fill up the villus until the whole structure breaks open and feeds into a large adenoma.

Another way to study cancer stem cells involves crossing in multicolor Cre in the background of the floxed APC-LGR5-Cre mouse. With this approach, when Cre is activated, APC is deleted and at the same time, that cell picks one of four colors. Preliminary evidence shows that, contrary to some reports in the literature, adenomas are monoclonal structures.

Building organs

One method direly missing in this field is a culture system for adult, healthy non-transformed epithelium. Using this GFP system to screen hundreds of combinations of growth factors has identified epidermal growth factor receptor (EGF-R), Spondin and Noggin in matrigel as an effective combination that allows the cells to grow into three-dimensional structures.

When they are passaged, the cells produce a more regular gut-like structure with a central lumen and multiple crypt-like parts. These domains have terminally differentiated cells of all types. This is surprising because textbooks maintain that the mesenchymal niche supports stem cells and provides growth factors. But in this case, the culture system doesn't instruct them to form any architecture.

By electron microscopy, the undeveloped cells in the

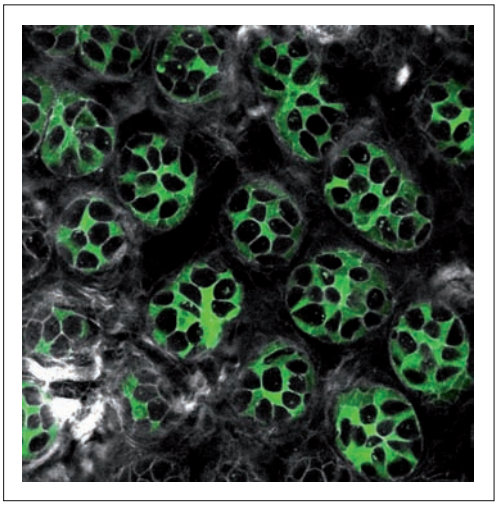

Figure 3
Paneth cells and LGR5 stem cells are physically close. Do Paneth cells build the niche?

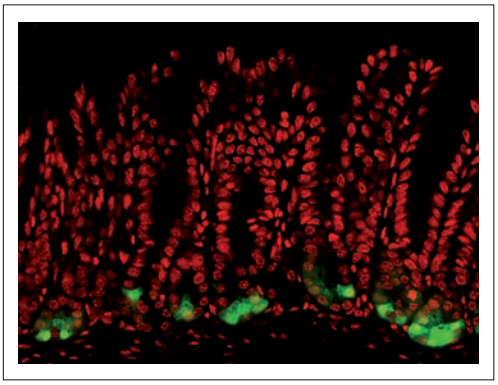

Figure 4
LGR5 is expressed at the base of the pyloric glands.

crypt don't have a well-developed bridge border, and there is a very close association between Paneth and stem cells. The stem cells and Paneth cells together make geometrical patterns so that Paneth cells avoid contact with other Paneth cells and stem cells with other stem cells.

Using CD24 as a marker for Paneth cells and LGR5 for stem cells, doublets of stem and Paneth cells, or of two stem cells or two Paneth cells — although they're very rare — can be sorted.

The plating efficiency of single stem cells is 2%, but plating heterologous doublets of stem and Paneth cells can take that efficiency up to 100%. Gene expression analysis shows that Paneth cells make all of the factors known to be necessary for stem cells, including EGF, TGF-α, Notch, Defa1, and, importantly, Wnt11 and Wnt3. They also make the candidate for LGR5 ligand.

These results suggest that the niche for stem cells is not mesenchymal, but is epithelial. In fact, the stem cells build their own niche[4].

To summarize, LGR5 stem cells don't adhere to the hypotheses in the field. They are not quiescent, don't divide asymmetrically and don't depend on a mesenchymal niche. This culture system has been shown to be useful for many other tissues, and does not develop chromosomal aberrations even after a year. A small biopsy from the stomach, colon or small intestine from individuals would also generate a complete, self-renewing intestinal epithelium, a "mini-gut", from a single adult LGR5+ stem cell.

In the stomach, stem cells are thought to sit halfway at the bottom of the isthmus. However, LGR5+ cells are found at the base of the pyloric glands. A single one of these cells can grow stomach organoids[5]; small intestinal organoids and colon intestinal organoids will also grow under same conditions, and maintain their identity for at least a year.

In the pancreas, LGR5+ cells have not been seen either during development or in adults. Ducts have been proposed to harbor stem cells, but they're not well studied. Islets can self-renew to some extent, but they do so by throwing beta cells back into cycle and, like livers, increasing cell mass.

Tying a rope around a duct damages the pancreas, and regenerates cells that are Wnt-responsive. This duct ligation induces the appearance of LGR5+ stem cells only in the buds of the pancreas that are repairing themselves.

Once again, the single cells makes organoids, in this case adult pancreatic ducts. They never stop growing, and go through a ten-fold expansion every week. Unlike in the gut, however, these cells don't form an entire organ with all the adult structures.

They instead grow progenitors that express early endocrine markers. When growth factors are removed, the organoids form islet-like structures that produce some glucagon. Experiments are under way to see whether, over the long term, they can also produce insulin.

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Interrogating cancer stem cell function in brain tumors

A report on a lecture by
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Brain tumors appear to have a hierarchical cellular organization, suggestive of a stem cell foundation. It is possible to enrich for tumorigenic properties in sub-populations of tumor cells in freshly dissociated brain tumors from humans and from genetically engineered mice, using prospective cell sorting for cell-surface markers. Adherent tumor stem cell lines have been developed with high efficiency using defined media from both human malignant glioma and experimental mouse brain tumors. These lines display stem cell properties and initiate representative tumors following orthotopic engraftment. Lines from different tumors exhibit divergent gene expression signatures and differentiation behavior that correlate with specific neural progenitor subtypes, suggesting distinct cancer stem cell phenotypes from different patient tumors. These human and mouse tumorigenic stem cell lines are valuable tools for probing the chemical and genetic mechanisms that regulate cancer stem cell self renewal and differentiation, as well as defining heterogeneity within the same stem cell pool and between stem cells from different individuals. ***Peter Dirks*** *discussed the possibility that normal stem cells differentially express neurosignaling pathways, creating a stochastically heterogeneous group of cells within the stem cell compartment.*

The pathways that are involved in brain cancer also regulate neural stem cell (NSC) function. Two papers published in 2008 analyzing the human glioblastoma multiforme (GBM) genome turned up the big five pathways: PTEN, epidermal growth factor receptor, p53, RB and NF1[1,2].

Each of these pathways when perturbed is known to cause a pre-neoplastic expansion of the NSC pool. For example, when clonogenic cells are isolated from the cerebellum, the frequency of clonogenic cells in an *in vitro* limited dilution assay increases as p53 is taken away.

The idea of tumor initiation from stem cells is not new. As early as 1964, researchers had shown that single cells from mouse models of teratocarcinoma can initiate tumors. The frequency of tumor initiation is much higher when the cells return to the environment they had been initially propagated in rather than to the subcutaneous space. These single cells generate tumors that show all germ layers, and recapitulate all properties of tumor stem cells.

The results of studies in human leukemias and solid tumors by a number of different laboratories have suggested that human neoplasms consist of a heterogeneous population of cells, a small number of which are undifferentiated, highly malignant stem cells, that are responsible for disease propagation.

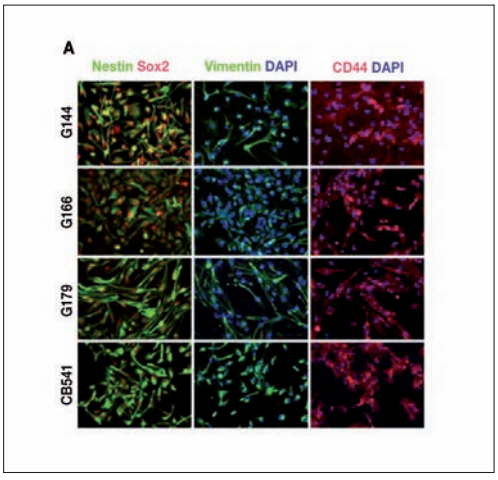

Figure 1
Stem cell lines express Nestin/SOX2 and combinations of other markers.

When fresh glioblastoma and medulloblastoma specimens are sorted for CD133, the marker enriches for a population of cells that can engraft in the mouse brain. In contrast, injections of thousands-fold more of cells that don't express this marker are not capable of initiating tumors[3].

By the time most human tumors are seen, they are well past the early neoplastic state and are full-blown tumors. Mouse models provide access to stages of the disease that are not accessible in human disease. They can also be useful for studying initiation, propagation and responses to therapy. In the mouse, CD133 is not an informative marker for brain tumor initiating cells, but CD15, also called SSEA-1, is useful. Still, CD15 is also an enriching marker, and does not isolate an extremely pure population.

For human samples, culture conditions have been established that enable populations of brain tumor precursors to grow out *in vitro*, traditionally as floating cell aggregates or spheres. More recently these conditions have been modified so that a purer population of precursor cells that don't express markers of differentiation can be efficiently derived. Cells in this population have a stem cell phenotype, and express Nestin and SOX2, but there is variation in the expression of cell-surface markers, so that no single cell surface marker can be used to identify tumor initiating populations.

For some tumor precursor lines, when about 100 GBM cells are injected into immuno-deficient mice, they migrate diffusely through the brain, which is a characteristic of glioblastoma, indicating that they recapitulate disease. These human brain tumor-derived NSC cultures provide an opportunity to probe some mechanisms involved in disease.

For example for some tumor precursor lines, treatment with a γ-secretase inhibitor (GSI) inhibits proliferation and/or survival of GBM precursor cells. GBM precursor lines generally express the activated form of Notch in the nucleus. GSI treatment can lead to loss of this expression of activated Notch in the nucleus. This is a dose-dependent effect, and up-regulates genes that specify differentiation programs such as Mash1.

The dominant negative form of the Notch transcriptional co-activator mastermind (DN-MAML) inhibits proliferation in a number of cell lines. The results suggest that there is some responsiveness to blocking Notch signaling, but it's not universal.

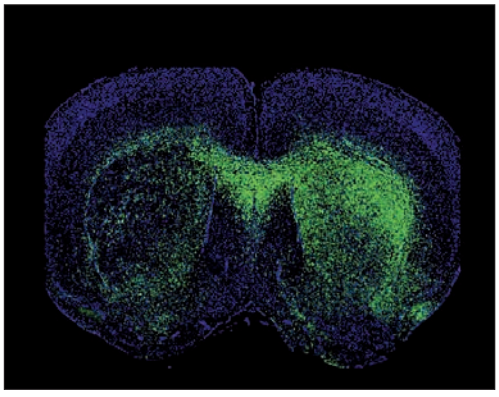

Figure 2
100 glioblastoma cells injected into immuno-deficient mice migrate diffusely through the brain.

Some studies have shown that Notch is important for lineage choice in the nervous system. In GBM, blocking Notch signaling with GSI promotes a lineage switch so that tumor cells begin expressing markers of neuronal differentiation. In conditions under which cells don't differentiate, GSI treatment leads to expression of b III tubulin+ neuron-like cells. These cells lose proliferative potential, assessed by BrdU labeling.

Ex vivo treatment with GSI has only a modest effect in enhancing survival of the mice. Although it's unclear where GSI acts, one strategy may be to shift cells into a neuronal differentiated compartment. Committing down this lineage may enhance the ability to treat these tumors.

Culture contrasts

Genetically engineered mouse models of brain tumors show that the tumors retain a hierarchy. For instance, in the well-characterized model of PTC+/- mice which get medulloblastomas that resemble human disease, fewer than 5% of the total cells express the markers Nestin and SOX2[4].

These cells don't grow very well as spheres, but when they grow out in adherent cultures, they express Nestin

and SOX2. When PTC+/- mice are crossed with Nestin-GFP mice, all the clones that arise are GFP+. In the case of primary cells, hundreds of thousands of cells have to be plated. But as p53 and PTC are taken away, the number of cells that need to be plated drops to 4,000 or 5,000 cells. The cells that are CD15+ can initiate growth *in vitro* in the absence of serum and generate CD15- cells, regenerating the heterogeneous culture. In contrast, fresh CD15- cells when plated do not grow robustly, and don't generate CD15+ cells. Once the cell lines are established, however, CD15 does not define a tumor-initiating phenotype, suggesting an adaptation to culture.

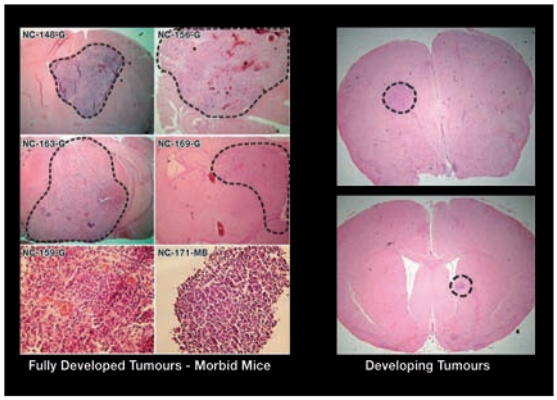

Figure 3
Phenotypically heterogeneous mouse gliomas.

In contrast to the hundreds of thousands of unsorted cells needed to create disease, just 10,000 cells from a CD15-enriched population can initiate tumors. At least in this model, there is a hierarchy in cells that can be identified by CD15 expression. This model will therefore be important for further development of anti-neoplastic agents that target tumors through the CD15+ population.

For a glioblastoma model, a p53-floxed mouse was crossed with Nestin-Cre. This is because when p53 alone is deleted, the mice get many other tumors, limiting the ability to study brain tumors. It has long been reported that when mice are given ENU, 100% of the mice develop brain tumors, mostly glioblastomas.

When mouse glioma stem cells are grown out in cell lines, they express immature and mature cell markers, with a few differentiated cell types that are not seen in human tumors. The cells can also be differentiated. For example, NC-166-G cells in serum express S100β and GFAP, but do not do so in EGF and FGF conditions.

The cell lines developed are tumorigenic and show an ability to generate tumors with lower cell number injections than unsorted populations. This provides an opportunity to study the different stages of the disease.

Microarray profiling of p53-WT and p53-deficient NSC compared with different mouse glioma cell lines shows that there are about 500 annotated genes that are down-regulated in p53-null compared with p53-WT, including TRP53 and its transcriptional targets. About 300 genes are up-regulated in p53-null compared with p53-WT cells. Comparing the glioma cells with non-tumor cells reveals a cancer profile of about 500 genes, including HOX genes and others in developmental pathways.

This approach makes it possible to explore the genetic and chemical space between mouse and human tumors. For instance, using the human glioma precursor lines and the mouse model, the BIOMOL library of neuro-modulatory compounds has been screened for drugs that target both human glioma cells and mouse cells. A smaller group of compounds can be tested on various animal models and xenografts.

Primed choice

When cells were isolated from the embryonic mouse brain, grown as spheres and then screened against a library of about 1,200 activated compounds known to penetrate into the nervous system, a high proportion of the 1,200 drugs turned out to be hits[5].

About 25% of the library comprises of compounds that modulate neuro-signaling pathways, including dopamine, acetylcholine, glutamate and serotonin. It is unclear whether these drugs act on the NSC or on differentiated cells that then feed back to the NSC.

NSC may express low levels of "promiscuous" expression of mature lineage genes, leaving the cell primed

in a state of indecision, and enabling it to respond to external cues in order to make fate choices. A given stem cell might exist in a precarious state in which a small perturbation up-regulates some programs and simultaneously suppresses others, prompting the cell to either differentiate or self-renew.

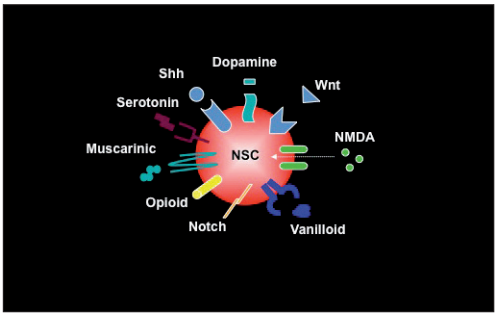

Figure 4
Alternative hypothesis: neural stem cells may be "primed".

In cultures of human NSC, every cell is equivalent for stem cell marker expression. RT-PCR analysis spanning a variety of neurotransmitter signaling pathways shows the expression of various functional neurotransmitters, including glutamate, acetylcholine, GABA, serotonin and dopamine.

Because many of these pathways have agonists and antagonists, it is also possible to measure transcriptional output related to signaling. For example, there is up regulation of NURR1, usually thought to be a dopaminergic-responsive transcription factor, in response to acetylcholine (ACh). There are similar results with dopamine and serotonin agonists, indicating that these cells do signal and cause downstream events.

To determine whether the cells differ in their expression of receptors from cell to cell, single cells are sorted into wells and analyzed with a multiplex PCR strategy. Using controls for both high-frequency and low-frequency genes, at the single-cell level not every cell expresses the same neurotransmitter genes. Dopamine pathway genes are quite rare, for example.

Shuttling states

When single cells are grown into colonies, the colonies recapitulate the differential expression of receptor pathways in the parental cells, suggesting that these cells are intrinsically programmed to create the heterogeneity of the primary culture.

Sorting specifically for one of these pathways could reveal whether, for instance, nicotinic receptor-positive cells remain committed to the nicotinic lineage, or whether they shuttle between different lineages.

Snake venom toxin, called α-bungarotoxin, binds to a rare population of CHRNA7+ neuronal stem cells, so sorting with fluorescent labeled α-bungarotoxin isolates pure populations of CHRNA7+ or CHRNA7- cells. Each of these populations can regenerate heterogeneity seen in the original culture, meaning that NSC heterogeneously express CHRNA7 in a reversible and equilibrating manner. These data suggest that there is shuttling between different states, at least for AChR.

Although NSC can be defined by some markers such as Nestin and SOX2, other signaling pathways may be differentially expressed, creating a stochastic heterogeneity. It's possible, for instance, that there is shuttling between different states from stem cell to stem cell, allowing the pool to function as a heterogeneous unit.

This could be a random process, so that a dopamine signal causes only a limited fraction of NSC to make a lineage choice, keeping other NSC competent to either proliferate or differentiate. Discreet shuttling between states could then allow more control than simultaneous responses to a signal.

There is some clue in the literature that a functional reason for this phenomenon may be that these chemicals are involved in influencing lineage choice. NSC treated with ACh, for example, more robustly differentiate into neurons. How the stem cell pool is organized may have important implications for treating cancer. If a cancer stem cell pool contains stochastic or cell-to-cell variability, or the cells shuttle between different states, it will be difficult to the target the right compounds to the right cell at the right time.

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Somatic diversity generated in neuronal stem cells

A report on a lecture by
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New neurons are born in the adult dentate gyrus of the hippocampus in the mammalian brain and then integrate into the circuitry and become functional. LINE-1, or long interspersed nucleotide elements-1, retroelements are active in somatic neuronal progenitor cells, providing an additional mechanism for neuronal diversification. Together with their mutated relatives, retroelement sequences constitute 45% of the mammalian genome, with L1 elements alone representing 20%. The fact that L1 can retrotranspose in a defined window of neuronal differentiation, changing the genetic information in single neurons in an arbitrary fashion, allows the brain to develop in distinctly different ways. These characteristics of variety and flexibility may contribute to the uniqueness of an individual brain. However, the molecular mechanism that regulates L1 expression in neuronal progenitor cells is not completely understood. L1s are likely to be silenced in neural stem cells due to SOX2-mediated transcription repression. Down-regulation of SOX2 accompanies chromatin modifications, such as DNA de-methylation and histone acetylation, which in turn may trigger neuronal differentiation. ***Fred Gage*** *suggested that characterization of somatic neuronal diversification will not only be relevant for the understanding of brain complexity and neuronal organization in mammals, but may also shed light on the differences in cognitive abilities.*

There are two systems of stem cells in the adult nervous system that have significant similarities and differences depending on their site of origin. One of the only two sites in the adult mammalian brain where adult neurogenesis occurs is a niche called the subventricular zone (SVZ). About 95% of the cells born here migrate out to the olfactory bulb and become what are called inhibitory neurons. These neurons rely on the neurotransmitter GABA for their signaling.

Cells born in the dentate gyrus (DG), in contrast, migrate very short distances and differentiate into excitatory neurons. There is debate as to which of these two populations constitutes the authentic stem cell. BrdU labeling, retroviruses and other tools can help identify the point at which a cell decides to become an inhibitory or excitatory neuron and the similarities and differences between these two areas of neurogenesis.

At day 3, the newborn cells in the adult brain are immature, with parallel branching processes. By day 7, they are polarized, with dendrites up and axons down. Over a period of a month, the neurons fully elaborate, develop synapses, make contact with their partners and make their way to the CA3, where they make unusual synapses. There is some evidence to suggest that these cells don't make new synapses, but rather compete with existing synapses. Cells that are born and make it through this process stably integrate into the circuitry.

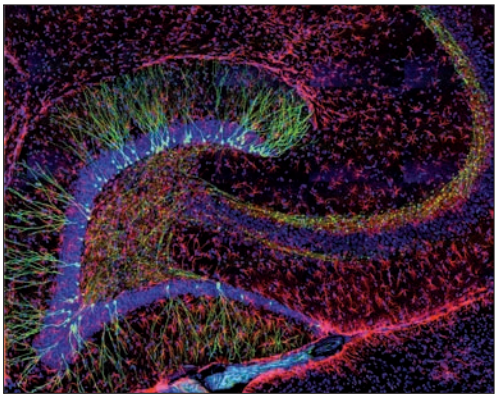

Figure 1
Adult neurogenesis occurs in only two sites in the adult mammalian brain.

When a mouse is injected with retroviruses, the virus only lasts a couple of hours *in vivo*, but the cells born during that two-hour period survive and, a couple of

months later, their dendrites and their axons can be seen integrated into the circuitry.

One view of this stem cell system is that there is a radial cell that is functionally quiescent. This radial cell becomes a rapidly amplifying cell — also called a non-radial cell — and then, if it survives amplification, differentiates into a fully born neuron. There are a variety of markers, mostly developed through histological markings, that define the cells at different stages.

Interestingly, although SOX2 is known to be important in induced pluripotent stem cells (iPSC), it persists in the adult brain, and fate-mapping studies reveal that it represents the self-renewing and multipotent neuronal stem cells in the adult hippocampus. Not having to add SOX2 may be one of the reasons it's easier to reprogram adult stem cells from the brain than from almost any other system in the body.

Neurogenic niches

In a reporter mouse, SOX2 is expressed exclusively in the SVZ and the DG. When radial and non-radial cells are directly isolated from the brain and expanded, SOX2+ cells express most of the phenotype of the non-radial cell, and not the quiescent radial cell, but they can give rise to all three lineages, at least *in vitro*.

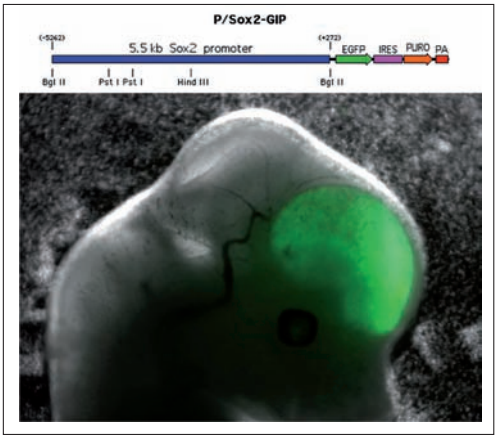

Figure 2
SOX2 expression represents self-renewing and multipotent neural stem cells.

To determine whether the cells have the same potential *in vivo*, a lentivirus carrying the SOX2 promoter is injected into ROSA26-GFP floxed reporter mouse. The lentivirus infects all or most cells, not just the dividing cells. If the animals are pulsed at various time points, there are cells that are SOX2+ even after two months, suggesting that they are self-renewing and maintaining themselves as progenitor cells. The same SOX2+ cells can also divide and give rise to PROX1+ neurons, which are specific for that region. Under rare conditions, the progenitors can also give rise to astrocytes.

In this model, the non-radial SOX2+ cells are stem cells that retain multipotency and self-renewal capacities at a single-cell level. By lineage tracing using low-titer retrovirus, these dividing non-radial cells periodically give rise to GFAP+ radial cells. Under conditions that induce proliferation of these cells — for example, stress or physical activity, which induces proliferation — radial cells can be activated into cycle and give rise to non-radial cells.

SOX2+ and another marker, DCX, both characterize the neurogenic niches. If SOX2-GFP mice are crossed with DCX-DsRed mice and the cells sorted for GFP and DsRed, microarrays show that, at the level of the stem cell, there are very few genes that differ between the two groups. There are more differences when the cells differentiate from an immature state into neurons[1].

Among the few genes that differ at the SOX2 level, one in particular differentiates between the two groups of cells. IGF2 is not expressed in the SVZ, but is expressed at high levels in the dentate granule cells. *In vitro*, an shRNA for IGF2 decreases the proliferative rate of the DG cells, but has no impact on the SVZ cells. This is an IGF2-mediated effect because it can be rescued by over-expressing IGF2 or just by adding it to the media.

At a later stage, when the cells are differentiating toward their intended fate, the SVZ cells express MASH1 and NGN2, which are important transcription factors that define inhibitory neurons, and are not expressed in the hippocampus.

If MASH1 is over-expressed in the DG cells, which normally turn into excitatory cells, these cells turn into oligodendrocytes, indicating that the transcription factor can redirect the fate of newborn cells. Putting MASH1 into the SVZ has no impact. It also has no effect *in vitro*, suggesting that the reprogramming is context-dependent.

LINE jumping

To determine the genes that are expressed in fully differentiated neurons, astrocytes and oligodendrocytes, clones of cells from the SVG and DG are purified and propagated *in vitro*. When these cells are differentiated into the lineages *in vitro* and analyzed by microarrays, the top 9 genes expressed in neurons are all LINE elements, or long interspersed nucleotide elements, from ORF2. PCR analysis confirms the high expression of LINE elements within these cells.

The LINE elements are non-LTR retrotransposons, which constitute 17% to 20% of the human genome. In the mouse, there are about 3,000 full-length or active elements. In humans, the number is much smaller, on the order of about 150[2].

In the normal life cycle of the L1 elements, the ribonuclear protein complex, or RNP, forms in the cytoplasm and reenters the nucleus only under conditions in which the nuclear membrane is broken. In those conditions, L1 can be inserted back into the genome by reverse transcriptase and it will express GFP.

When this construct is over-expressed in cells *in vitro*, only neural precursor cells show the insertion, and mesenchymal stem cells, hematopoietic stem cells and others do not. Inverse PCR of cloned individual cells shows that many of the insertion sites correspond to genes expressed in the central nervous system.

In L1-EGFP transgenic mice with this reporter construct, cells expressing GFP are seen in the neurons of the cerebellum, the amygdala, the hypothalamus and pretty much everywhere in the adult brain, but not in other organs.

There is never any jumping or mobility in stem cells. Just as in the neural progenitor cells, SOX2 seems to be important for suppressing L1 activity in the stem cells: as L1 activity goes up, SOX2 expression decreases[3].

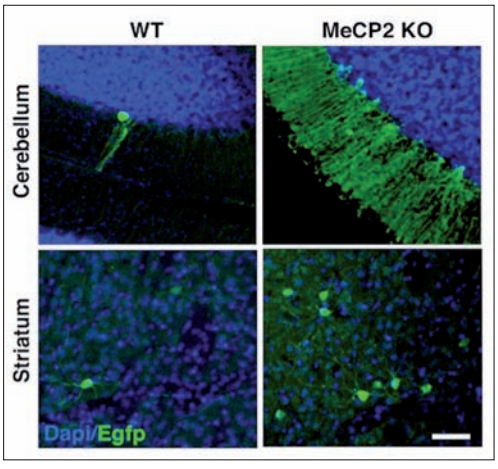

Figure 3
There are more EGFP+ cells in the brains of MeCP2-knockout mice.

There are also histone marks associated with the L1 promoters, as well as CpG islands surrounding the 5' UTR. siRNA for MeCP2 dramatically increases activity of the reporter construct, suggesting that SOX2 is not an exclusive regulator. A MeCP2 knockout mouse also shows a dramatic increase in L1 activity. SOX2 inhibits part of this activity, but not all, suggesting that both MeCP2 and SOX2 are required for L1 silencing in neuronal stem cells, and the two together may form a repressor complex.

If the MeCP2 knockout mouse is crossed into the L1-EGFP mouse, there is an unexpected increase in EGFP+ cells in the brains of these MeCP2 knockout mice, particularly in the striatum, cerebellum and olfactory bulb.

The L1-EGFP transgene is a single element and there are about 3,000 endogenous elements in mice. If the L1 elements insert into neurons, then neurons should have more DNA than do other tissues in the same animal. There should also be more detectable insertions in the brains of MeCP2 knockout mice compared with wild type brains.

If brain cells, fibroblasts and cells from other tissues are taken from wild type mice, MeCP2 knockout mice, and MBD mice, using TaqMan amplification to quantify the DNA, there's no difference in the amount of DNA in the fibroblasts. But there is a dramatic increase in DNA in neuroepithelial cells, and even more so in the MeCP2 knockout mice than in MBD mice.

There's an increase in the amount of L1 activity going up the phylogenetic hierarchy to humans. Using about 80 picograms (pg) of DNA in each reaction, the hippocampus and cortex consistently show more ORF2 content than do the liver and heart.

By a rough calculation, each reaction contains 80 pg of DNA and the human cell genome has 6.6 pg of DNA, equaling approximately 12 cells for each reaction, and an estimated 80-300 insertions per cell. This estimate needs to be confirmed by other methods.

In humans, MeCP2 loss causes Rett syndrome, and there are consistently more ORFs in the brain and heart than in other tissue from patients with Rett syndrome, as well as a significant difference in the heart and brain between patients and controls.

In this model, SOX2, MeCP2 and the HDAC control element regulate neurogenesis, maintaining the cells in a stem cell state and keeping L1 activity silent. When that's relieved and the cell differentiates into a neuron, there's a short period in which the nuclear membrane is open and L1 insertion can occur. In conclusion, neural progenitor cells can support L1 retrotransposition and L1 can affect neuronal gene expression with consequences for the cell.

Primate evolution

Chimps and humans are different, and there are significant differences in the brain in terms of brain size, language and cognitive function. One way to compare the species is to use iPSC generated from fibroblasts taken from a range of old-world and new-world monkeys.

This approach provides an opportunity to gain insights into human evolution using primate stem cells. It's difficult to obtain good quality tissue samples from apes, but iPSC are a long-lasting resource of ape cells. They constitute a good system to study cell behavior, including neuronal differentiation and behavior. The system also allows for screening tests that cannot be performed in live animals.

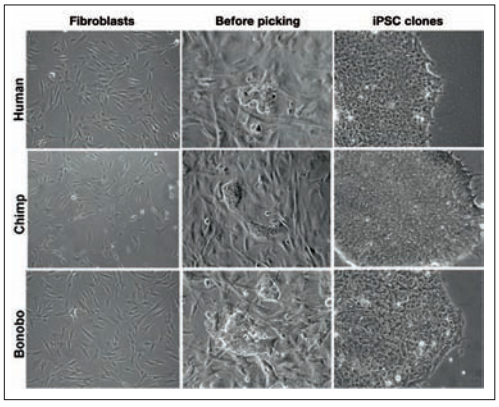

Figure 4
Reprogramming primate fibroblasts.

However, there are significant caveats. For example, there are differences between human and chimps that may rely on specific cell types or on environmental components that can't be produced *in vitro*.

If the fibroblasts from different species are reprogrammed with a retrovirus using the human transcription factors OCT4, SOX2, c-MYC and KLF4, they form colonies and maintain some of the key features of iPSC.

There was a fusion event between bonobos and humans at chromosome 2 and 3, so the apes have more chromosomes than do humans. By karyotyping, humans have 46 chromosomes, whereas chimps have 48. Both sets of cells express pluripotency markers such as Nanog and Tra-1-81, they form teratomas in nude mice and can differentiate *in vitro* into beating cardiomyocytes and neurons. The mitochondrial control region 1 is the same size in chimps and humans, but the sequences are different. The human L1 construct can infect both humans and apes, although how much of the L1 inserts and where has not yet been determined.

One of the major differences between humans and chimps is that humans don't make an enzyme that converts a specific sialic acid on the cell surface. Instead of the enzyme Neu5Gc, humans make an excess of Neu5Ac. This is actually an issue with co-culturing human embryonic stem cells and mouse fibroblasts.

Bonobo and chimp cells make NeuGc when they differentiate into neurons. When the cells are differentiated further for 2 months under these conditions, neurons develop synapses and spines and function appropriately under voltage clamp conditions.

One gene that is interesting for further study is neuropsin, which was shown recently to be uniquely alternatively spliced uniquely in humans. Humans have two versions of the protein, whereas chimps and lesser mammals only have one[4]. The two forms of the gene are expressed in human placenta, but also in the prefrontal cortex and the hippocampus. Neuropsin is important for spine formation, so the difference in humans and chimps may influence how spines are formed during development. In conclusion, iPSC can be generated from chimps and bonobos using the same methods as for human iPSC, and these iPSC can be induced to functional neurons *in vitro*. This species-specific neuronal derivation is a new tool to search for evolutionary changes in primate brain function.

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Not all cancers follow a stem cell model

A report on a lecture by
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Some cancers appear to follow a cancer stem cell model, in which tumorigenic cells are rare, phenotypically distinct from the vast majority of other tumor cells, and robustly hierarchically organized. In other human and mouse cancers, including melanoma, however, tumorigenic capacity is a common attribute of cells and there is no clear evidence of hierarchical organization. The growth and progression of these cancers does not appear to be driven by rare cancer stem cells (CSC). Optimizing xenotransplantation assay conditions, including the use of more highly immunocompromised NOD/SCID IL2Rγnull mice rather than NOD/SCID mice, can increase the detection of tumorigenic cells by orders of magnitude. In these optimized assays, more than 25% of cells from stage III and IV melanomas have the potential to form tumors after transplantation. By marker analysis also, melanoma lacks the obvious hierarchical organization observed in cancers that follow a stem cell model. Melanomas thus contain phenotypically diverse tumorigenic cells, and heterogeneity appears to arise mainly from reversible phenotypic changes in tumorigenic cells. ***Sean Morrison*** *proposed that some cancers like melanoma do not follow the CSC model, and so it will not be possible to cure them by targeting rare CSC.*

The heterogeneity of tumors has been known for years. According to the stochastic or clonal evolution model of cancer, the intrinsic differences between tumor cells are driven by ongoing genetic change that can be positively or negatively selected. Nonetheless, this model holds, many cells with many different phenotypes within a tumor are capable of proliferating extensively and of forming a tumor, and therapies must therefore try to kill every cell in the tumor.

An alternative view initially emerged in the 1960s and 1970s, and revived in the 1990s with some work on hematologic malignancies (see Dick, page 61) and breast cancer. According to this model, a tumor can be understood in much the same terms as a normal tissue. Like normal tissue, tumors have a small sub-population of stem cells that are capable of self-renewing extensively, giving rise to phenotypically diverse progeny with less proliferative potential.

These cancer stem cells (CSC) are uniquely capable of forming new tumors, but they can undergo an aberrant process of epigenetic change to convert into phenotypically diverse progeny with little or no ability to proliferate. The fundamental prediction based on this model is that cancer can be treated effectively by targeting this sub-population of CSC, rather than going after all the cells.

Some cancers clearly follow this CSC model, but others fail this prediction, and follow a more complicated progression.

The data from mouse models of acute myelogenous leukemia (AML) are consistent with the CSC model. Conditionally deleting PTEN from adult hematopoietic cells generates both AML and acute lymphoblastic leukemia (ALL) in mice. If 2 million bone marrow cells from those mice are transplanted into wild type

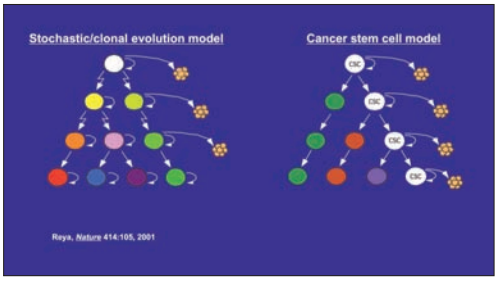

Figure 1
Different models of tumorigenesis.

recipients, half of the recipients develop AML and the other half of the recipients develop AML and ALL.

In contrast, if 20,000 myeloid blast cells are transplanted, half of the recipients develop AML and die, but the other half never develop disease. That says that, at least in the case of histo-compatible transplants into mice, not every cell is capable of proliferating extensively and causing disease.

Instead, a sub-population of cells is enriched with the capacity to transfer disease. When cells with surface markers similar to hematopoietic stem cells (HSC) are sorted out, and 10 to 15 of those cells transplanted into mice, 4 of 25 mice develop AML. The rest of the mice engraft with donor hematopoietic cells, but never develop disease.

These results suggest that cells with a primitive phenotype in this mouse are about 400-fold enriched for leukemogenic activity as compared with the unfractionated population[1]. This is consistent with others' results. All the activity is not within the stem cell population (see Dick, page 61), however. There is some activity in mature myeloid cells, but much much less than in the stem cell pool.

Transferring disease

AML is not the only cancer that follows a stem cell model. A model of chronic myelogenous leukemia (CML) was created by conditionally activating HIP1/PDGFβR and AML1/ETO translocations, each of which are knocked into their endogenous loci and conditionally activated with Mx1-Cre by deleting a stop codon.

When both translocations are activated at the same time, mice develop leukemia within a week. The disease looks like CML and when the mice are treated with imatinib, they're very responsive. Their spleen decreases down to normal size.

Even if large numbers of bone marrow cells from the mice are transplanted, they are rarely able to transfer disease into wild type recipients. But if relatively small numbers of cells with a phenotype similar to that of normal HSC are sorted, those cells are readily able to transfer disease, demonstrating that cells with this primitive phenotype are highly enriched for leukemogenic activity.

CSC are a lot less sensitive to imatinib than are other tumor cells. In this mouse model, if bone marrow cells from untreated mice are transplanted, very few of the mice engraft with CML. If the mice are treated with imatinib, the prediction would be that it should be more difficult to transfer disease from this healthy mouse than from a sick mouse. But, in fact, the imatinib-treated mice transfer disease far more readily than untreated mice.

This suggests that imatinib treatment is enriching the leukemogenic cells in these mice by orders of magnitude. The same is true if cells with a phenotype similar to that of HSC are sorted out. Those cells are much more able to transfer disease when treated with imatinib than when the mice are untreated.

This system directly confirms the difference in sensitivity of the leukemogenic cells versus the differentiated CML cells to imatinib[2]. It also provides an opportunity to study what would render the leukemogenic cells more sensitive to imatinib treatment, potentially identifying things that could be combined with imatinib to make it more effective.

Rare populations

Some cancers do follow a stem cell model, and that group clearly includes CML and AML and some solid cancers. For example, there is compelling evidence that some breast cancers, epithelial cancers and some brain tumors follow the CSC model. But it is unclear how generally applicable the model is.

Melanoma had been proposed to follow the CSC model, with only 1 in a million metastatic melanoma cells

being tumorigenic, and targeting that rare sub-population was the desired goal.

Taking tumors from 7 different patients, single-cell populations are transferred into NOD/SCID mice. Consistent with the prevailing hypothesis, about 1 in a million cells form a tumor in NOD/SCID mice 8 weeks after transplantation. If the mice are allowed to live longer, to 6 months instead of 2 months, however, these odds rise to 1 in a 100,000 cells, suggesting that tumorigenesis assays should not be stopped too early. Nonetheless, the results are consistent with the idea that tumorigenic cells are very rare.

If the cells are transplanted into highly immunocompromised NOD/SCID IL2Rγ^{null} mice, however, there is a 170-fold increase in the number of tumorigenic cells. Mixing the cells with matrigel prior to injection gives another 20-fold increase.

NOD/SCID mice, although they have no B cells or T cells, do have functional NK cells. There is a powerful xenogeneic immune response that's mounted against many human cells that get transplanted into those mice. As a result, the frequency of tumorigenic cells is being underestimated, particularly in melanoma.

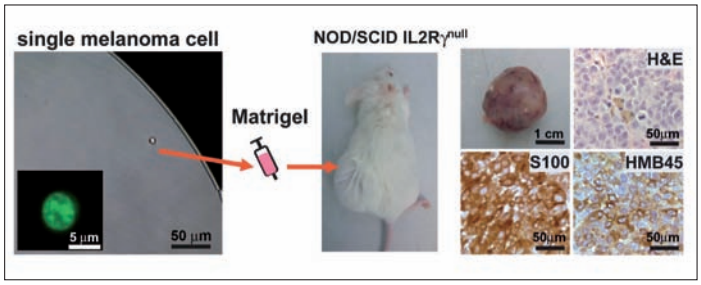

Figure 2
Single melanoma cells mixed with matrigel and injected into immunocompromised mice initiate tumors.

To assess the magnitude of the difference, single-cell suspensions were made from 3 tumors obtained from patients. Using the same single-cell suspension, side-by-side injections were introduced either into NOD/SCID mice or into NSG mice with matrigel. This makes an enormous difference.

Three injections of 50,000 cells into the NOD/SCID mice didn't form any tumors, but from the same single-cell suspension, 4 of 6 injections of only 5 cells with the modified assays led to tumor formation. Instead of being rare, cells with a tumorigenic capacity in fact seem to be quite common under these modified assay conditions[3].

A large number of tumors from other patients have been tested, in some cases expanding the cells first in xenografts, in other cases transplanting the cells directly from patients. Most of these tumors are stage III tumors, and a few are stage IV. Many are metastatic, and some are primary cutaneous melanomas but, in all cases, the results are the same.

In every case, there is a very high frequency of cells with tumorigenic potential. On average, 1 in 9 cells with the limited dilution analysis on xenografts, or 1 in 4 when they're directly from patients, lead to tumors. For the first time in cancer biology, it is possible to literally take single cells from patients, put those single cells into mice, and study human tumorigenesis *in vivo*, opening up many questions to more precise study.

Unequal abilities

One possible explanation for these results is that there is a hierarchy, with different classes of tumorigenic cells. Some tumor cells have unlimited tumorigenic capacity, and some cells have a more limited capacity. Although these cells are not equal, in a single transplant, they would all read out as having tumorigenic potential. To test this hypothesis, single cells from individual tumors are taken from patients and transplanted into mice. Once they generate a tumor, 100 cells from the tumor are serially transplanted to a second and third generation.

It turns out that none of the tumors generated is a dead end. In every case, the tumors are indefinitely serially

transplantable. And in every case, unless the mouse is euthanized, the tumor will grow and kill the mouse first.

Metastatic melanoma has a much higher frequency of tumorigenic cells than in cancers that follow the CSC model. But this doesn't prove that melanoma doesn't follow the CSC model. It is possible, for instance, that 30% of the tumor cells have tumorigenic capacity and 70% lack that capacity.

To address whether there are intrinsic differences in melanoma cells, 50 markers in melanoma have been evaluated. Of these, there are 17 markers that frequently identify phenotypic heterogeneity among melanoma cells, and they can be used to identify a sub-population of melanoma cells with a higher tumorigenic capacity.

But when the positive fraction and negative fraction for many such markers are sorted and injected into mice, none of these markers distinguishes tumorigenic from non-tumorigenic cells. Every fraction sorted so far has high tumorigenic capacity, even when only 10 cells are injected.

CD133 has been suggested as a marker to identify melanoma stem cells, although there is no functional data to support that assertion. But using this assay, 10 cells from the CD133+ and CD133- fractions both readily form tumors. ABCB5 is another marker claimed to be a CSC marker, but just 10 cells from the ABCB5- fraction almost always form a tumor, just as in the ABCB5+ fraction.

p75 is a marker that has been used to purify neural crest stem cells. A *Cancer Research* paper published in January 2010 used a realistic mouse model of melanoma with BRAF and PTEN mutations, and also found that single cells from a mouse melanoma can readily transfer disease. When the researchers sorted p75+ versus p75- cells, both fractions formed tumors, but the p75- fraction generated more tumors, leading them to conclude that p75- cells have more tumorigenecity than p75+ cells.

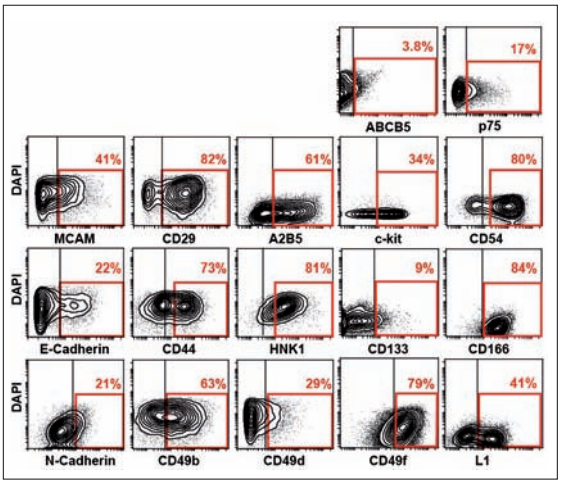

Figure 3
Phenotypic heterogeneity among melanoma cells.

But using the assays described above, even injecting 10 cells, from 4 independent tumors, readily generates tumors from from both p75+ and p75- fractions. There is no correlation between p75 staining and frequency of melanoma initiating cells.

One possibility is that a less sensitive assay detects less activity in the p75- fraction. Another possibility is that much of the data is obtained from xenografted melanomas, and as the p75 marker falls apart after xenografting, it's most useful in fresh cutaneous melanomas.

Recapitulating heterogeneity

One of the fundamental arguments in the CSC model is that tumorigenic cells recapitulate the phenotypic heterogeneity of the tumors from which they derive. When CD133+ and CD133- cells are sorted from tumors, both fractions readily form tumors. In fact, both fractions form a tumor that recapitulates the heterogeneity of CD133 in the original tumor.

For instance, if the original tumor had 2.5% CD133+ cells, the new tumor shows roughly the same proportion, regardless of whether it arises from CD133+ cells or from CD133- cells.

This capacity to recapitulate the phenotypic heterogeneity of a tumor is not necessarily a unique property

of a CSC. Some cancers may have many cells that are capable of recapitulating the phenotypic heterogeneity of the tumors from which they derive.

The arguments about recapitulating heterogeneity had been based on superficial analyses of a couple of markers. But the tumors are indistinguishable when looking at CD133, p75, α-2 integrin, MCAM, α-6 integrin, A2B5 and CD44. Even looking across a large panel of markers, there's no difference in phenotypic diversity within tumors that arise from cells that express different markers at the outset, and have sometimes been assumed to be intrinsically different.

In the clonal evolution model, heterogeneity is driven by irreversible genetic changes occurring within the cells. In the CSC model, heterogeneity is driven by irreversible epigenetic changes akin to differentiation, generating post-mitotic derivatives from rapidly dividing progeny. In melanoma, there appear to be many markers being turned on and off in a reversible way within the cells. The phenotypic diversity in the cells seems mostly driven by these reversible changes in marker expression[4].

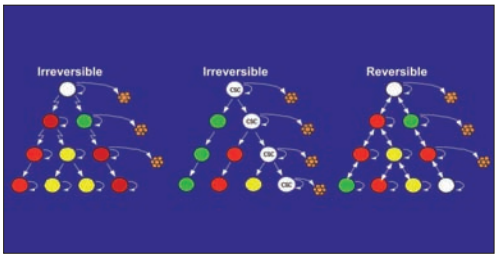

Figure 4
Phenotypic differences among cancer cells are not necessarily associated with functional differences or irreversible changes.

Although there is substantial heterogeneity, none of the markers seem to distinguish tumorigenic cells from non-tumorigenic cells. In every case, no matter what fraction is transplanted, they always recapitulate the phenotypic diversity of the tumor from which they derive. There may be other cases in which heterogeneity is similarly largely driven by reversible changes.

If the heterogeneity were driven by genetic changes, the cells would not be able to recapitulate the heterogeneity of the tumors from which they derive. That is, if every cell within the patient tumor is genetically different from every other, single cells taken from a patient's tumor and transplanted into a lot of different mice should make tumors that are all genetically distinct from each other. If there is a fair amount of genetic stability within the patient's tumor so that every cell is essentially genetically the same, then these tumors won't differ from each other.

To get a better handle on the rate of genetic change, single cells from the A1 generation of tumors are taken and transplanted. SNP genotyping of all these tumors reveals that the tumors are all very genetically similar to each other, at least at the level of copy number variations.

However, even if melanoma doesn't follow the CSC model, the stem cell perspective can help understand how cancer works, particularly for phenomena like metastasis.

In summary, observations in melanoma are different than what the CSC model predicts. First, melanoma has a high, not low, frequency of tumorigenic cells. This is very different from AML, which has a low frequency of tumorigenic cells with very robust hierarchy, and obvious markers that distinguish the leukemogenic from non-leukemogenic cells.

So far at least, there are no markers that robustly distinguish tumorigenic from non-tumorigenic cells, and there is no evidence of hierarchical organization. No matter what sub-population is sorted out, melanoma cells with many phenotypes can all recapitulate the phenotypic heterogeneity of the tumor from which they derive. The heterogeneity of tumors is driven by reversible phenotypic changes and irreversible genetic changes rather than by the epigenetic hierarchical changes envisioned by the CSC model.

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PART II: Understanding stem cells complexity

Implications of cancer stem cells in brain tumor biology

A report on a lecture by
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Existing therapies against brain tumors do not take into account the complexity of the tumors. In particular, they are not based on a full understanding of cancer stem cells (CSC), which have a close relationship to their microenvironment. For instance, the vasculature in CSC-derived tumors is markedly different, which is important because there are therapies available that specifically target the vascular endothelial growth factor. Hypoxia, which is prominent component of the tumor microenvironment, induces non-stem cells to express several CSC markers. In particular, hypoxia prominently affects the expression of HIF2-α, which seems to be important only in CSC. Several signaling pathways, including Notch and PI3K/AKT are also important in regulating CSC. CD133 is often used as a marker for stem cells, and, in fact, is useful even in primary tumors taken straight from the operating room. The expression of another marker, integrin α-6, is stronger than CD133 in predicting tumorsphere formation and tumor growth. Although integrin α-6 is not an easy target because it's stable, there are integrin inhibitors in clinical trials that may prove to be useful therapies. ***Jeremy Rich*** *argued CSC are at the center of a nexus, and must not be studied in isolation.*

Each year, there are 18,000 cases of primary central nervous system tumors, including glioblastoma. With nearly 13,000 deaths per year, brain tumors are the leading cause of cancer death in children. But therapies against this disease have been ineffective, in part because most approaches have failed to account for the diversity of cells within a tumor. The viewpoint on stem cells, in particular, needs to be broadened.

Stem cells are usually judged by these criteria: persistent self-renewal, sustained proliferation tumor initiation/propagation, rarity within a tumor, stem cell marker expression and the ability to differentiate along multiple lineages. But both normal stem cells and cancer stem cells (CSC) have additional properties that should be considered.

For example, the ability to move, the ability to evade the immune system and the ability to have a relationship with blood vessels are all important. The relationship to a niche and promotion of angiogenesis are closely related.

There are lessons to be learned from looking at other diseases for which failure of therapies is endemic. One of these is tuberculosis, which kills 1.6 million people a year. An epigenetically created sub-population of tuberculosis infection, called the latent infection, is relatively resistant to therapies. As in cancer, the reason for resistance is that permissive conditions are created to stimulate growth and, not surprisingly, a large number of drugs developed essentially target proliferation.

Epigenetic regulation identifies new molecular targets that may preferentially target the sub-population, but are ineffective against the bulk infection. This non-replicative persistence is an epigenetic state that is regulated by microenvironmental conditions almost identical to that of CSC.

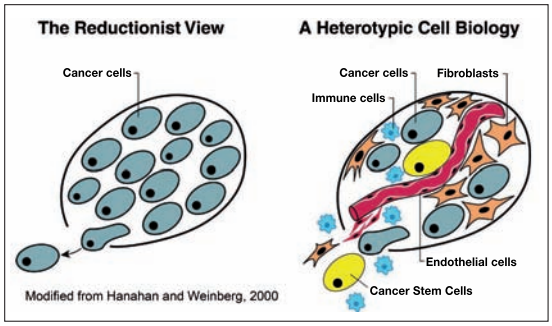

Figure 1
How can we better model cancer?

These different states can be seen as attractor states in a non-linear dynamic relationship, and a cell has the ability to adapt to these different fates because there are relative points of stability. As the tumor cell progresses, there's likely to be an interplay between the epigenetic state and genetic state. Blocking one pathway that a cell takes to assume the epigenetic state could open up another pathway, however.

Reliable marker

To characterize brain tumor stem cells at a population level, CD133 is a useful marker for primary tumors right out of the operating room. Xenografts are also useful as long as they're short-term and can maintain the hierarchy. These cells form cell-culture artifacts, and the external surface of the spheres express high levels of many other stem cell markers.

Using a laminin-based substrate, it is possible to maintain a homogeneous, less differentiated population of these cells, but some differentiation events still occur. Interrogating a variety of stem cell markers shows that the bulk of the markers do not asymmetrically segregate. CD133 is actually the only marker that consistently identifies asymmetric cell divisions.

Removing growth factor augments asymmetric division and increases symmetric divisions aimed toward differentiation. In contrast, cyclopamine, which blocks hedgehog signaling, leads to a decrease in asymmetric cell division. This is consistent in several different tumors.

Also of interest are two markers that have been commonly used in brain tumors: CD133 segregates with cell survival, whereas CD15 marks proliferation.

Radiation, the single most effective non-surgical therapy for brain tumors, increases the expression of CD133. This is not an induction due to radiation, but rather because there is an increase in cell numbers. The net effect is that if a constant number of cells is maintained, there is a decreased latency in tumor growth.

This is because of a difference in cell death, so that cells undergoing apoptosis and segregation enrich for the ability to survive after radiation. Mechanistically, this is not because of a difference in the ability to resist damage, bur rather in repairing damage.

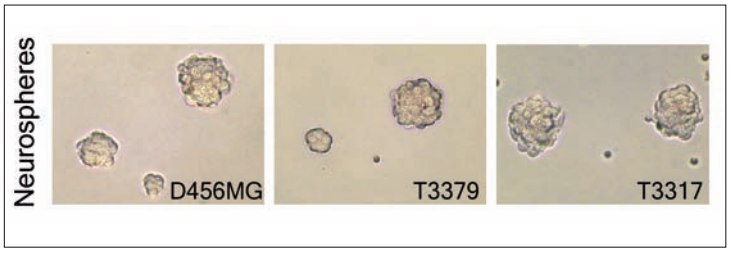

Figure 2
CD133+ tumor cells form spheroids.

The comet assay, in which populations of cells are differentially labeled, shows that cells derived from CD133+ cells start to repopulate the tumor. Radiation that patients receive essentially has no impact on the ability of these cells to generate secondary tumors.

This seems at least in part to be linked to dysregulation of the DNA damage checkpoint response. Transient activation of this checkpoint is beneficial for cell survival. There are drugs available that sensitize cells to the checkpoint by blocking the CHK1 and CHK2 kinases.

Notch inhibition

Notch's role in CSC doesn't relate to the DNA damage checkpoint response, but to other survival mechanisms. Radiation itself induces the activation state of the Notch pathway.

γ-secretase inhibitors can sensitize CSC to radiation[1]. In CD133- cells, Notch activation is completely dispensable. There is no activity in combination between the radiation and the γ-secretase inhibitor, which

presents a challenge in the clinic.

Brain tumor stem cells are a product of their environment and require their environment to be contained. The most prominent aspect of this is the vasculature. Under the microscope, tumors derived from CD133+ cells have a dramatically different and increased vascularity, compared with rare tumors derived from CD133- cells. This appears at least partly to be because of a secreted factor. Migration, tube formation and other *in vitro* measures of angiogenesis are differentially regulated in CD133+ conditioned media.

The factor that stands out most dramatically in this media is vascular endothelial growth factor (VEGF). This is important because there are substantial efforts, including in neuro-oncology, against VEGF. The VEGF inhibitor bevacizumab potently blocks the effects on endothelial cells of the conditioned media from CD133+ cells. Others have confirmed these studies and shown that SDF-1 mediates an additional mechanism.

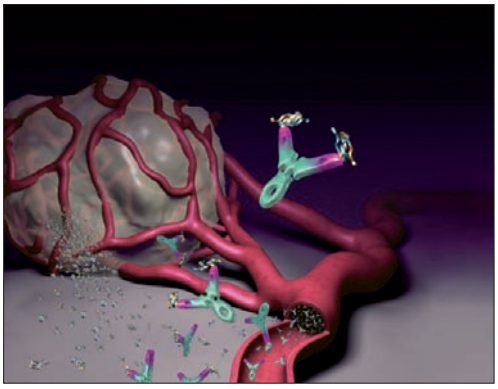

Figure 3
The VEGF inhibitor bevacizumab blocks angiogenesis in CD133+ cells.

Bevacizumab appears to specifically block angiogenesis in the CD133+ population, whereas the CD133- derived populations are resistant to its effects. In the clinic, the drug seems to reverse vascular integrity, and there are changes in the patients' quality of life.

Others have shown that there is a perivascular niche in which the CSC seem to reside, and that the drug might also be targeting this niche.

Hypoxia is one candidate for what might be instructing cells in this niche to express more VEGF, but HIF1-α levels were actually higher in the non-stem cells. However, HIF2-α has very consistently proven to be a factor. Normoxia, characterized by oxygen levels of between 2% and 5%, stabilizes HIF2-α, explaining the co-localization of HIF2-α and a perivascular group of CD133+ cells as well as perinecrotic areas.

Interestingly, this is not a mechanism shared by neural progenitor cells, suggesting there may be a therapeutic index. The net effect is that both HIF1-α and HIF2-α play a role in CD133+ derived cells, and their effect is additive. If both are knocked down, VEGF levels decrease further, whereas HIF2-α is dispensable in non-stem cells.

HIF1 and HIF2 also both play a role as seen in the serial neurosphere formation assay. The data suggest that HIF1-α is clearly expressed in all the populations, but the addition of HIF2-α allows cells to become more stem-like. Targeted knockdown shows that HIF1 and HIF2 both play a role in the proliferation and survival of stem cells, whereas HIF1 is the only one that's relevant in the non-stem cells.

The *in vivo* setting shows similar effects with a VEGF knockdown as with a HIF knockdown. With a selection assay, which is difficult to do because HIF knockdown is toxic to cells, viable cells show little or no tumor initiation. The importance of HIF2-α is not just an animal artifact. It is possible to segregate patient survival based on HIF2-α alone.

Non-stem cells don't form tumors, they're adherent and they don't express stem cell markers. But just with hypoxia alone, there is induction of HIF2-α, OCT4, Nanog and c-MYC. Very often, HIF2-α is prominently affected.

If a non-degradable form of HIF2-α is introduced into cells that do not fulfill CSC criteria, they start to self-renew. It is also possible to get tumor growth and induction of other stem cell markers just by HIF2-α expression. By manipulating microenvironmental conditions, a cell that fails to live up the CSC criteria can be changed to fulfill all criteria for CSC[2].

Required regulators

AKT signaling has the ability to induce apoptosis in the CD133+ population, but is dispensable in the CD133- population. PI3K inhibitors have the same effect. AKT inhibition has a dose-dependent effect on self-renewal, as measured by neurosphere formation, and on invasion in the CD133+ population.

One regulator for the CSC phenotype that is not shared by normal stem cells is L1CAM. The bulk of cells are positive for CD133 and L1CAM, which is a cell adhesion molecule. Targeted disruption of L1CAM results in the induction of cell death, specifically in the CSC-enriched population. This allows for the targeting of these tumors *in vivo*.

There is also a very clear enrichment of c-MYC expression at the RNA and protein levels in the CD133+ fraction. Targeting c-MYC induces apoptosis, blocks self-renewal and attenuates the ability to form tumors.

Most recently, something called A20 has received a lot of attention as a tumor suppressor in the lymphoma literature. A20 both de-ubiquitinates and ubiquitinates proteins that regulate TNF-α signaling and a variety of other pathways.

In this case, the effect is the opposite of that seen in lymphoma. There is an enrichment in mRNA level and in protein in the CD133+ fraction. Targeting A20 induces apoptosis and alters the activation state of the NF-κB pathway, a key regulator of CSC[3]. Interestingly, TNF-α can induce in the non-stem cells an amount of cell death that is not shared with CSC. *In vivo*, the targeting factor extends lifespan and reduces tumor initiation.

There is an interaction in the normal stem cell situation between the extracellular matrix and the vasculature that's very similar to that seen in CSC. One of the key regulators of this is laminin, which binds to the receptor integrin-α6, either paired with β-1 or β-4. β-1 is seen in the tumors, but in the specimens, integrin α-6 is expressed in the peri-vascular region.

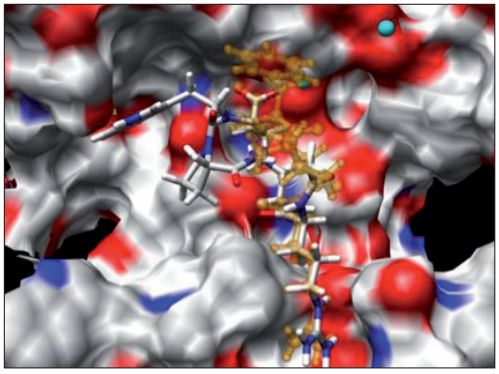

Figure 4
Integrin inhibitors such as celingitide may prove to be useful therapies.

At the the mRNA level, integrin α-6 is strongly differentially expressed, and the cells can be binned on the basis of its expression. In fact, integrin α-6 expression is stronger than CD133 in predicting tumorsphere formation and tumor growth[4].

However, integrin α-6 is not an easy target because it's so stable. If it is targeted with RNAi, it's difficult to get high levels of knockdown, but it is still possible to extend lifespan. A neutralizing antibody also has some effects. *In silico*, there is an inverse relationship to patient survival. There are integrin inhibitors in clinical trials that may prove to be useful therapies.

Autocrine loops

There are also some autocrine loops that can be targeted. One of them is EPO, which has been controversial because although it alleviates anemia, it also potentially stimulates tumor growth.

The EPO receptor is preferentially expressed in CSC. It is possible to sort cells using the EPO receptor, although it's not as good as integrin α-6. Nonetheless, this sorting enriches for neurosphere formation and tumor growth. Targeting the EPO receptor results in a loss of cell survival, induction of apoptosis and decrease in self-renewal. Again, there is an inverse relationship between patient survival and EPO receptor expression.

Non-stem cells can signal to stem cells. The receptors for interleukin-6, both gp130 as well as the canonical IL6-R receptor, are expressed in the CSC-enriched population.

Interestingly, expression of the ligand is higher in the non-stem cells. Targeting the receptor shows an attenuation of neurosphere formation, suggesting disruption of an autocrine loop, both *in vitro* and in *vivo*[5].

One of the downstream targets for IL6 signaling is STAT3. Targeting this extends the lifespan and there is an inverse relationship with patient survival, suggesting that these may be a surrogate marker of CSC frequency. The ligand antibody blocks the ligand and shows decreased tumor growth and induction of cell death.

Intriguingly, Genentech has received FDA approval for an IL6-R antagonist, tocilizumab (Actemra), that is active and tolerated for rheumatoid arthritis. This drug may be useful for some cancers, although probably not for brain tumors.

In conclusion, CSC cannot be considered in isolation, but must be seen as communicating with their microenvironment. Hypoxia is a dominant regulator of this interaction, regulating HIFs in part through PI3K/AKT. HIF2-α binds and stabilizes c-MYC containing complexes. There are some key downstream regulators, including STAT3.

In a way, CSC are at the the center of a nexus. Studying the cells *ex vivo*, or even in the best *in vivo* assays, doesn't recapitulate the true nexus. Studies that intend to capture the cells' complexity must take into account immune interactions, as well as interplay with non-CSC.

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Niche-initiated oncogenesis

A report on a lecture by
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The microenvironment in which tumor cells reside is a recognized modulator of tumor cell behavior. Whether that environment can participate in the induction of cancer is less clear and difficult to investigate, in part because of the limited understanding of the cell types comprising stroma. The hematopoietic system has been informative in defining heterologous cells in the microenvironment that can serve a regulatory role, including the regulation of hematopoietic stem cells. Specific mesenchymal cells in bone have been shown to serve as niche components for stem cells in the bone marrow stroma. Genetically modifying subsets of osteolineage cells perturbs stem cell function and causes disordered hematopoiesis. The resulting myelodysplasia is microenvironment-dependent and results in the emergence of a frank leukemia with distinctive secondary genetic abnormalities. The multi-step process of oncogenesis may include an initiating step in heterologous cells that comprise stroma. The leukemia when transplanted only engrafts in recipients who have the genetically altered osteolineage cells, suggesting that the interaction between the microenvironmental cells and the hematopoietic cells is capable of initiating malignancy and appears to be necessary for its maintenance. ***David Scadden*** *proposed that the dependence on interaction between cell types offers the potential for intervention at the points of interaction in treatment and prevention strategies.*

The concept of stem cells has a reasonable utility in thinking about cancer for a number of reasons. It provides a conceptual framework within which to regard tumor heterogeneity. It assigns specific cell processes corresponding to a normal cell. And it also represents a tissue disorder, one that places a stem cell in the context of the inter-relationship between parenchyma and mesenchyma.

Parenchyma is a longstanding biological concept, and is considered to be the functional part of an organ. This is in contrast to the mesenchyma or stroma, which refers to the structural tissue of organs. But in fact, this paradigm needs to be rethought. In developmental biology, for instance, epithelial-mesenchymal interactions are among the most important phenomena in nature.

In the developmental context, mesenchymal cells provide critical instructive cues, and are permissive of the kinds of interactions necessary to achieve tissue size and function. They affect things such as the morphology of tissue and the way that cells are patterned in the tissue by their differentiation state. Ultimately, mesenchymal cells also have an impact on organ size. The mesenchymal-parenchymal interactions dictate the length of a limb, for example.

In the adult, the mesenchyma is important for hematopoiesis. There are a couple of studies that also indicate the importance of the non-hematopoietic compartment. For example, bone marrow injected into soft tissue can ectopically generate bone and bone marrow. It's very difficult to modify a hematopoietic stem cell (HSC) *ex vivo* without bone marrow stroma. The bone marrow stroma contains a population of mesenchymal stem cells that can form multi-lineage cells.

The interaction of bone and bone marrow and HSC can serve as a model for parenchymal-mesenchymal interactions. One of the advantages of this system is that there are well-defined subsets of mesenchymal cells in the skeleton that can help identify some of the key players, and the nature of their interactions.

Also available is a fine-grained analysis of the hematopoietic system, including a series of assays to look at function. These systems can ask questions about the nature of a regulatory relationship between mesenchymal (skeletal) cells and parenchymal (hematopoietic) cells in the marrow, focusing more on the adult, whether the mesenchyma is as dynamic as the the hematopoietic system, whether mesenchymal cells contribute to disease and whether they can be therapeutically targeted to alter parenchymal cells.

Defined bones

There are well-defined stages of osteolineage differentiation but, beyond that, the mesenchymal hierarchy in bone is crudely defined. *In vitro*, the so-called mesenchymal stem cell has the ability to form bipotential chondrocyte osteoblast progenitors and osteoblast adipocyte progenitors, but it is not clear this also occurs *in vivo*.

Within the context of this lineage map, there are tools available based on differential expression of genes in the osteolineage. There are also mice that show differentiation stage-specific expression of markers or of Cre recombinase in order to activate or delete genes at particular stages in this developmental cascade.

The collagen 1 promoter, which is found on predominantly in osteoblast lineage cells, can help study the physical relationships in the regulatory microenvironment. A physicist helped design an instrument that captures the interactions between the cell types in high resolution and high speed in living animals, looking through the skull into the marrow space.

The osteoblastic cells and the micro-vasculature form a tightly woven web. Osteoblasts can be seen wrapping right round the vessels, so there is a close contiguity of the different cell types at the endosteal surface. HSC are also in close proximity, nestled in between the osteolineage cells and micro-vasculature.

Because this is a confocal system, it can measure with some precision the distance between a stem cell and other elements such as the mesenchymal cells and the osteoids. It can also help assess whether there is a cell-autonomous way in which sub-populations of hematopoietic cells at various stages of differentiation organize in the marrow.

There seems to be an inverse relationship between differentiation and distance: the more primitive the hematopoietic cell, the closer it is to the osteoblast lineage. Engrafting stem cells locate closer to these mesenchymal cells, whereas cells that are further away divide more rapidly. Conditions of stem cell self-renewal are associated with the cells being closer to the mesenchyma.

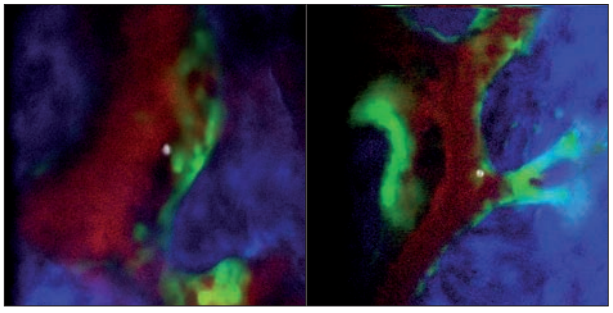

Figure 1
A single hematopoietic stem cell (white) observed in the bone marrow cavity of an anesthetised mouse.

When the collagen 2.3 Kb promoter is used to ectopically express or to over-express a constitutively active parathyroid hormone receptor in these osteoblastic cells, there is an increase in osteoblastic cells and in the number of HSC, suggesting that the osteoblastic cells are capable of modifying the number of primitive cells[1]. Deleting the BMP receptor-1A more broadly in the hematopoietic compartment has the same effect[2].

The same promoter can be used to drive thymidine kinase. A thymidine kinase analog disrupts the survival of the osteoblastic cells and results in a dispersal of the primitive cells out of the marrow space[3]. This suggests that some osteolineage mesenchymal cells contribute to an HSC niche. There are other models in which modifying mesenchymal cells doesn't have an effect on the stem cells (see Morrison, page 23), so this may not be characteristic of all osteolineage cells, but under some conditions, these cells can function as niche cells.

Dynamic states

In their activated state, mesenchymal cells show differential expression of Jagged1. Stem and progenitor cells in the animals show evidence of increased activation of Notch. When these cells are placed *ex vivo* on a transgenic feeder layer, they have increased support compared with the wild type feeder layer, which can be abrogated by adding a γ-secretase inhibitor. These mesenchymal cells seem to have dynamic production of molecules that are capable of regulating the parenchyma near them.

Hematopoietic cells have varying half lives, but are very dynamic in their response to different conditions. The bone matrix also turns over very rapidly, but it is unclear whether osteoblastic cells do. A pulse-chase experiment to measure the half lives of mesenchymal cells can address whether the same is true of them.

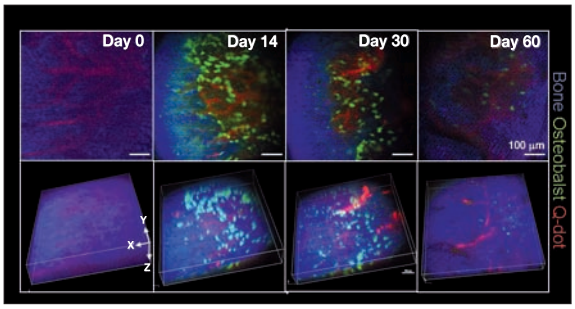

Figure 2
Pulse-chase experiments show that the osteoblast population has a fairly short life.

The osteocalcin promoter driving Cre excises the stop codon in front of a constitutively driven EYFP. A sequential confocal imaging technique can then look at osteoblast cells once they are pulse-labeled with a chase of varying intervals. The virtue of this approach is that it is possible to go back to the same animal repeatedly, and because of geographic landmarks, it is also possible to get fairly precise relocalization.

The pulse-chase experiments show that this osteoblast population has a fairly short life life, which is unexpected. They do not appear to be replaced by division of themselves. Instead, an immature population replaces the osteoblast lineage for long periods of time, suggesting that it is a stem progenitor cell.

The mesenchyma cells then appear to turn over rapidly and modulate the genes that regulate the parenchyma. One question, therefore, is whether it is possible to take advantage of that information to therapeutically target them.

Expressing a cognate ligand, a fraction of the parathyroid hormone, increases the number of stem progenitor cells. *In vivo*, it also increases the animal's tolerance for sequential chemotherapy, and the ability to tolerate a stem cell transplant. With a limiting number of stem cells, the animals have a high mortality if they just get the cells. But if they get the cells plus thus parathyroid hormone, which drives the mesenchymal compartment, there is a big impact on the outcome of the animal.

That has resulted in two clinical trials. One trial aims to enhance donation of stem cells in patients who had previously failed bone marrow donation for their hematologic malignancy. The other is for patients receiving an allotransplant of cord blood, which normally engrafts poorly.

Far-reaching effects

It is clear from these data that there is a role for mesenchymal cells of osteolineage in the regulation of HSC in the adult. It may be possible to therapeutically target mesenchymal cells, or at least the sub-population that is of osteoblast lineage. There is also now a model that suggests that mesenchyma can contribute to disease.

In this case, the Osterix promoter can be used for a more immature population and osteocalcin for a more mature population to assess whether modifying something in the osteolineage changes the support of hematopoiesis.

When the Osterix-Cre promoter is used to delete Dicer1, the mutant animals are born viable. They form

mesenchymal colonies but don't differentiate very well to osteoblast cells. There is a modest but significant decrease in the number of osteoblasts.

In terms of architecture by micro-CT, the tubercular bone looks about the same and the cortical bone is, if anything, slightly increased. However, the way that collagen fibrils are arrayed seems to be a little more disorganized, as does the laying down of osteoid.

Although the deletion is only in the mesenchymal lineage, hematopoietic cells are modified in many lineages. The number of total leukocytes, red cells and platelets are all decreased and, among the leukocytes, both lymphoids and myeloid cells are decreased. This is associated with some change in the cells' morphology, such as dysmorphic nuclear organization.

There is also dysplasia in the marrow space. Megakaryocytes, which are usually quite substantial in size with multi-lobulated nuclei, end up being very small with relatively compact nuclei. In the primitive cell compartment in the marrow, the cells proliferate above the progenitor level, and there is an enriched stem cell population. There is also a fair amount of cell death, increased marrow vascularity and abnormality of B lymphocyte development. In mouse model terminology, this would be considered a myelodysplasia.

If the hematopoietic tissue from these animals is transplanted into a genetically wild type background, there is a complete reversion of the hematopoietic phenotype. The morphological changes, overall cell numbers and increased cell death all revert back to normal. Similarly, if a genetically normal hematopoietic compartment is transplanted into these different microenvironments, despite the normal features of the donor mice, they acquire features of dysplasia.

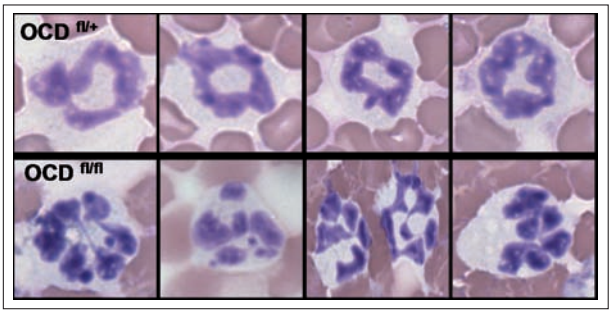

Figure 3
Dysplastic changes in blood.

These results suggest that there is something dictated entirely by the microenvironment. If the osteocalcin promoter driving the Cre is used, some of the same bony changes are visible, but there is no change in the hematopoietic phenotype. This indicates that, within the mesenchymal compartment, there are specific subsets of cells that have a particular relationship with the parenchymal component.

Complex disease

The features observed in the mouse are seen in a common blood disease called myelodysplastic syndrome, a disease of considerable complexity. In the U.S., there are as many as 20,000 cases diagnosed each year. Some individuals with the disease have a clearly defined genetic abnormality, but up to 60% of patients have no genetic abnormality in the hematopoietic cells. The disease is associated with age, and has poor prognosis and limited therapeutic options. Some people with the syndrome develop acute myelogenous leukemia.

Among the animals in which there is a genetic change in the mesenchymal cells, about 2 to 3 of every 100 animals develop an infiltrative process. These animals score positively for myeloid markers and infiltration of soft tissue within 6 weeks of age. In humans, a hematopathologist would score this as a subtype of leukemia called M4/M5, which is associated with leukemia and soft tissue infiltration.

Tumors from these animals have intact Dicer1, which was deleted in the bones of the animals but not in the tumors. By comparative genomic hybridization, it's clear that the tumors are clonal. Interestingly, a few tumors have a common area of amplification on chromosome 14.

If the marrow from the animals is transplanted into wild type recipients, there is no engraftment. But if the

marrow is transplanted into a recipient with an abnormal genotype, a few engraft with what appears to be leukemia. These observations suggest that this malignancy can emerge and retain some sensitivity to the environment in which it resides.

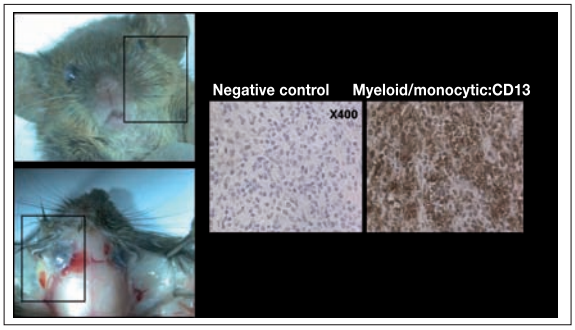

Figure 4
Fatal infiltrative process in Dicer1-deleted mice.

Osteoprogenitor cells from the mutant animals can be cultured *ex vivo* and support hematopoietic cells. This *ex vivo* system can be used to address some mechanistic questions. For example, Dicer1-deleted osteoprogenitors *ex vivo* increase hematopoietic progenitor proliferation and alter megakaryocyte differentiation. Compared with wild type controls, Dicer1-deleted osteoprogenitors have 656 differentially expressed genes.

Among these is SBDS, a gene associated with a rare autosomal human disease called Shwachman-Bodian Diamond syndrome. Children with this syndrome have a high incidence of bone marrow failure in association with skeletal abnormalities and some exocrine pancreatic insufficiency. They are also at high risk of myelodysplasia and acute myelogenous leukemia.

The SBDS gene product is expressed in all tissues, but is not very well characterized. It's found in the nucleolus, and seems to bind to some ribosomal units as well as to some proteins associated with DNA repair[4]. It may also play a role in modifying programs of genes. Knockdown of the gene with shRNA in hematopoietic cells alone shows only a minimal phenotype[5]. Deleting the SBDS gene from osteolineage and mesenchymal cells recapitulates the vast majority of the features of the Dicer1 deletion.

Overall, there appear to be differentiation-stage specific perturbations in mesenchymal cells that can induce complex hematological disorders in surrounding parenchymal cells. In particular, changes in osteolineage cells can induce hematologic disorders. Interestingly, primary genetic changes in mesenchymal cells in the microenvironment, which are clearly very distinctive, can initiate secondary neoplastic disease of a heterologous cell type.

In this model, the multistep process of oncogenesis can begin with a mesenchymal cell. And subsequent events, maybe in a heterologous cell type, can lead to malignancy. In the end, even with this highly malignant disorder, there still may be some partial niche dependence.

There are some implications of this model. If this does happen in humans, it might be possible to target the relationship between mesenchymal support cells and the cells undergoing the dysplasia and neoplasia. Transplant studies suggest that these may be necessary for maintenance of the malignancy, in which case, targeting that interaction could help treat cancer.

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Maintenance of embryonic metabolic networks in tumors

A report on a lecture by
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Tumors metabolize glucose at a higher rate than do normal adult tissues, and a smaller fraction of the glucose taken up by tumor cells is metabolized by oxidative phosphorylation. This is similar to the form of metabolism carried out in normal tissues under conditions of hypoxia, but unlike normal tissues, tumors undergo hypoxic metabolism even in the presence of high oxygen. Cancer cells typically revert to an embryonic form of metabolism that diverts intermediates in glycolysis into the synthesis of amino acids, nucleotides and lipids rather than synthesis of ATP. This shift in glucose metabolism in cancer cells can be driven by activation of the phosphoinositide 3-kinase (PI3K) pathway, as demonstrated by high rates of fluoro-deoxyglucose uptake into tumors that are initiated by mutant PI3K. Cancer cells invariably express a form of pyruvate kinase (PK-M2) that is normally found in embryonic tissues and in cells that have the capacity for self-renewal. Replacing PK-M2 in cancer cell lines with PK-M1, which is found in most adult tissues, increases oxygen consumption and impairs growth. PK-M2 expression is driven by activation of the PI3K pathway and is mediated by hypoxia-inducible factors. ***Lew Cantley*** *discussed the role of PKM2 and other metabolic enzymes in maintaining high rates of growth in cancer cells.*

Approximately 80 years ago, Otto Warburg noticed that cancer cells have lower oxygen consumption than typical cells. These cells instead consume much higher rates of glucose, producing lactate in the process.

This observation, known as the 'Warburg effect', dominated the cancer field for more than 50 years. The discovery of oncogenes diverted interest from cellular metabolism, but many oncogenes are proving to control enzymes of metabolism[1].

The two currencies cells use in order to grow and survive are ATP and NADPH. ATP supplies the energy needed for cell survival; when cells run out of ATP, they rapidly die. ATP is required to maintain ion gradients across the plasma membrane, as well as for many other housekeeping events.

However, when the cell needs to grow, it needs the reducing potential of NADPH to synthesize fatty acids, nucleic acids, protein and other substances. NADPH is also needed to protect the cells from oxidative stress by eliminating reactive oxygen species (ROS). Stem cells, as protectors of the genome, must particularly ensure that NADPH levels are high, and ROS levels low.

The cell must continually decide whether to use the nutrients it takes in to make ATP or NADPH, because production of the two is mutually exclusive. In fact, oxidative phosphorylation, which produces ATP, also generates large quantities of ROS.

In the Warburg experiment, a normal tissue when placed in a hypoxic environment switches to glycolysis and increases its glucose uptake. This is because of the hypoxia-inducible factor or HIF, which increases the levels of all enzymes of glycolysis and increases lactate production. If the same normal tissue is placed in a high-oxygen environment, it switches to oxidative phosphorylation, requires less glucose and makes less lactate.

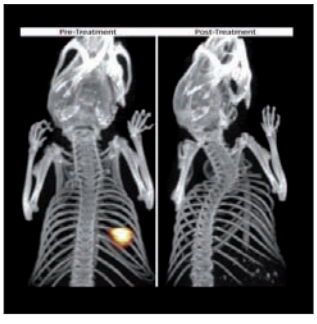


Figure 1
PIK3CA-driven lung tumors have high glucose consumption.

A cancer cell, in contrast, maintains high glucose consumption and lactate production regardless of whether it is placed in a low- or high-oxygen setting. This is seemingly a paradox because the cancer cell seems to be choosing an inefficient way of making ATP, but the reason for this is becoming clearer.

Not all cancers experience this shift in metabolism. In the clinic, roughly 75% to 80% of tumors can be visualized with fluorodeoxy-glucose positron emission tomography (FDG-PET), which indicates whether a given tumor is addicted to glucose. The remaining tumors grow at very high rates, but don't rely on glucose. These tumors instead use glutamine, fatty acids or other nutrients. Tumors with a mutated PI3K pathway — for example, loss of PTEN or activating mutations in PI3K — are invariably FDG-PET positive.

For example, a mouse with a PIK3CA-driven lung tumor has high glucose consumption and is avidly PET-positive. After two days of treatment with the PI3K inhibitor, BEZ235, the tumor remains, but is no longer PET-positive, suggesting that PET positivity is dependent on PI3K expression[2]. Likewise, with a DOX-inducible PIK3CA, removing DOX eliminates the PET signal in a day.

This is also true in human disease. For example, a gastrointestinal stromal tumor with a mutation in KIT tyrosine kinase — which directly binds to PI3K and activates it — is PET-positive. Sunitinib turns off KIT and although the tumor persists, glucose uptake decreases rapidly. Ultimately the tumor shrinks, though this treatment alone rarely results in a cure.

Conserved functions

PI3K probably regulates glucose uptake through HIF1-α, even in the absence of hypoxia. AKT phosphorylation eventually increases both message and protein level of HIF, so if this pathway is mutated, hypoxia is not required. AKT also turns on FOXO and many of the targets of FOXO involved in redox control.

AKT also phosphorylates GSK-3 and β-catenin, which raises the levels of MYC and SREBP1 — MYC mainly through activation of genes involved in amino acid uptake and glutamine metabolism, and SREBP1 through controlling genes involved in fatty acid and cholesterol synthesis. AKT phosphorylates and activates hexokinase, which affects phosphorylation of glucose, and phosphorylates and activates PFK2 which synthesizes fructose 2,6 bisphosphate, that in turn activates PFK1 to stimulate fructose 1,6 bisphosphate (FBP) synthesis.

The major function of the PI3K pathway, which is conserved all the way from worms and flies to humans, is to regulate glycolysis and speed up glucose uptake. However, at the same time that tyrosine kinases like PDGF-R and EGFR-R are activating PI3K, they're unexpectedly turning off the last step in glycolysis, pyruvate kinase.

There are two different genes that encode pyruvate kinase (PK), the last step in the glycolytic pathway: the L gene, which is expressed in the liver, and the M gene, expressed in muscle and all other tissues. PK-M is alternative spliced into two forms.

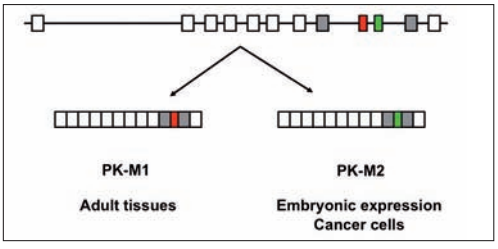


Figure 2
PK-M isoform expression is regulated by alternative splicing.

Muscle expresses the M1 isozyme, and embryos have the M2 isoform. Most, if not all, cancers exclusively express PK-M2, meaning that they revert to or never progress past the embryonic form. Interestingly, fat, a tissue that continues to grow in adulthood, maintains PK-M2. Every laboratory cell line also exclusively expresses PK-M2.

The difference between M1 and M2 is a single exon that underwent duplication during evolution. PK-M2 has maintained the ability to be regulated by FBP. PK-M1, in contrast, is a late-evolving form that has lost this ability. As a result, PK-M1 is frozen in a high-activity state, whereas PK-M2 has low activity even in the presence of its allosteric activator, FBP[3].

If M2 is blocked with shRNA and M1 is introduced in its stead, cells still grow perfectly well in plastic. But just by changing one exon of one gene, this experiment partially reverses the Warburg effect, and the cells consume more oxygen and generate less lactate.

Although the cells grow well in plastic, they don't grow well in a mouse xenograft. PK-M1-expressing cells eventually generate small tumors, but the cells in the tumor re-express PK-M2, suggesting that tumors need PK-M2 to grow *in vivo*[4].

Exclusive pathways

When PK is in its highly active form, M1, the glycolytic pathway is going at full blast. As a result, intermediates in the pathway cannot be diverted into other pathways, such as the pentose phosphate shunt to make ribose and NADPH, glycerol needed to make triglycerides and lipids and amino acids, particularly serine. This is an efficient way to make ATP, and is ideal for non-dividing tissue such as brain and muscle.

Fat and cancer cells express PK-M2, which is designed to have slow activity, allowing glycolytic intermediates to flood into other pathways and make protein, DNA, RNA and lipids. But these cells run the risk of not being able to produce enough ATP.

Figure 3
M1 rescue cells exhibit lower tumorigenicity than do M2 rescue cells in xenograft studies in nude mice.

ATP inhibits phosphofructokinase1 (PFK1), the regulating step in this pathway. When the cell has high levels of ATP, PFK activity is low; if ATP levels drop, AMP activates PFK1. More recently, AMP kinase has been shown to phosphorylate PFK2 and stimulate production of fructose-2,6-bisphosphate and further activate PFK1. The fructose-1,6-bisphosphate produced by this acute activation of PFK1 then activates PK-M2, allowing rapid synthesis of ATP and thereby bringing the cell back to energy homeostasis.

Growing cells need only about 50% more ATP than do stationary cells in an organism. In growth mode, the cell's real demand is for NADPH. For example, fatty acid synthesis requires 7 ATP molecules, 14 molecules of NADPH and 8 of acetyl CoA to produce a 16-carbon fatty acid. Glycolysis of 4 glucose molecules generates 8 acetyl CoA molecules through pyruvate dehydrogenase, and 8 ATPs from PK-M2, but no NADPH.

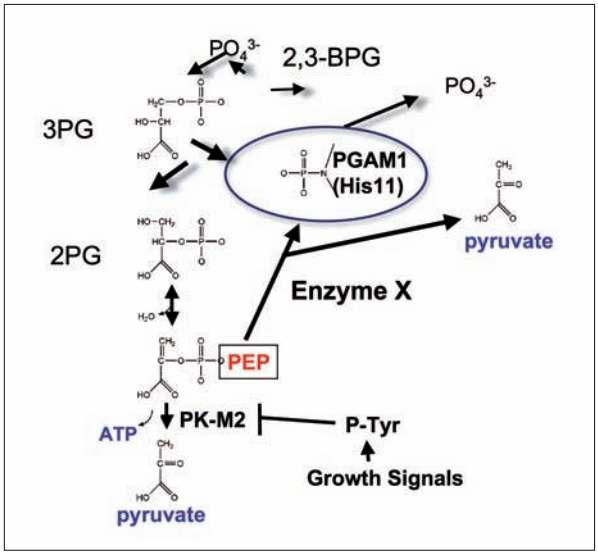
Diversion of glucose-6-phosphate into the pentose phosphate shunt allows synthesis of NADPH. To produce enough NADPH from the pentose phosphate shunt to make a 16-carbon fatty acid requires that 7 glucose molecules be diverted into this pathway. If the carbon atoms from these 7 glucose molecules re-enter glycolysis, they can generate more than sufficient ATP and acetyl-CoA than are needed for synthesis of the fatty acid, without the need for oxidative phosphorylation.

This system evolved early when the earth contained an anaerobic environment, so it doesn't rely on oxygen. In fact, oxidative phosphorylation produces 36 ATP molecules per glucose consumed, but it burns all the carbon atoms into carbon dixoide, and doesn't generate the acetyl CoA or NADPH needed for fatty acid synthesis. In addition, the high level of ATP generated by oxidative phosphorylation turns off PFK1, slowing down the pathway. This may be the reason that PK-M2 evolved to have low activity unless cells are in energy stress, and why cancer cells need the M2 rather than the M1 form of the enzyme in order to grow in an *in vivo* environment.

Alternative splicing

Embryos exclusively express PK-M2 and, during development, most tissues switch to PK-M1 as they mature. C2C12 myoblasts normally express PK-M2, but grown in the appropriate conditions for a week, the cells differentiate into myocytes, fuse and generate large cells that resemble myocytes of mature muscle. These cells now make roughly equal amounts of PK-M2 and PK-M1.

Figure 4
A glycolytic futile cycle with no net ATP synthesis



The most dramatic difference in these cells is in HNRNP A1 and HNRNP A2, and in PTB, another HNRNP family member. The levels of these proteins drop as the cells begin to differentiate, correlating with the switch from PK-M2 to PK-M1. Knocking down the A1 or A2 gene alone doesn't have any effect, but knocking down both, or PTB, suppresses PK-M2 and results in a shift to the M1 isoform. HNRNP itself is regulated by MYC, and turning down MYC may allow the cell to switch to the differentiated state.

Knocking down HNRNP also has other effects on metabolism. In a glioblastoma cell, for instance, knocking down A1, A2 or PTB results in a 50% reduction in lactate production. The switch from PK-M2 to PK-M1 reduces lactate by only 20%, suggesting that there is a coordinated effect of a number of enzymes in glycolysis that mediates the Warburg effect.

Stem cells are protectors of the genome. Because they continually replicate throughout life, they must ensure that they are not exposed to ROS. Oxidative phosphorylation is the most efficient mechanism for generating ATP, but at the cost of generating large quantities of ROS, so stem cells minimize oxidative phosphorylation, especially when entering the cell cycle. This limits their ability to make ATP.

This may also explain why cancer stem cells are particularly sensitive to salinomycin, a potassium ionophore that depolarizes the plasma membrane and activates the Na, K-ATPase, leading to ATP depletion.

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Epithelial-mesenchymal transition and the stem-cell state

A report on a lecture by
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During development, the epithelial-mesenchymal transition (EMT) enables epithelial cells to acquire motility and invasiveness and to translocate to other parts of the embryo. By activating previously latent developmental programs, notably an EMT, carcinoma cells may acquire many of the phenotypes of neoplastic cells found in high-grade malignancies. Carcinoma cells that have undergone an EMT are more resistant to therapies than the bulk of cells in a tumor. These resistant cells, exhibiting the properties of cancer stem cells (CSC), can regenerate new tumors once therapy is halted. Unexpectedly, when a mammary epithelial cell is forced through an EMT, the product is not a mesenchymal cell, but instead a cell that exhibits mesenchymal markers and, in addition, many of the traits of an epithelial stem cell, including the ability to seed mammospheres, to exhibit cell-surface markers associated with stem cells, and to seed a new tumor. Floating cells from monolayer cultures of immortalized mammary epithelial cells are able to reseed new monolayers, but retain certain stem cell-like characteristics. Single-cell clones of these cells spontaneously convert non-stem cells into stem-like cells in culture, and the same thing may also occur in living tumors. ***Robert Weinberg*** *argued that therapeutic strategies directed toward the elimination of CSC may need to recognize the ability of non-CSC within a tumor to spontaneously regenerate new CSC.*

Normal human mammary epithelial cells from a reduction mammoplasty, when propagated in one culture medium and transformed, yield an adenocarcinoma. When the same cells, propagated in an alternative culture medium, are transformed by an identical set of alterations, they yield a squamous cell carcinoma. These two sets of cells have an identical set of introduced mutations, so the differentiation program that preexist in each cell type prior to transformation is an important determinant of their biology.

The expression profiles of tumor cells (BPLER vs. HMLER) are closely correlated, in each case, with the pre-existing differences between their respective relatively normal cells-of-origin (BPE vs. HME). Although cancer cells undergo a profound change in their biology upon neoplastic transformation, the differentiation program of the normal cell-of-origin can affect the ability of derived cancer cells to metastasize.

For example, although BPLER cells are metastatic, HMLER cells transformed in the same way are not metastatic when implanted in identical conditions or even contra-laterally in the same mouse. What's more, the two sets of cells have dramatically different rates of tumor-initiating cells. As few as 10 cells of the BPLER cells suffice to seed a tumor xenograft, whereas about a million cells of HMLER are needed to accomplish the same thing.

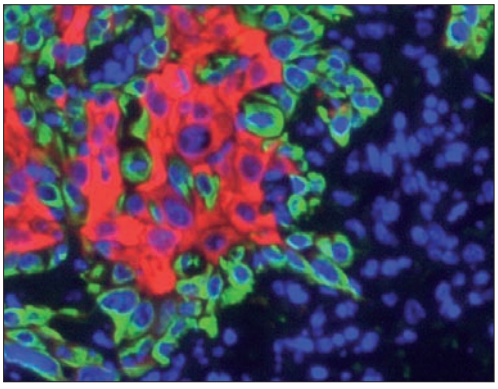

Figure 1
The behavior of a BPLER breast cancer xenograft in mouse host.

The entire process of metastatic dissemination — often termed the invasion-metastasis cascade — is complex. Indeed, it is as complex biologically as the steps that led to the formation of the primary tumor. Initial steps

involve the physical translocation of a cell from a primary tumor to a distant organ site. The last of these multiple steps, often termed colonization, requires the disseminated cells to adapt to the microenvironment of a foreign tissue.

One solution to understanding this complexity at the mechanistic level comes from studying the epithelial-mesenchymal transition (EMT) program. Many primary tumors harbor islands of epithelial cancer cells that are surrounded stromal cells. Carcinoma cells at the outer edge of the islands often shut down the expression of epithelial cell markers and instead express mesenchymal markers. Such cells are juxtaposed directly with the mouse stroma, suggesting that contextual signals that they receive from this stroma are important for the activation of their EMT program.

In a more general sense, the EMT program is activated during a variety of embryogenic steps, including gastrulation in both the sea-urchin and the fly, emigration from the primitive neural crest, and ostensibly dozens of other steps in embryonic morphogenesis. It is a complex, multi-faceted program involving multiple changes in cell properties[1]. Many of the acquired cellular phenotypes confer advantages on cancer cells, allowing them to exhibit highly aggressive phenotypes[2].

An EMT program can be activated by any one of a half-dozen or more pleiotropically acting transcription factors, including Twist, Snail, Slug, Zeb1, Goosecoid and FOXC2. These factors operate on various stages of embryogenic morphogenesis. There may be subtle variations of the program activated by each of the factors, but they all seem to drive to the same endpoint.

One of the transcription factors, called Twist, enables mouse breast cancer cells to metastasize to the lung from a subcutaneous site of implantation. When Twist is shut down, the number of metastases is reduced by 85%. The residual metastases all express Twist, indicating that Twist function is necessary for the metastatic ability of the cancer cells. However, whether Twist is sufficient to convert a non-metastatic to a metastatic cell is still unclear.

Important origins

A striking example of the importance of the differentiation program of normal cells of origin derives from the study of normal human melanocytes. When a variety of normal human epithelial cells are taken through a transformation protocol, they yield primary tumors that are weakly metastatic, if at all.

If the identical genetic changes are experimentally induced in normal melanocytes, those changes suffice to transform the cells, as before, but in addition yield primary tumors that generate hundreds of metastases to the lung, liver, spleen and intestine. These contrasting behaviors provide a dramatic demonstration of the fact that the preexisting differentiation program of the normal cell-of-origin is an important determinant of metastatic behavior.

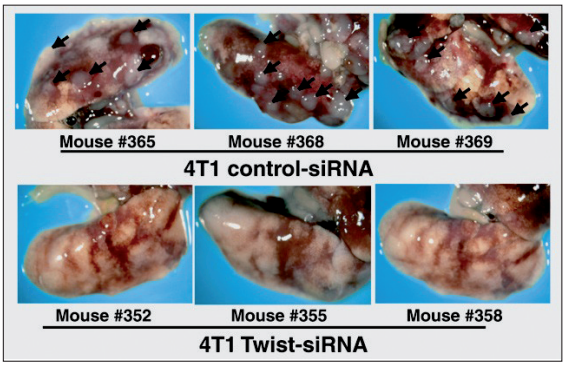

Figure 2
Twist is essential for 4T1 tumors to metastasize from the mammary gland to the lung.

The melanocytes in the skin derive from the embryonic neural crest and use the transcription factor Slug to enable an EMT and migrate from this site to sites elsewhere in the body of the embryo. The same transcription factor, Slug, is expressed at levels 1000-fold higher in experimentally transformed melanocytes compared with, for example, RAS-transformed breast cancer cells.

This indicates that the facility of turning on one of these transcription factors is highly tissue dependent, and that such proclivity to express a

transcription factor like Slug may in turn explain the dramatically contrasting behaviors of various human tumor types. In the case of the experimentally transformed human melanocytes, when Slug is shut down through the use of an siRNA, it yields a 93% reduction in the metastatic phenotype, indicating its central role. The early embryonic program utilizing Slug is resurrected by transformed melanocytes to enable their metastasis.

The various EMT-inducing transcription factors act on different cohorts, but they intercommunicate, and often induce expression of one another. It is plausible, but hardly proven, that every aggressive human carcinoma utilizes different combinations of these transcription factors, at least transiently, in order to acquire the traits associated with high-grade malignancy.

Among immortalized human mammary epithelial cells growing in culture, the non-stem cells are $CD44^{lo}CD24^{hi}$ whereas the stem cells are $CD44^{hi}CD24^{lo}$. These stem-like cells seem to perpetuate themselves in culture indefinitely and do so outside of the specialized niche that they normally occupy in living tissue. In fact, the induction of EMT by long-term exposure to TGF-β triggers an *en masse* migration of cells from the non-stem cell to stem cell position.

This can be demonstrated more persuasively through the ectopic expression of either Snail or Twist, two EMT-inducing transcription factors. Once again, there is an *en masse* migration from the non-stem cell to stem cell position, and the conversion from epithelial to mesenchymal cell morphology in monolayer culture[3].

If quantitative RT-PCR is used to measure the levels of different mRNA expressed in these cells, stem cells express about $1/200^{th}$ of the epithelial marker E-cadherin, but between 40-fold and 200-fold more fibronectin, vimentin and N-cadherin, which are all mesenchymal markers. What's more, they over-express to comparable extents four EMT-inducing transcription factors, FOXC2, SIP1, Twist and Snail.

The same kind of dichotomy exists with human reduction mammoplasty cells. Expression of the SIP1 EMT-inducing transcription factor is 150-fold higher in the putative stem cells relative to their non-stem-cell counterparts. Because these cells have not been modified experimentally in culture, these associations already suggest an intimate relationship between the EMT and the stem-cell state.

If that's so, epithelial tissues in the mammary gland and possibly in many other epithelial tissues possess many mesenchymal attributes. What's more, the EMT may be the main route through which non-epithelial cells enter into the stem-cell state, rather than simply a side door through which they do so. Spontaneous or forced expression of these transcription factors in differentiated epithelial cells may also convert non-stem cells into stem cells under a variety of conditions.

Distinct differences

When stem and non-stem cells are separated, the putative stem cells form a mesenchymal monolayer and form mammospheres in 3-dimensional culture. The non-stem cells form cobblestone islands in monolayer culture and fail to form mammospheres. Induction of an EMT by Snail or Twist induces mammosphere formation, which is associated with stem cells.

The resulting mammospheres have at least two different kinds of mammary epithelial cells, cytokeratin K8 (luminal) and K14 (myoepithelial) If all this is confirmed, the EMT program holds two dangers of cancer patients: it can confer on cancer cells the ability to physically disseminate and to self-renew. The latter trait, in turn, would seem to be essential to the ability of disseminated cancer cells to found a new metastatic colony.

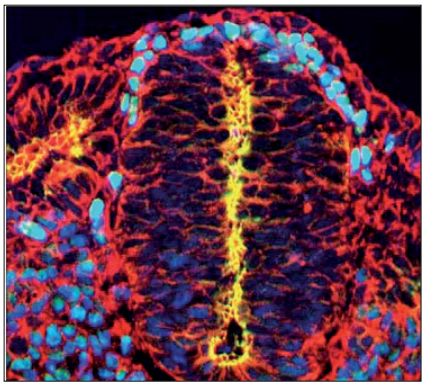

Figure 3
Slug plays a prominent role in the emigration of cells from the neural crest.

Still, more compelling proof is required that the EMT program creates stem cells, rather than cells that merely have many of the superficial attributes of stem cells. CD49 and CD24 serve as useful markers to separate normal mouse mammary epithelial cells into two populations, a putative mammary stem cell (MaSC) population and a non-stem cell population. If these cells are injected into a cleared mammary stromal fat pad, the putative stem cells generate entire mammary ductal trees but look mesenchymal in monolayer culture. In contrast, non-stem cells look epithelial in monolayer culture but fail to produce ductal trees *in vivo*.

The putative stem cells also show a 10-fold up-regulation of N-cadherin, Vimentin and Slug, but not Twist, and a 10-fold lower expression of E-cadherin. A mammary gland implantation assay can assess whether these are indeed MaSC. Cells that have undergone an EMT — through the actions of Slug, for example — can be mixed with control cells to see which of the two sets populates a cleared stromal mammary fat pad.

Control cells, infected with an RFP control vector, show minimal amount of ductal tree formation. If cells induced to undergo an EMT by Tet-inducible Slug for a week before implantation are injected into a stromal mammary fat pad with equal number of the RFP controls, both sets initially generate roughly the same number of cells.

This is important because it argues against the notion that Slug merely allows the implanted cells to survive better the trauma of initial implantation. After 7 weeks however, there is an up to a 100-fold increase in the mammary gland-repopulating activity of the Slug-induced MaSC, compared with controls.

Transient Slug exposure also increases the ability of cells to create solid or ductal-lobular mammospheres in 3D culture. Conversely, shutting down Slug expression in mammary epithelial cells decreases the cells' ability to form mammospheres by a factor of 20 or 30. Endogenous natural expression of Slug by these cells seems to be essential for their ability to form mammospheres.

Targeting stemness

Existing breast cancer therapies often wipe out the bulk of the non-stem cells in a tumor, which creates the illusion of success because of the debulking of the tumor. But the stem cells may persist and regenerate the tumor once therapy is halted. Accordingly, models for cancer stem cells (CSC) have been created by forcing cancer cells through an EMT by blocking E-cadherin. The resulting cells are mesenchymal by a variety of attributes, including increased expression of vimentin and N-cadherin. These cells have been used in a high-throughput screen to identify compounds that selectively hit CSC relative to non-CSC.

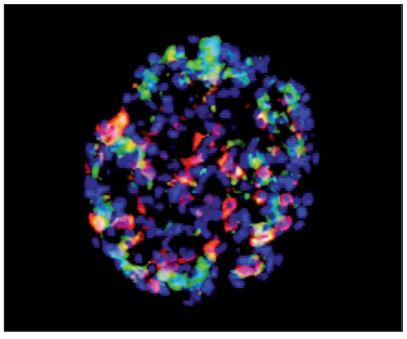

Figure 4
Mammospheres are cytokeratin 8+ (green) and 14+ (red), an indication of bipotentiality.

In a high-throughout screen of a library of 16,000 compounds, doxyrubicin appears to kill the non-stem cells at about 10-fold higher than the stem cells. The same is true of paclitaxel, once again showing preferential killing of non-stem cells versus the stem cells. Screening for compounds that preferentially kill CSC gave 22 hits. Of these, salinomycin shows about a 10-fold preferential killing of putative CSC versus non-CSC.

In one tumor cell population, assessed by FACS, stem cells typically comprise a minority of about 5% of the total tumor population. After treating with the standard chemotherapeutic taxol derivative, about 70% of the surviving cells are stem cells.

In contrast, if the tumor is treated with salinomycin, only 0.2% of the surviving cells behave like stem cells. Salinomycin may not be useful therapeutically, but it offers a proof-of-principle that it is possible to select compounds that preferentially kill stem cells. What's more, with 22 hits in a library of 16,000 compounds, these stem cell-targeting agents are not rare.

If these populations are treated *ex vivo* for one week with these compounds, then allowed to expand for two

weeks *in vitro* and planted into a mouse, salinomycin treatment results in approximately a 100-fold decrease in tumor-initiating cells. 4T1 mouse breast cancer cells show a similar decline in tumor-initiating cells.

Control tumors express high levels of vimentin and N-cadherin, but salinomycin treatment shuts down expression of these proteins and substantially increases E-cadherin, suggesting that the surviving cells are largely epithelial and, therefore, are non-stem cells.

Floating stems

Floating populations of cultured HME cells, dubbed 'flopcs', grow well when taken from a culture dish and placed in a fresh dish. The flopcs maintain a more mesenchymal phenotype in monolayer, expressing lower levels of E-cadherin, and are biologically and biochemically different from the bulk populations from which they arose.

They tend have lower expression of ESA, or epithelial specific surface antigen, in consonance with their being more stem-like. Flopcs also have a greater proportion of cells that are $CD44^{hi}$ than do normal HME cells, and are ostensibly more closely allied with stem cells. Both $CD44^{lo}$ and $CD44^{hi}$ populations form mammospheres with acini.

When single-cell clones of $CD44^{lo}$ and $CD44^{hi}$ populations are isolated from heterogeneous flopc populations, each of these clones has at least a minor population of $CD44^{hi}$ cells. That suggests that epithelial cells in monolayer always include a subset of stem-like cells, which are able to maintain themselves in culture even in the absence of interaction with their normal biological niche.

As expected, $CD44^{hi}$ cells can regenerate $CD44^{lo}$ cells. But unexpectedly, $CD44^{lo}$ cells also produce cells of the $CD44^{hi}$ phenotype. The rate of this differentiation changes depending on how cells are genetically altered — by putting in the large T antigen or the RAS oncogene, for instance. Introducing these highly transforming events seems to progressively lower the barrier to retro-dedifferentiation.

One potential reason for this result might be that $CD44^{hi}$ cells are the products of the selective outgrowth of a contaminating subpopulation of cells present there in these single-cell clones from the beginning. But that notion is weakened by the fact that stem cells proliferate more slowly than non-stem cells do.

If the different flopc clones are transformed after they have been isolated as single-cell clones, the $CD44^{hi}$ clones in one case yields 344 metastases per mouse, compared with one or no metastases from $CD44^{lo}$ clones or the bulk HME population. This indicates that the phenotype of the cells prior to transformation by RAS, including of the *de novo* $CD44^{hi}$ cells, is far more metastatic than that of the bulk population transformed in parallel.

What's more, the tumor-initiating potential of the $CD44^{hi}$ cells is about 1000-fold higher than that of the bulk population from which the flopcs originally arose. The untransformed $CD44^{hi}$ cells also partially reconstitute a mammary gland when implanted into the mouse mammary fat pad.

If immortalized mammary epithelial cells can generate $CD44^{hi}$ cells, and if contextual signals can induce tumorigenic cells to undergo an EMT and create $CD44^{hi}$ immortalized cells, it follows that a variety of non-stem cells can be converted into stem cells. In fact, whenever a cell is induced to undergo an EMT, it may be entering into a stem cell or a quasi-stem cell state. This suggests that populations of more differentiated epithelial cells can spontaneously de-differentiate into stem-like cells.

When CSC are killed through some type of therapy, the assumption is that the tumor eventually melts away because its source of regeneration has been cut off. But what happens, if, instead, the surviving non-stem cells generate new CSC? That would obviously complicate therapy and necessitate the development of agents that target both stem and non-stem cells[5].

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PART III: Targeting cancer stem cells

Wnting leukemia stem cells down a Notch

A report on a lecture by
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An emerging theme in the therapeutic recalcitrance of leukemia is the subversion of self-renewal and survival pathways by committed progenitors following aberrant differentiation of hematopoietic stem cells. In particular, leukemia stem cells (LSC) have co-opted multiple stem cell signaling pathways, resulting in relative quiescence, resistance to apoptosis and increased self-renewal capacity. The relative importance of these pathways varies depending on the leukemia subtype. A group of disorders known as myeloproliferative neoplasms, characterized by over-production of specific types of blood cells, provides a valuable model for studying the molecular evolution of LSC. Individuals with these disorders have a propensity to develop acute leukemia, and many of them carry an activating mutation in the tyrosine kinase JAK2, which initiates the disease and sets the stage for leukemic transformation. In blast crisis chronic myelogenous leukemia (CML), enhanced self-renewal of LSC is associated with the activation of β-catenin or sonic hedgehog pathway signaling through functional disruption of GSK3-β, a major common negative regulator. In CML, the relative importance of another self-renewal pathway, Notch, is still unclear. However, in human T-cell acute lymphoblastic leukemia, Notch1 deregulation is the predominant mechanism driving self-renewal and therapeutic recalcitrance. ***Catriona Jamieson*** *discussed the importance of combining different molecularly targeted therapies to block the abnormal differentiation, self-renewal, survival and homing capacity of cancer stem cells.*

The past decade has seen remarkable advances in drugs to treat leukemias. Despite this progress, however, most leukemias are considered incurable, in part because of their proclivity to relapse after therapy. Unbridled activation of stem cell pathways following hematopoietic stem cell (HSC) differentiation may lead to malignant progenitor reprogramming and the generation of leukemia stem cells (LSC).

These LSC evolve as a result of sequential mutations in pathways that regulate differentiation, self-renewal, survival and homing.

From a therapeutic perspective, LSC pose several specific challenges. For example, it's important to know whether a therapy is most effective when the cells are in their infancy, or when they fully express the abnormalities that alter their differentiation and self-renewal capacity.

An important paradigm for dissecting the molecular evolution of LSC is a group of disorders known as myeloproliferative neoplasms, first described in 1951. People with these disorders make too many of one type of blood cell in the bone marrow. For instance, individuals with polycythemia vera make too many red cells, those with essential thrombocythemia make too many platelets, those with chronic myelogenous leukemia (CML) make too many white cells, and people with primary myelofibrosis show scarring of the bone marrow.

In all of these disorders, there is clonal involvement of a multipotent progenitor. The patients share marrow hypercellularity and extramedullary hematopoiesis. The most vexing problem for patients is that they have a 36-fold higher propensity to clot than the general population. As a result, they have strokes, and then hemorrhagic conversion of those strokes, resulting in serious sequelae.

Patients also have varying propensities for evolution to acute leukemia, making it important to study these diseases. When untreated, some myeloproliferative neoplasms such as CML progress to leukemia 100% of the time, whereas others do so only 15% of the time. It's important to understand how these leukemias are initiated, and what molecular events drive the generation of LSC.

The initiating events in the leukemias that emerge from these illnesses seem to occur at the level of the HSC. In normal hematopoiesis, the long-term HSC is the only cell with robust self-renewal capacity. As the cells make the commitment to differentiate along the myeloid lineage, however, they lose the self-renewal capacity.

In the context of these myeloproliferative neoplasms, an activating mutation in the tyrosine kinase JAK2 could act as a clonal marker to study initiation of events. The JAK2 mutation V617F is expressed in more than 97% of people with polycythemia vera, and in about 50% of people with essential thrombocythemia or myelofibrosis.

Clonal transmission

The JAK2 mutation is expressed at the level of the HSC and, as previously suggested, is transmitted clonally through to the progeny, the common myeloid progenitors, the granulocyte macrophage progenitors (GMP) and the megakaryocyte erythroid progenitors[1]. In the U.S. alone, about 100,000 people have JAK2-driven myeloproliferative neoplasms.

In culture, HSC from JAK2-driven polycythemia vera have a propensity to form red blood cells, indicative of JAK2-driven lineage priming. TG101348, a potent and selective JAK2 inhibitor, decreases the cells' capacity to form red blood cells, and the HSC adopt full multi-lineage differentiation potential. In most patients, a 300 nanomolar dose of the inhibitor inhibits the overt propensity to give rise to red blood cell colonies at both the level of HSC and progenitor[2].

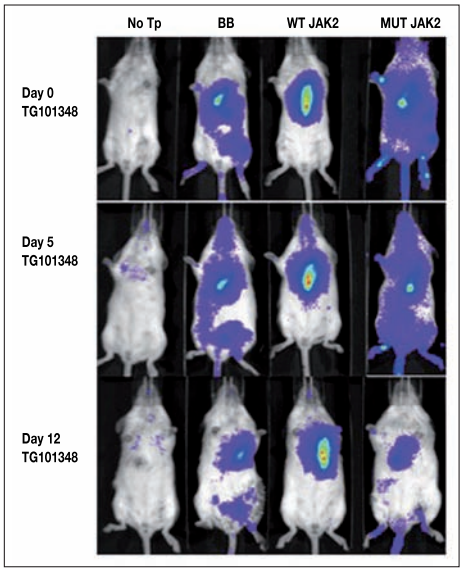

Figure 1
Selective inhibition of JAK2 V617F-driven erythroid engraftment.

Compared with a wild type JAK2 control, lentiviral transduction of primitive progenitors with mutant JAK2 leads more robust red blood cell differentiation typical of polycythemia vera. The JAK2 V617F-expressing cells are also the most sensitive to TG101348.

The gold standard test of the capacity of these genetically engineered human progenitor cells to recapitulate polycythemia vera is to transplant them into immunocompromised mice. To enable non-invasive tracking of engraftment, wild type JAK2 or mutant JAK2 transduced cord-blood progenitors or polycythemia vera progenitor cells from patient peripheral blood are lentivirally transduced with luciferase. The human progenitors are transplanted intra-hepatically into neonatal RAG2-/-γc-/- mice, which permit high-level human hematopoietic engraftment. This provides a remarkably robust model of human hematopoiesis because the T and B cells that derive from the cord blood progenitors are functional[3].

Non-invasive *in vivo* imaging (IVIS 200, Caliper) shows bioluminescent engraftment of polycythemia progenitors, which can be tracked starting at about 4 weeks after transplantation providing an indispensable model for assessing efficacy of JAK2 inhibitor therapy. Although there is sustained

bioluminescent engraftment in mice treated with vehicle for two weeks, those treated with a highly selective JAK2 inhibitor (TG101348; 120 mg/kg) lose their bioluminescent engraftment.

Following this, the JAK2-treated mice show a reduction in human glycophorin A expression compatible with reduced red blood cell production. Importantly, this also corresponds with a decrease in JAK2 mutant allele burden in these mice. The results suggest that treatment with a selective JAK2 inhibitor reduces bioluminescent engraftment of JAK2+ progenitors, the propensity of these progenitors to engraft red blood cells as well as the JAK2 mutant allele frequency.

If the mutant JAK2 is over-expressed in cord blood progenitors and transplanted using the same system, those cells are more sensitive to the inhibitor than are the wild type JAK2-expressing human progenitor cells in the spleen and in the liver.

The propensity of polycythemia stem and progenitor cells to differentiate along the erythroid lineage may be explained, at least in part, by the relative increase in GATA1 expression, an erythroid transcription factor, compared with PU.1, a myeloid transcription factor. This imbalance between GATA1 and PU.1 also occurs when mutant JAK2 is over-expressed in the cord blood progenitor cells. If the cells are treated with JAK2 inhibitor, they have decreased P-AKT, decreased phosphorylation of GATA at serine 310, and also decreased phosphorylation of STAT5.

Effective inhibition

One of the reasons TG101348 is such a potent inhibitor of the pathway is that it targets two modes of enhancing erythropoiesis: by preventing GATA phosphorylation at serine 310, and translocation of phospho-STAT5 to the nucleus.

The inhibitor has very similar effects in a mouse model of JAK2 mutation-driven polycythemia vera that goes on to develop myelofibrosis. At the same dose, 120 mg/kg, the inhibitor improves survival of the mice, decreases spleen size, lowers the white blood cell count and hematocrit, and decreases the JAK2 mutant allele burden.

All these results became the basis for a clinical trial that began in February 2008. Because the trial investigators were all involved in the preclinical work, they were aware that there is a dose response and that the trial must push through the side effects. The preclinical model predicted toxicity, which was anemia. The main effect of the drug is on red blood cell production, more than on white blood cell or platelet production.

Another predicted side effect is a decrease in spleen size. All patients had at least a 50% reduction in spleen size, and some had a 100% reduction in spleen size.

One patient with a very high allele frequency of the JAK2 V617F mutation was about to transform to AML. Following treatment for 12 months, the patient's fibrosis was almost entirely cleared, with no side effects except for mild anemia. Unexpectedly, another patient, who does not have a JAK2 mutation, had a very fibrotic marrow but by 6 months, also started to clear out the fibrosis.

What drives the response to the JAK2 inhibitor still needs to be sorted out. But the

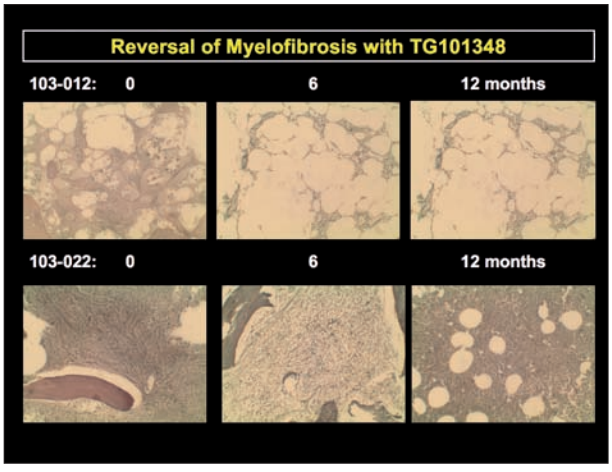

Figure 2
Reversal of myelofibrosis with TG101348.

clinical trial results argue that over-expression of JAK2 or the expression of V617F at the level of HSC triggers fibrosis. Patients who received an adequate dose in the trial also showed a decrease in JAK2 mutant allele burden. Treating people early, at the stage of myelofibrosis, may therefore change the natural history of the disease.

Overall, the preclinical data suggest that these preclinical models have predictive value. Testing human cells, particularly those that are responsible for initiating and propagating disease, may help predict not just efficacy, but toxicity in a clinical trial.

Lineage bias

Each year, CML is diagnosed in 1 in 100,000 people in the U.S. The disease is fairly well defined in terms of initiating events, and the BCR-ABL gene is known to be necessary and sufficient to initiate CML.

To test how important BCR-ABL really is in the eventual pathogenesis of the disease, a BCR-ABL lentiviral vector, with the EF1 promoter, is used to drive copGFP expression. CD34+ cells that are differentiated from human embryonic stem cells are transduced.

When those cells are transduced and transplanted into the same RAG2-/-γc-/- knockout mouse, BCR-ABL is sufficient to sustain engraftment in the primary transplant setting, but not in the secondary transplant setting. This suggests that BCR-ABL does not enhance self-renewal of these stem cells and underscores the need for additional mutations that drive blast crisis transformation.

There is a myeloid lineage bias in CML, and that bias becomes more prominent as patients progress to blast crisis. In the chronic phase, BCR-ABL is primarily expressed in HSC, but in blast crisis, they amplify BCR-ABL in the GMP population. They also activate β-catenin.

The Wnt/β-catenin pathway has an important role in stem cell self-renewal as well as malignant transformations. Work defining the Wnt pathway (see Clevers, page 9) has shown that when not phosphorylated by the destruction complex, β-catenin can translocate to the nucleus, bind LEF and TCF and activate transcription of key target genes. Non-phosphorylated β-catenin accumulates in the nucleus of HSC. In blast crisis CML, GMP harbor inappropriately high levels of β-catenin in the nucleus.

By LEF/TCF reporter assay, β-catenin is transcriptionally active and drives GFP expression in the resulting colonies. FACS analysis demonstrates sustained β-catenin activation following transplantation of these malignant progenitor cells into RAG2-/-γc-/-, giving rise to blast crisis CML, which is typified by BCR-ABL leukemic engraftment in the bone marrow, liver, spleen, thymus and the formation of BCR-ABL expressing myeloid sarcomas.

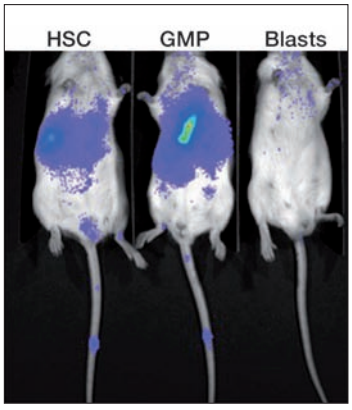

Figure 3
Quiescent blast crisis LSC are enriched in the GMP population.

The myeloid sarcomas are bioluminescent and composed almost entirely of human myeloid cells. They seem to subvert the microenvironment and become quite well vascularized. Myeloid sarcomas occur in patients with blast crisis CML who have not received or are resistant to BCR-ABL inhibitor therapy. These patients are rarely eligible for hematopoietic cell transplantation.

In eligible patients, an allogeneic hematopoietic cell transplant, the survival rate after 5 years is typically between 5% and 40%, depending on co-morbidities. Unfortunately, the acquisition of self-renewal and survival promoting mutations makes it difficult to suppress the malignant clone. So it is important to treat patients early, before they have had a chance to undergo blast crisis transformation.

The population of cells with the most robust leukemic transformation

potential is the GMP population, not the blasts or the HSC. Surprisingly, pre-labeling the cells with DiR, a lipophilic dye, reveals that GMP home to the bone marrow and then become quiescent. The spleen cells have a smaller quiescent fraction, the liver less so, and those in the myeloid sarcomas even less so. This underscores the idea that the microenvironment can educate these cells to become quiescent, thus enabling them to evade BCR-ABL inhibitor therapy.

Patients who activate β-catenin can be classified into two sub-populations: those with less aggressive disease and those with more aggressive disease.

Among those with more aggressive disease, 57% have a missplice in GSK3-β, which is the most predominant spliced isoform in the leukemic GMP population. This wouldn't have been apparent in a mouse model because the splicing rate is higher in humans than in mice. This cell type and context-specific splice isoform alters the function of GSK3-β, which regulates stem cell self-renewal and survival, by deleting the kinase domain and making it very difficult to phosphorylate and therefore inactivate β-catenin.

Over-expression of full length GSK3-β in these cells reduces β-catenin expression, both *in vitro* and *in vivo*, and decreases the replating capacity of these cells. A β-catenin inhibitor called MCC-3011, a compound derived from marine sponges, decreases LSC engraftment by 50% as a single agent[4,5] without significant weight loss. The data suggest that targeted Wnt/β-catenin inhibition may be safe and effective.

Combination treatment

In addition to GSK3-β role in phosphorylating and inactivating β-catenin, it also inactivates GLI1, a critical sonic hedgehog pathway target gene. Transgenic CML mouse models have shown that the sonic hedgehog pathway is essential for the maintenance of cancer stem cells in a CML mouse model. PF-04449913, a Smoothened antagonist made by Pfizer, also reduces GLI1 expression in blast crisis LSC *in vitro*.

To assess whether treatment with the Smo antagonist alone or in combination with a BCR-ABL inhibitor decreases human CML stem cell engraftment, LSC are transplanted intrahepatically into newborn mice and then treated with vehicle, the Smo antagonist, dasatinib — which is a potent inhibitor of BCR-ABL — or the combination. After 2 weeks of treatment, the liver and bone marrow are harvested, the cells are analyzed by FACS and BCR-ABL Q-PCR, and serially transplanted.

After 10 days of treatment, only the mice treated with combination of dasatinib and the Smo antagonist have a significant reduction in leukemic engraftment. Mice treated with single-agent therapy quickly succumb to leukemia, arguing for a combination molecularly targeted strategy. That was also borne out by the decrease in myeloid sarcoma formation, both in the primary and secondary transplantation setting, with the combination.

The combination has no effect on HSC replating, a surrogate measure of HSC self-renewal, suggesting that sonic hedgehog is dispensable for normal human hematopoiesis. Dasatinib inhibits normal HSC replating, but the Smo antagonist doesn't add to that effect.

In summary, PF-04449913, alone or in combination with dasatinib, blocks CML blast crisis LSC engraftment, at least in a humanized mouse model. The treated cells seem to lose their self-renewal capacity, but normal HSC replating doesn't seem to be detrimentally affected.

These results have prompted a multi-center phase I clinical trial, which aims to assess whether inhibiting LSC helps patients with advanced-phase CML. Participants recruited to phase Ib of this trial receive a combination of the Smo antagonist and dasatinib.

Expression of BCR-ABL at the level of the HSC initiates chronic phase while β-catenin activation and BCL-2 overexpression occurs at the level of the GMP (LSC) population in blast crisis CML.

At low doses, a BCL-2 antagonist called Apo-Gossypol induces differentiation of the LSC population *in vitro*,

and begins to normalize the progenitor commitment of these cells.

Apo-Gossypol reduces engraftment of the LSC population, showing a decrease in CD45+ cells. Interestingly, it also alters the splice isoform ratio of MCL1 promoting expression of a pro-apoptotic splice isoform rather than an anti-apoptotic splice isoform.

The more potent 97C1 derivative almost completely abrogates LSC engraftment in the bone marrow, but engraftment persists in the myeloid sarcomas and in a few cells in the spleen.

These data suggest that in the chronic phase of myeloproliferative neoplasms, initiating events occur at the level of a HSC. In CML chronic phase, there is BCR-ABL expression at the level of a HSC; in JAK2-driven neoplasms, it's JAK2 V617F.

Over time, mutated progenitors acquire over-expression of BCL2, thereby expanding the pool of progenitors, which subsequently acquire self-renewal capacity.

T-ALL order

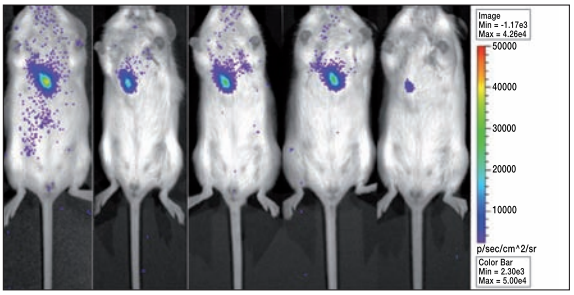

Figure 4
TP-20K Lin+ cells from T-ALL patient lose engraftment.

Another leukemia frequently driven by activation of a self-renewal pathway is pediatric T-ALL. The relative importance of the Notch1 self-renewal pathway in CML is unclear, but Notch1 activating mutations are detectable in more than half of T-cell acute lymphoblastic leukemia (T-ALL) cases.

If the CD34+/CD4-/CD7- population of cells and the lineage positive population are transduced with lentiviral luciferase and then tracked *in vivo*, the lineage positive population loses its engraftment capacity over 10 weeks. In contrast, the CD34+/CD4-/CD7- cells persist and home to the bone marrow.

To look at the functional molecular hierarchy among these cells, the cells are further sub-fractionated into a CD34+CD2+ lineage-negative population and 1,000 cells are transplanted. These cells have activating mutations in Notch, which enhance their self-renewal capacity, leading to a faithful recapitulation of pediatric T-ALL.

T-ALL CD34+ cells from pediatric patients over-express Notch target genes including HES1, and they over-express Notch at the cell surface. By CGH analysis, reveals additional mutations and deletions in CD34+ cells with the most robust transplantation potential.

Different patients vary depending on how many mutations they acquire but it's the cell type and context specificity of those mutations that matters. In addition to Notch mutations, some patients harbor PTEN deletions or mutations in PIK3R1. The mutations are more difficult to detect in the bulk population compared with the CD34+ fraction. When the cells are transplanted into secondary recipients, the exact same mutation is found in the engrafted CD34+ LSC population.

A γ-secretase inhibitor, which inhibits both Notch1 and Notch2, does not eradicate the cells even after a 3-week treatment. In patients, γ-secretase inhibitors are also known to cause intractable diarrhea. However, a targeted Notch1 monoclonal antibody given for 3 weeks reduces leukemic engraftment in the marrow of patients, even those with a high level of Notch1 expression.

Although approximately 56% of patients with pediatric T-ALL activate the Notch pathway through HD or PEST mutations, additional mutations, such as PTEN deletions, render LSC less susceptible to Notch inhibition. This underscores the importance of combining molecularly targeted therapies to block the abnormal differentiation, self-renewal, survival and homing capacity of these cancer stem cells.

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Stem cell biology meets cancer research

A report on a lecture by
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University of Toronto, Toronto, Canada

The cellular and molecular basis for the heterogeneity within a tumor is not well understood. The cancer stem cell (CSC) model postulates that heterogeneity arises because the tumor is organized as a cellular hierarchy sustained by a CSC at the apex. Clonal assays and prospective cell purification have shown that acute myelogenous leukemia follows a CSC model, in which the stem cells are the only ones capable of self-renewal and of generating rapidly proliferating progenitors and terminal leukemic blasts. These leukemia stem cells (LSC) are not functionally homogeneous but, like the normal hematopoietic stem cell compartment, comprised of distinct hierarchically arranged classes. Although there are a number of well-known problems with xenotransplant assays, recent improvements to this model have shown that LSC represent a rare sub-fraction of leukemia blasts. Based on expression analysis, gene signatures derived from LSC and normal hematopoietic stem cells turn out to be the strongest predictor of overall survival compared with signatures obtained from bulk leukemia blasts. Together these data support the hypothesis that the biological determinants that underlie stemness in both normal and leukemic cells are predictors of poor outcome, and are potential targets for novel therapy. ***John Dick*** *discussed the relevance and importance of CSC beyond experimental models.*

Experiments with mouse models of differing levels of immunodeficiency can change the observed frequency of tumorigenic cells, to the point where certain cancers like melanoma do not seem to follow the cancer stem cell (CSC) model. These new assays challenge the field to assess and, in some cases, re-assess, whether CSC can be prospectively isolated using the most sensitive xenograft models and methods of transplantation available.

Xenograft models are most advanced for the leukemias, especially for acute myeloid leukemia (AML) because of work that's been done to detect normal hematopoietic stem cells (HSC). As with normal HSC engraftment, intravenous transplant methods for AML leukemic stem cells (LSC) show that highly deficient NSG or NOG mice are much more sensitive in stem cell detection compared to the widely used NOD/SCID mice. However, the use of CD122 to deplete residual natural killer cells in NOD/SCID mice reduces this difference.

Transplantation using the intrafemoral injection (IF) technique results in a large increase in HSC or LSC detection irrespective of the recipient. Detailed studies to compare NSG/NOG with CD122-treated NOD/SCID mice, all transplanted by IF, have shown that NSG mice are only a few fold better in detecting HSC compared with NOD/SCID mice. This new model therefore does not not result in a major revision of the conclusions drawn from NOD/SCID, in contrast to the situation with melanoma (see Morrison, page 23).

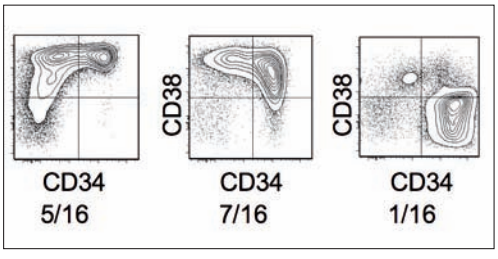

Figure 1
CD34/CD38 profiles vary in chronic myelogenous leukemia.

Many phenomena associated with cancer have been demonstrated in cell lines or animal models, but eventually, have been found not to translate to humans. The same may prove to be true of CSC. Ultimately, the way to

prove CSC's relevance is to isolate them, identify a pathway that is important in the cells, develop an inhibitor of the pathway, test it in clinical trials and change the course of the disease. There is some promising early data supporting this approach, including on CD47 (see Weissman, page 65), and a couple of clinical trials[2,3].

A second interim option might generate data quicker. Under the assumption that CSC drive tumors and that their ability to resist chemotherapy or radiation causes them to persist, it is their properties that determine whether patients survive. If that is the case, then the biological properties of CSC, translated from genetic signatures, should be more predictive of clinical outcome than just sampling the bulk tumor. That is a testable hypothesis.

Precise assays are available to test this idea in the hematopoietic system. Others have tested it in breast cancer, but that model lacks the precision of markers needed to cleanly distinguish CSC.

Functional readouts

As a first step to determine the gene expression signature of LSC from AML, LSC and non-LSC from multiple AML samples are isolated and each fraction analyzed for gene expression patterns. In the literature, some papers predict that LSC are limited to one particular cell surface fraction (CD34+CD38-). The problem in AML, however, is that the CD34 and CD38 profiles vary along a continuum. Identifying whether a specific cell fraction contains LSC requires using the most sensitive assay available. Looking at all 4 quadrants based on CD34 and CD38 expression of 16 different samples, leukemia initiating activity is found in all cases in the CD34+ CD38- fraction. But in about half of the samples, the CD34+CD38+ fraction also contains LSC.

In one case, LSC activity is found in all fractions, indicating again that AML is heterogeneous between individual samples. The frequency is variable, ranging from 1 in 1000 to 1 in 1,000,000 cells. Overall, despite the use of the most advanced LSC assay methods, the data establish that AML is organized as a hierarchy that follows the CSC model.

To carry out bioinformatics to generate signatures, the gene array data for all fractions from the 16 different samples are assembled into two bins — those that contain LSC and those that do not — and then compared for specific patterns of gene expression. The result is a 25-gene signature of genes over-expressed in LSC compared with non-LSC fractions.

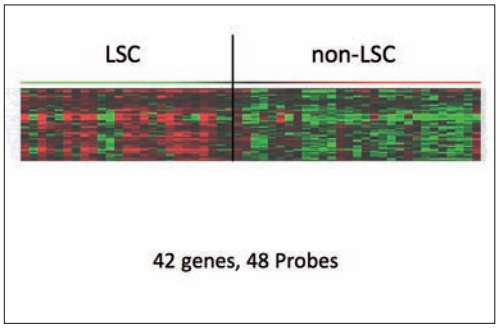

Figure 2
Gene signature for leukemia stem cells.

To assess the clinical significance of the signature, public gene expression data were obtained from160 cytogenetically normal AML samples associated with long-term survival. These samples were divided into two populations — those that have high expression of the top 25 probes of the LSC signature, and those that have low expression.

The samples with high expression of the LSC signature turn out to be remarkably predictive of a bad outcome compared with those with low expression of LSC genes, which show significantly better overall survival.

In a parallel set of experiments, using the same approach with normal HSC and non-HSC reveals a normal HSC signature of 120 genes. These 120 genes are differentially highly expressed in the HSC fraction compared with fractions composed of only progenitor cells or mature cells. This signature also shows the same high prediction for poor outcome in the same AML patient data set.

Comparing HSC genes with LSC genes using Gene Set Enrichment Analysis reveals that HSC signatures are highly enriched within the LSC signature, revealing the close similarity between these two stem cell

types. Over-expression and silencing lentivectors are being generated for the genes in these signatures to create the tools to functionally determine their role in governing LSC and HSC properties.

These observations suggest that biological programs that underlie stemness in both leukemia and normal blood are predictors of poor outcome. It will be interesting to determine the pathways that these signatures of stemness predict.

To extend the leukemia studies into more samples and more tumor types, the Canadian province of Ontario is setting up a large province-wide program to interrogate CSC and non-CSC from hundreds of samples across 10 different tumor types.

Why does stemness translate into poor outcome? It is possible that in the leukemia blasts, there is a disassociation between self-renewal and maturation, or that the elements of a stem cell program persist in mature cells.

Genetic diversity

In the clonal evolution model of cancer initiation and progression, one genetic hit in a cell confers a selective advantage, and is probably followed by a series of hits that are selected for. This model has been a large driver in cancer biology research for many decades, and is sometimes viewed in opposition to the idea of functional heterogeneity or hierarchy in a tumor, as the CSC model predicts.

It is unclear whether in this model, every cell is susceptible to a second hit, or whether there is some heterogeneity, and one cell is more susceptible to a second or third hit. This question can only be answered using clonal functional assays that assess at the level of single cells.

The general view of CSC is also that they are a homogeneous entity. But in leukemia, there is some evidence to suggest that LSC have different potencies — that they have higher or lower self-renewal, for instance, or can sustain grafts at greater or lesser amount[2]. But it is not known whether, at the clonal level, CSC are genetically identical, so these hierarchy models must take genetics into account.

Because AML is so heterogeneous genetically, the question of clonality and functional assays has been addressed in lymphoid leukemias, which are more genetically defined. Using DNA copy number alteration profiling, previous studies have shown that BCR-ABL has on average about 4 amplifications and 4 deletions. There are overall about 6 known lesions that are associated with the disease, providing a cleaner genetic space in which to work.

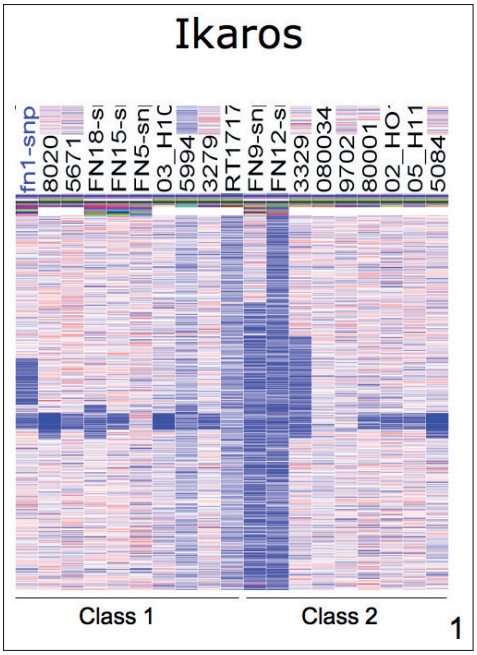

Figure 3
BCR-ABL1 lymphoblastic leukemia is characterized by the deletion of Ikaros.

One of those lesions is ikaros, which is almost obligatorially deleted in a large proportion of the BCR-ABL samples, followed by CDKN2A/2B and PAX5.

Samples were transplanted into three different mouse models with increasing levels of immune deficiency. The experiments always use IF injection, and measure engraftment of the injected bone, as well as the other sites to which cells can migrate.

Two classes

Among the 21 samples transplanted, there are two very distinct growth patterns. One class makes the mice sick, killing them all within 12 weeks, whereas the other mice are alive at that time point. Some of the second class of mice lived to reasonably long times.

Analysis of the leukemic load in the injected femur, the marrow, spleen and peripheral blood shows that the more aggressive class of tumors make the mice sick because they're growing massively and spreading broadly. In contrast, the less aggressive mice grow well in the marrow but don't spread much to the periphery, and some don't seem to grow at all.

The stem cell frequency in these two classes is markedly different by many orders of magnitude. Some have a frequency of less than 1 in 100 cells, whereas others have a frequency of about 1 in 10,000. Typically, the non-aggressive class has a frequency on the order of one in hundreds of thousands to millions. This difference in stem cell frequency seems to correlate with the differential aggressiveness in mice.

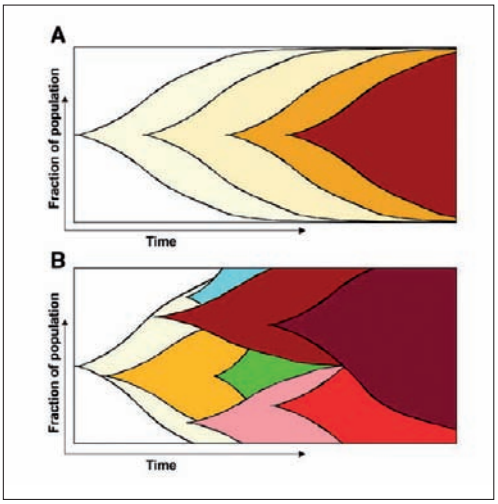

Figure 4
Monoclonal vs. multiclonal model for clonal succession in cancer.

Genetically, almost every sample in both classes has either a homozygous or hemizygous deletion in ikaros. But only class 1 samples, which are the aggressive tumors, have CDKN2A deletions. Many more of the class 1 samples also have deletions in PAX5, compared with class 2 samples.

The aggressive leukemias engraft and grow in all types of immune-deficient mice, even if the mice are not irradiated first. The leukemia-initiating cells in this class are permissive in their capacity to engraft less immunodeficient mice.

The non-aggressive samples are more selective, and do not engraft NOD/SCID mice. They do, however, engraft into a more immunodeficient mouse like NSG. This suggests that something restricts the growth of these cells that is linked to their response to innate immunity.

If a sample that does not grow in a non-permissive recipient is engrafted in a permissive mouse, and then put back into the non-permissive recipient, it starts to grow. The cells seem to adapt or acquire properties that allow them to grow in this less permissive environment.

One aspect that changes in these tumors seems to be the limiting dose. The frequency of LSC increases with secondary transplant, meaning that there is a change in the ratio between LSC and non-LSC.

Comparing the samples before and after secondary transplants suggests that samples might acquire copy number abnormalities during serial transplantation. For example, one sample shows a deletion in a particular region of chromosome 13, suggesting an outgrowth of clones with different genetic aberrations.

This isn't just a mouse phenomenon, however. Similar changes are seen in human sets of samples, suggesting that these copy number abnormalities are not just an artifact of the xenotransplantation model, but are clinically relevant.

Other researchers have done single-cell analysis on patient samples and made a convincing argument that there is little evidence for traditional clonal evolution. Instead, there is evidence for branched evolution, with clones at independent levels of evolution. With the help of clonal analysis and functional readouts, animal models can begin to link function to single-cell clonal events[4].

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Normal and neoplastic stem cells

A report on a lecture by
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Self-renewal is the principal property that distinguishes stem cells from their daughter cells. When stem cells divide they give rise to stem cells (by self-renewal) and progenitors (by differentiation). The balance between self-renewal and differentiation is what generates and then maintains tissues, and enables them to respond to injury or other stressors. Studies identifying hematopoietic stem cells and progenitors have made hematopoiesis one of the best systems for studying the molecular changes in cell fate decision-making and oncogenesis. What's more, it serves a paradigm for finding preclinical and clinical platforms for tissue and organ replacement and regeneration, Stem cell isolation and transplantation is the basis for regenerative medicine. Self-renewal is dangerous, and therefore strictly regulated. Poorly regulated self-renewal can lead to the genesis of cancer stem cells (CSC), the only cells within a tumor or leukemia that have the ability to self renew and therefore, the cells that maintain the cancer. Thus, it is predicted that CSC elimination is required for cure. This prediction necessitates profoundly different approaches to cancer research, compelling investigators to prospectively isolate CSC and to characterize the molecular pathways regulating their behavior. ***Irving Weissman*** *discussed the need to develop truly effective therapies that target CSC.*

Stem cells are defined as those that, at the single-cell level, can both self-renew and differentiate. In the hematopoietic system, the stem cell can not only give rise to more stem cells but, through various steps of differentiation, generate everything that's in the blood.

When hematopoietic stem cells (HSC) were first isolated, they showed that stem cells do not express markers of the lineages they are to become. But they have a distinct set of markers, none of which is enough on its own to isolate the cells. In the young adult mouse bone marrow, these cells number roughly 1 in 15,000. In both mice and humans, the identity of the HSC is proven in a bone marrow or HSC transplant, in which limited numbers of cells save the host and give rise to donor-derived blood-forming cells of all types, including stem cells.

HSC can migrate out into the bloodstream and become part of a circulating or re-circulating pool. Once a cell enters the bloodstream, it remains there for less than a minute for 90% of the cells, and less than 5 minutes for the rest. After it leaves the blood vessel, it homes highly selectively to go into the sinusoids that line the bone marrow, the liver and the spleen, which are the hematopoietic sites of a mouse.

Every day, about 30,000 stem and their multipotent progenitors go into the bloodstream of a mouse. The cells use integrin $\alpha 4$-$\beta 1$ to home to VCAM1+ vessels, bypass the sinusoidal macrophages with CD47, and use their CXCR4 receptor to home to SDF-1. Cancers of these cells also use this same system to metastasize.

Most HSC are in G0 or resting phase. Between 3% and 8% of the cells each day go into cell cycle, seemingly at random. They then go through multipotent progenitors which, at the single-cell level can give rise to virtually all of the cells of the blood system, but don't self-renew.

After several weeks in this stage, they divide and go into either the myeloid or lymphoid pathway through a common myeloid progenitor or a common lymphoid progenitor. The common myeloid progenitors give

rise to granulocyte monocyte progenitors (GMP), one of the levels at which a myelogenous leukemia, but not normal GMP, can have a self-renewing cell.

Aging phenotype

Mice start off with a set number of HSC, which give rise to lymphoid and myeloid progenitors at a balanced rate. By the time mice are 2 years old, however, they have about five to ten times the number of HSC, which give rise to more myeloid cells — granulocytes and monocytes — than to lymphoid cells. When old stem cells are transplanted into young animals, they retain the old phenotype and have a myeloid bias.

HSC of the old phenotype over-express myeloid transcription factors, and 17 of the top 32 are genes that are found in human myelogenous leukemia to be either mutated or translocated. As a class of cells, they up-regulate genes involved in leukemias that tend to occur in older people.

There are two ways to think about this phenomenon. The Lamarckian view suggests that the young adult cells become old age cells and, as they age, they change their transcriptome and somatically inherit that acquired characteristic.

A second way to look at it, however, is that the young adult cells are already diverse clones of cells, some of which are myeloid-biased, others of which are lymphoid-myeloid balanced and so on. In that case, there is a Darwinian selection of the cells that have the phenotype right for the age.

In the Lamarckian model, every stem cell has to be regulated so that its change in transcriptome is appropriate to the animal. In the Darwinian model, cells have their known transcriptome and don't change it, but they do whatever they do whenever they're called into cell cycle.

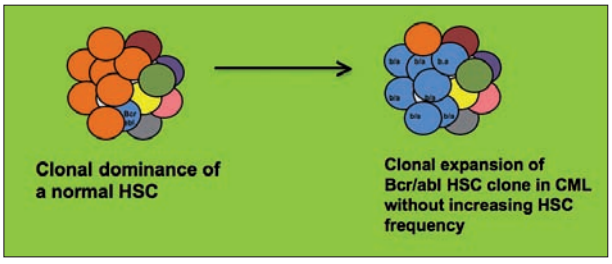

Figure 1
Clonal selection of hematopoietic stem cells in the development of CML.

In a young mouse, there are a rare few cells with CD150 at high levels that are found in a predominant number of old mice. Those cell give rise to the higher number of stem cells and a myeloid-biased output if transplanted. All of the available evidence suggests that clones of stem cells are diverse from the beginning and are selected as the animals age[1].

When AML cells from patients with the AML-1/ETO+ translocation are transplanted into immunodeficient mice, the cells that are CD34+/CD38- and harbor the leukemic stem cell (LSC) are the multipotent progenitors that lack Thy-1, rather than the stem cells that have Thy1. Comparing stem cells from many patients, anywhere from 5% to 40% of the stem cells that have Thy1 harbor the AML-1/ETO+ translocation, but they are normal on transplantation and give rise to normal multi-lineage cells *in vitro*.

In the pre-leukemic phase, AML-1/ETO, which is necessary but not sufficient for leukemia, is present in a subset of HSCs, and because they're self-renewing cells, is fixed in that population. Translocation into a multipotent progenitor doesn't give rise to leukemia, and the cells have a normal lifespan[2].

Cumulative events

The cell that harbors BCR-ABL is probably at or near the initiating cell in the HSC pool. Before myeloid blast crisis, that clone comes to dominate the clones of stem cells without measurably increasing the frequency of stem cells.

When that pre-leukemic cell leads to a frankly leukemic cell, however, it does go beyond the bounds of cell

numbers. In most cases of AML, the LSC arises at the multipotent progenitor, but in myeloid blast crisis of chronic myelogenous leukemia (CML), it emerges at the level of the GMP.

The model then is that stem cells give rise to blood cells, and in that whole population of cells, the HSC is the only self-renewing cell and the only cell that can go through the minimum progression seen in virtually every myelogenous leukemia in mouse or humans. A number of rare independent events seem to accumulate in sub-clones of the stem cells. It's possible to see in a patient a LSC with all the relevant events, but also cells with the progression of events.

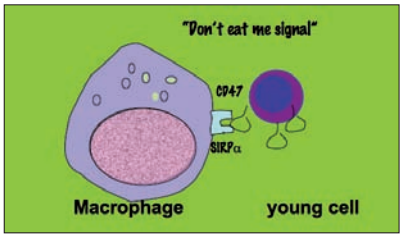

Figure 2
Mouse AML marrow cells over-express CD47.

There are a variety of mutations that can occur at the level of stem cells, including BCR-ABL, AML-1/ETO, JUN-B and JAK2. The cells express anti-senescence and anti-apoptotic factors. Myeloid leukemias also evade T cells and macrophage eating. And, at least in CML going to blast crisis, they activate or retain self-renewal pathways.

According to the leukemia CSC hypothesis based on these observations, the cell is still trying to produce progeny. Chemotherapy is based on reducing the tumor load in the model system, but if these cells are on average 5% of the tumor, it may not affect them at the same rate as it affects the daughter cells[3].

Compared with the daughter cells, these LSC or CSC are resistant to radiation-induced, free radical-induced DNA damage. The cells also have transporters to take small molecule drugs out. As a result, available cancer drugs have more effect on the daughter cells than on the CSC.

AML cells express a molecule called CD47, which serves as a "don't eat me" signal: its presence on a young red blood cell (RBC) tells a macrophage the cell is too young to be eaten. It conveys this signal through the SIRP–α receptor, which has immunoreceptor tyrosine-based inhibitory motifs that activate SHP phosphatases and prevent phagocytosis[4].

If CD47 is knocked out, even the youngest, newborn RBC would be eaten by macrophages. HSC from a mouse that lacks CD47 expression cannot be transplanted into a wild type mouse because they are all eaten at the entry points of the macrophages in the sinusoids.

In normal mice, CD47 is present everywhere in the body, on all circulating cells. It's not present on resting HSC, but when the cells are mobilized into the cell cycle, they first up regulate CD47, then go into the bloodstream, bind to VCAM1+ vessels, pass the macrophage-lined sinusoids, and within 2 days revert CD47-lo status.

This seems to be a mechanism by which cells can emigrate from one place to another via the bloodstream. Most stem cells, including HSC, brain stem cells and osteochondral stem cells are moving cells. It's important to look at moving programs in these cells for genes that might be involved in, for instance, the epithelial-mesenchymal transition.

Risk factor

CD47 is an important molecule in human leukemia, and is expressed broadly on all AML subtypes. By microarray analysis of AML patient samples, those with the highest 30% levels of CD47 were dead in 5 years, no matter what the therapy. And among those in the lowest 30%, about half were alive after 7 years, suggesting that CD47 is an independent risk factor for outcome in leukemia.

In order for CD47 expression to be effective, the macrophage also has to have an "eat me" receptor. If a tet vector making CD47 is inserted into a cell line, the cells grow outside of the macrophages and are not phagocytosed. Cells that don't have CD47, in contrast, are taken up by, eaten and killed in the macrophages.

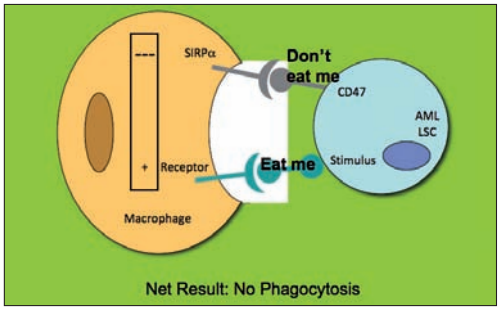

Figure 3
Increased expression of CD47 on human AML is associated with a worse clinical outcome.

If human AML cells taken directly from patients in the clinic and added to human macrophages in a dish, adding an anti-CD47 antibody — and only an anti-CD47 antibody — results in the LSC being inside the human macrophages.

If patient AML LSC samples are pre-coated with the blocking anti-CD47 antibody, HSC don't transfer the disease because they are eaten by macrophages. If the antibodies bind to the cell surface but don't block the CD47 SIRP-α interaction, the cells grow as a tumor.

The same is true of primary human leukemias taken from patients. If the cells are treated beginning at about 3 to 4 months after transfer to NSG [T, B and NK-deficient mice] with 100 micrograms/day of anti-CD47 for 14 days each day, the tumor disappears from the bloodstream, and from the bone marrow in most animals. Before treatment, the bone marrow is effaced with leukemia, but after 2 weeks of treatment, all that seems to be left are a few residual HSC. This bone marrow does not transfer the disease to another animal.

In immunodeficient mice with non-Hodgkin's lymphoma, neither the anti-CD47 antibody nor rituximab cures the mice on its own, but the combination of the CD47 antibody and rituximab given together cure virtually every mouse.

If human bladder cancer "stem cells", isolated by their tumor-initiating property, are transplanted into either RAG-γ or NOD/SCID-γ mice, T- B- and NK-deficient mice, the tumor transfers only with CD44+ cells, not the CD44- cells that are made in the tumor. When the tumors grow as spheres or nodules, the CD44+ cells are at the rim of the growth. The cells co-stain with CD47. Coating the cells with anti-CD47 therapies results in the cells being eaten by human macrophages.

Common marker

CD47 is also a risk factor for ovarian cancer in humans. In both ovarian and bladder cancer, the two antibodies that block CD47 signaling and SIRP-α enable phagocytosis of the cancer cells. If an ovarian tumor is transplanted and treated with either anti-CD47 antibodies or IgG controls, the anti-CD47 antibodies can eliminate the ovarian xenograft tumors. Antibodies targeted to CD47 can also eliminate breast cancer xenograft tumors.

These results indicate that CD47 is a common marker that's up-regulated on cancer cells, expressing a "don't eat me" signal. LSC from AML or from the acute phase of CML express CD47. Bladder cancer stem cells, ovarian cancer cells, non-Hodgkin's lymphoma, glioblastoma and medulloblastoma brain tumors, malignant melanoma, breast cancer and colon cancers have all been found to over-express CD47.

It is possible to transplant human surgical sample melanomas separated by CD271. The minimum number required to transplant is usually 100 cells, and cumulatively with doses of 100-10,000 surgical sample human melanoma cells, 25 of 33 CD271+ cells transfer, compared with only 3 of 34 for CD271- cells. This is highly significant. As the tumor is passaged, more and more of the CD271- cells can transfer the tumor.

Interestingly, very few tumor CD271+ cells express all 3 of the classical melanoma tumor antigens, MART1, MAGE, and the tyrosinase HMB45, whereas these antigens are expressed on the CD271- populations in the same tumors. This goes a long way to explain why these antigens have not resulted in any functional immune therapies.

Anti-CD47 antibodies would be a great therapeutic option if CD47 were not present on normal cells. Giving mice 2 to 5 times the tumor-effective dose of anti-CD47 clears a HoxA9:Meis 1 mouse leukemia with little to no toxicity.

The antibody does not seem to affect normal cells even though it binds in all tissues. The prediction is that there is an "eat me" signal expressed on cancer cells and not on normal cells, and the "eat me" and "don't eat me" signals work together to determine phagocytosis of the target.

When a cell is stressed and undergoing abnormal divisions, it activates or expresses sugars or proteins that are not normally on viable cell surfaces, creating an "eat me" signal. That happens in tumor cells, but those tumor cells that also have a "don't eat me" signal are selected. When these cells are treated with an antibody that blocks the "don't eat me" signal, the "eat me" signal is seen and the cells get eaten.

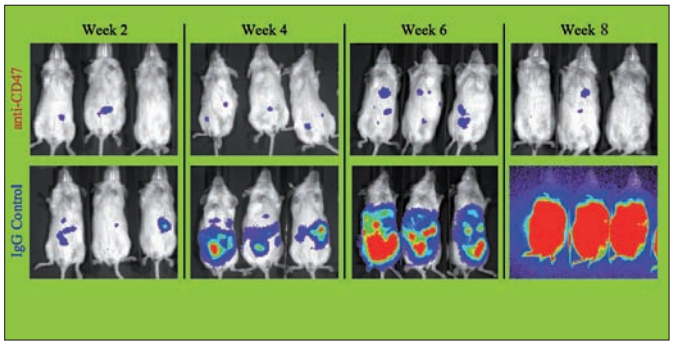

Figure 4
CD47 antibodies can eliminate ovarian cancer xenograft tumors.

This hypothesis puts the macrophage, and not the NK cell, as the most important front-line defense not just against leukemias, but against carcinomas.

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Targeting self-renewal in breast cancer stem cells

A report on a lecture by
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Human breast cancers appear to be hierarchically organized and driven by a cellular subset that displays stem cell properties. The dysregulation of self-renewal pathways in stem cells may be a key event in carcinogenesis. These tumor-initiating or cancer stem cells have been shown to be relatively resistant to available therapies, but inhibitors of self-renewal pathways may be able to specifically target them. CXCR1, a cellular receptor for the inflammatory cytokine interleukin-8 is over-expressed in breast cancer stem cells. Recombinant IL-8 stimulates the self-renewal of cancer stem cells as demonstrated by tumorisphere formation and by the Aldefluor assay. In vitro, a small-molecule CXCR1 inhibitor selectively targets breast cancer stem cells. One of the most significant clinical advances in the treatment of breast cancer has been the development of HER2 blocking agents. Recent evidence suggests that the remarkable efficacy of these agents may be due to their involvement in regulating breast cancer stem cells. Another important stem cell regulatory pathway frequently dysregulated in breast cancer is the Notch pathway. Inhibition of Notch signaling utilizing γ-secretase inhibitors is able to target breast cancer stem cells, and is being tested in clinical trials. ***Max Wicha*** *discussed the need to reevaluate clinical trial design and the strategies used to develop adjuvant treatments.*

The mammary gland is a beautiful developmental system because it undergoes postnatal development during pregnancy, lactation and involution, and also because it's so important clinically for the development of breast cancers. There has been a great deal of progress in elucidating the markers that might define some of these components. Although the markers are not as defined as for the hematopoietic system, they are modeled after both the hematopoietic and neuronal literature.

One of the goals of research in this arena is to understand the hierarchy in the normal gland in order to elucidate the pathways that regulate the self–renewal of stem cells, and the progression of differentiation to early progenitors, late progenitors, and the cells that constitute the mature gland. There's accumulating evidence that cancers can arise from stem cells or from progenitor cells, each of which may produce different molecular subforms of breast cancer (see Visvader, page 95).

Over the past 8 years, a series of both *in vitro* and mouse models have been developed to study human mammary stem–progenitor cells and interrogate stages of differentiation of the human mammary gland. There are also systems that use normal human mammary cells, taken from women who have had reduction mammoplasties. These models have provided the basis for understanding self–renewal pathways because the same pathways are deranged in carcinogenesis, and to develop targets for therapeutic intervention.

The assays used are borrowed from the neuronal literature. Like neurosphere–based assays, they rely on spherical structures dubbed mammospheres. When primary cells are put into suspension culture, only a small fraction survive. Stem cells and progenitor cells in various stages of differentiation can be assayed by putting them under conditions in which they differentiate, or in matrigel in which they undergo morphogenetic differentiation.

More recently, modified systems have been developed in which primitive cells can be reintroduced into NOD/SCID mice or NOG/SCID mice. The mammary gland can be humanized with mammary fibroblasts in

order to induce the regeneration of mammary glands. And mammary cells can be transplanted into cleared fat pads (see Visvader, page 95). All of these have been very useful and have been modeled after mouse mammary biology.

For primitive cells, aldehyde dehydrogenase (ALDH) is a functional marker that marks primitive cells in mammary and many other tissues. ALDH belongs to a family of about 30 enzymes, which mark both stem and progenitor cells. The enzymes also play a functional role by metabolizing retinol to retinoic acid, which is necessary for stem cell differentiation.

A simple assay called the aldefluor assay isolates cells with increased activity of all isoforms of ALDH. Aldefluor is a fluorescent substrate that's freely diffusible across the cell membrane. Under ALDH action, it becomes ionized and trapped in the cell. There is also a specific inhibitor that blocks ALDH, and helps set the gates of the flow cytometer.

Aldefluor assay

Interrogated for stem and progenitor properties, cells within the aldefluor+ population form mammospheres, whereas the aldefluor– ones don't. If the cells are put into a mammary fat pad that has been preconditioned by human mammary fibroblasts to make it into a human microenvironment, the aldefluor+ cells form glands whereas the aldefluor– cells don't.

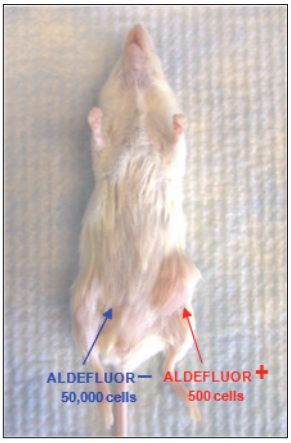

Figure 1
Aldefluor+ population and tumorigenicity.

The same assay can be used to isolate tumor–initiating cells from human breast cancers. These studies rely either on primary tumors taken directly from patients or on tumors that have been passaged in NOD–SCID mice, but that have not been put into tissue culture.

With serial deletions of 50,000, 5,000 or 500 cells put into contralateral sides of a mouse, 500 aldefluor+ cells grow tumors, whereas even 50,000 aldefluor– cells don't. In primary tumors, aldefluor+ cells constitute between 1% and 5% of the population, but not all subtypes of breast cancer have this aldefluor+ population.

Tumors derived from aldefluor+ cells have cells that are both aldefluor+ and aldefluor–, regenerating the heterogeneity of the initial tissue. Essentially, all aldefluor+ human breast tumors are able to be grown as xenografts. Interestingly, many aldefluor– cells, which are almost all estrogen receptor (ER) positive, are harder to culture.

Cancer stem cells (CSC) are defined operationally, as cells that can self–renew as well as differentiate into cells that have lost their self–renewal capacity.

When aldefluor+ cells are compared with previously discovered markers such as CD44+/CD24–, used to isolate the tumor initiating cells in breast cancers, there is an overlapping but non–identical population. Cells that share all three markers are even more tumorigenic, with 20 cells forming tumors in NOD/SCID mice, and as few as 5 cells forming tumors in NOG/SCID mice[1].

A particular isoform of ALDH, called ALDH A1, is expressed in stem cells, making it easier to find these cells in human tissues. This marker identifies both stem and progenitor cells, so it enriches for stem cells, but does not signify stem cells exclusively.

In a series of almost 500 tumors, a few very aggressive tumors are full of cells that stain with the marker. In most tumors, these cells comprise 1% to 5% of the population and tend to be on the leading edge of invasive edge of the tumor. And then some tumors don't stain at all[2]. In those tumors, virtually all the cells are ER+.

The expression of this marker is not an independent variable for bad prognosis, but there is a positive correlation with the grade of the tumors, and a very strong negative correlation with ER and progesterone receptor. Tumors that are ALDH+ are highly proliferative. There's also a very strong correlation between ALDH expression and HER2.

Molecular typing

Even though primary tumors are used for much of the work on the mammary–initiating cells, many cell lines are also hierarchically organized. Because cell lines are adapted to tissue culture conditions, it is not possible to extrapolate directly when looking at the influence of the microenvironment. But cell lines can be used for mechanistic studies that are then verified using primary tissues.

There are also cell lines derived from each of the known molecular subtypes of breast cancer. For example, of 34 cell lines, all of the ones that are ALDH– are ER+, luminal type A cell lines. This correlates with clinical data. Basal cell lines have an ALDH+ population, which are the tumor–initiating cells. ALDH+ cell lines generate both ALDH+ and ALDH– cells, recapitulating a ratio that's characteristic of each cell line. In contrast, ALDH– cells do not generate ALDH+ cells.

Comparing the gene expression profiles of both ALDH+ and ALDH– populations in a series of cell lines reveals that many of the genes regulated in the ALDH+ populations are known stem cell regulatory genes. Genes that are up regulated in ALDH– populations are involved in oxidative metabolism and cell signaling.

One gene expressed in ALDH+ cells that has important clinical relevance is CXCR1. Another is RAD51, which is involved in DNA repair. This may be related to observations from a number of different groups that CSC have more efficient DNA repair capacity than do the bulk tumor cells.

CXCR1 is one of two receptors for interleukin 8 (IL-8). IL–8 is associated with ER– breast cancers, and the level of IL–8 in serum correlates with a poor outcome and high metastasis.

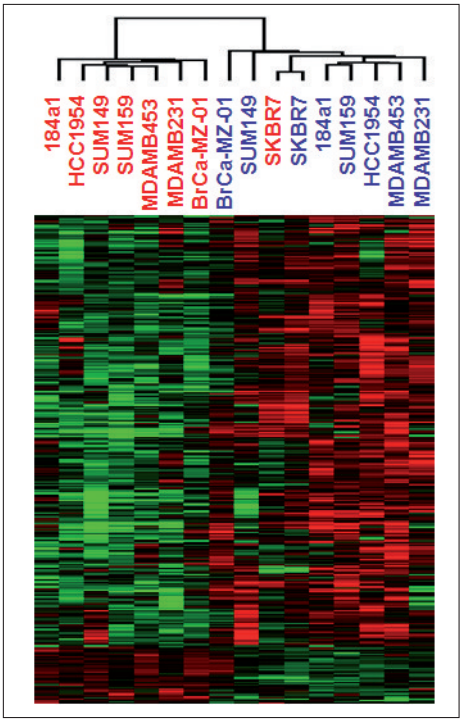

Figure 2
Breast cancer stem cell gene expression signature.

In four different cell lines, a CXCR1 antibody shows that CXCR1 is exclusively expressed in a subset of aldefluor+ cells, with virtually no expression in the aldefluor– population. Recombinant IL–8 increases tumor sphere formation in a dose-responsive manner.

IL–8 correlates with metastasis in patients. In invasion assays, within a given cell line, most of the invasive capacity is determined by the ALDH+ population, not the ALDH– population. What's more, if IL–8 is added into the bottom chamber, only the aldefluor+ cells become more chemotoxic. Injected into the left ventricle of NOD/SCID mice, the aldefluor+ cells are highly metastatic, whereas the aldefluor– cells have much less metastatic capacity, if any.

Analysis of microRNA expression shows that both LET–7, which is very well studied, and MIR93 are down regulated in the ALDH+ populations. If cells are exposed to tetracycline followed by MIR93 induction, there is a marked reduction in the CSC population. This effect is not because of apoptosis, but because the cells differentiate into an ALDH– phenotype.

Inducing MIR93 has little effect on the gene set of ALDH– cells, but ALDH+ cells turn on a series of genes at the very early time points, involved in virtually all of the pathways known to regulate stem cells, including Notch, hedgehog, AKT, BMI1 and STAT3.

The hypothesis is that a quiescent stem cell is negative for the parent gene MCF7, it is not proliferative and doesn't express MIR93. When proliferation is turned on in these cells, MYC induces E2F, which turns on MCM7, which then turns on MIR93. This causes the cell to proliferate and differentiate.

Adjuvant therapy

When MIR93 is induced in an established tumor, non-tumor initiating cells can continue to proliferate, but only for a few cycles. Still, this results in a tumor that continues to grow, so in a clinical trial, it would appear as if the patient had progressed. Even if the stem cells are knocked down and the tumor has few tumor-initiating cells, the size of the tumor is determined by the bulk population. Combining traditional therapy with stem cell-targeting agents would hit both populations.

Adjuvant therapies are very different than treating bulk disease in the clinic. When stem cells are knocked down early on, the tumor doesn't grow, and the treatment blocks both tumor growth and metastasis. Tumors grow back after chemotherapy treatment is stopped, just as in patients in the adjuvant setting, but the stem cells are knocked down, they don't grow back.

These observations suggest that the endpoints both in preclinical models and in clinical trials need to be reexamined. Tumor regression has been a marker of efficacy, and has led to several therapies that can shrink tumors, but don't increase patient survival. For example, for breast cancer and many other diseases, longevity of patients doesn't correlate with tumor regression. CSC may be resistant to therapy, so therapies must target CSC while sparing normal stem cells that are needed for survival.

In a clinical trial for patients with large breast cancers, the participants got neoadjuvant chemotherapy up front. Stem cells were measured before and after in order to assess the effect of therapy on the cells. When patients were given chemotherapy, as the tumors shrank down, the percent of stem cells actually increased.

Interestingly, this is very different in patients who have HER2–amplified tumors and get a HER2 blocking agent, lapatanib. In those cases, as the tumor shrinks down, the number of stem cells also decreases.

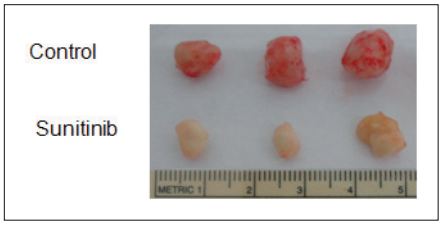

Figure 3
Sunitinib delays tumor growth.

In the neoadjuvant setting, only a complete pathologic rate translates to a good outcome for patients. Patients who have a 95% reduction in tumor size don't do any better than those who show no reduction of tumor. However, there's another reason that CSC increase after chemotherapy. When tumors are treated with chemotherapy or FAS antagonist, the bulk tumor cells make IL–8. In addition, the FAS ligand kills surrounding cells by a bystander effect.

But the IL–8 binds to CXCR1 and has two effects. It triggers self–renewal, working through an AKT/β–catenin loop, and it also blocks apoptosis. It blocks FAS–induced apoptosis because focal adhesion kinase, which is activated by CXCR1, blocks FADD, which is the signaling molecule that signals FAS death.

This series of events also occurs during involution of the normal mammary gland, when the bulk of cells undergo apoptosis mediated by FAS and falling hormones. IL–8 stimulates and protects the normal mammary stem cell, which is why a woman never runs out of stem cells in subsequent pregnancies.

In a tumor, this process can be blocked with a monoclonal antibody to CXCR1. A small molecule inhibitor called reparataxin, developed by an Italian company to treat graft reduction after transplant, blocks Il–8, which is involved in the immune response[3].

When tumors are treated with chemotherapy alone, or with chemotherapy plus reparataxin, the big difference is in the stem cell population. Chemotherapy increases the number of stem cells, whereas the combination knocks them down.

Just as predicted with the miRNA experiments, the results are more dramatic when the therapy is given earlier. If the cells are injected into the left ventricle of a mouse and treated with reparataxin alone, it significantly blocks metastasis. Tumors that are resistant to this treatment are ones that have PTEN deletions, because they activate AKT independent of CXCR1.

Oxygen's effects

There is substantial literature on normal stem cells in hypoxic environments in stem cell niches. Embryonic stem cells remain undifferentiated longer under low oxygen levels, and adult stem cells often reside in hypoxic niches. When breast cells are grown at 2% oxygen compared with 21% oxygen, there is a significant increase in all the breast stem cells, including in primary growths. Hypoxic tumors are often resistant to conventional therapies and poorly differentiated.

Approved anti–angiogenic agents are expensive, but clinical results with these drugs are modest. Most of the therapies delay tumor growth and increase patient survival by only a few months. Two interesting articles in *Cancer Cell* last year showed that anti–angiogenic agents make tumors more aggressive[4,5]. The papers suggest that the drugs perhaps work on the stem cell component to increase stem cells. It is possible that these anti-angiogenic therapies stimulate CSC through induction of tumor hypoxia.

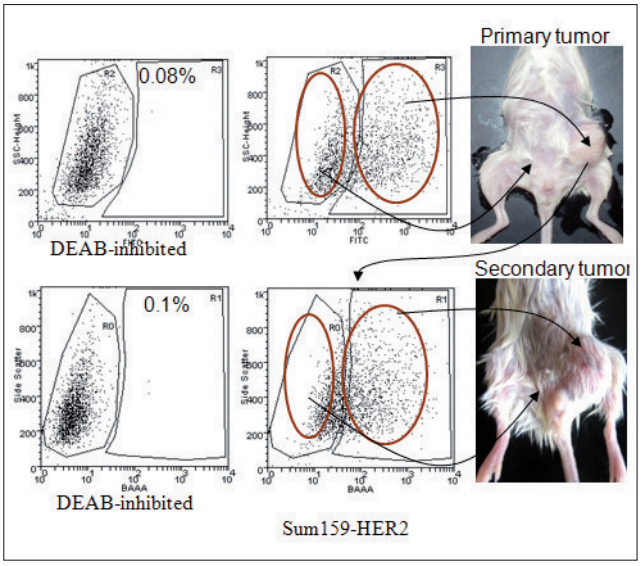

Figure 4
HER-2 regulates breast cancer stem cells.

Sunitinib, a multi-kinase inhibitor, is not a pure anti-angiogenic agent, but works as a potent angiogenic agent. Given early or late, it blocks tumor growth. It also increases the number of stem cells, as seen by the increased tumor-initiating frequency.

As seen by ALDH staining and hydroxy probe staining, sunitinib increases the number of CSC in the hypoxic areas. This suggests that, in the clinic, anti-angiogenic agents should be combined with anti-stem cell agents.

HER2-blocking agents are probably the biggest clinical advance against breast cancer. Women who take herceptin, trastuzumab or lapatanib have a 50% reduction rate in their cancer recurrence rate.

Herceptin was probably the first cancer stem cell agent, although when it was developed, it clearly wasn't appreciated as such. When transfected into cells, HER2 increases the aldefluor+ population. Even though all the cells express HER2, only the aldefluor+ cells make tumors, whereas the aldefluor– cells don't. HER2 over expression drives the stem cells, making a larger component of stem cells. Trastuzumab or herceptin, which block HER2, essentially knock down stem cells, which explains why they are so effective.

These drugs were supposed to only be effective in patients who have amplified HER2. But several women who had been misdiagnosed and did not have HER2 amplification benefited just as much or more from the

HER2 blocker as those with the amplification[6].

This may be because tumor shrinkage in advanced disease is a marker of bulk population, whereas adjuvant recurrence is a marker of stem cells. When a patient has amplified HER2, herceptin shrinks the tumors. In the adjuvant setting, HER2 is expressed only in the stem cells and not in other cells, so herceptin knocks them down even when HER2 is not amplified.

Immunochemistry might help predict which patients would benefit from trastuzumab in the absence of amplification, as those are the ones who express HER2 in the stem cells.

When it is not amplified, HER2 is regulated through pathways like Notch. Notch turns on HER2 expression, suggesting that Notch is an important regulator of stem cells. There is preclinical data showing that if tumors are treated with chemotherapy alone, the number of stem cells increases, but a γ-secretase inhibitor that blocks Notch knocks down the stem cells.

In a phase I clinical trial, adding the Notch inhibitor significantly knocks down the tumor-forming capacity or the number of CD44/CD24 cells. The inhibitor is well tolerated, and gastrointestinal toxicity is schedule-dependent.

Phase II of the trial will be a randomized trial of chemotherapy alone compared with chemotherapy plus the γ-secretase inhibitor DLL–4. There are other trials planned with a combination Notch and Hedgehog inhibitor, and the compound reparataxin. These different trials should soon reveal whether knocking out stem cells benefits patients.

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PTEN, stem cells and cancer stem cells

A report on a lecture by
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The PTEN tumor suppressor gene is one of the most frequently mutated genes in human cancers. PTEN and its controlled pathway regulate stem cell homeostasis, and PTEN loss is associated with cancer stem cell (CSC) transformation and, ultimately, tumorigenesis. Conditional deletion of PTEN leads to enhanced self-renewal capacity and G0-G1 cell cycle entry, which yield stem cell expansion. But the consequences of PTEN loss in stem cells may be cell lineage-dependent. In the PTEN null acute T-lymphoblastic leukemia model, at least two subsequent spontaneous alterations, namely β-catenin activation and a TCRα/δ-c-MYC translocation-mediated c-MYC over expression, lead to the formation of leukemia stem cells. These results provide strong evidence that the molecular and genetic events involved in 'multiple-hit' tumorigenesis are likely to take place at the level of CSC. A recent study on glioma tumor stem-like cells suggests a novel role of the PTEN/PI3K/AKT pathway in regulating the side population phenotype and ABCG2 activity. Combination therapies with inhibitors for PI3K or AKT may significantly synergize with drugs that are substrates of ABCG2 in inhibiting CSC. ***Hong Wu*** *emphasized the need to understand the nature of molecular mechanisms that lead to the formation of CSC, and the development of drugs that target these dysregulated pathways.*

Genetic studies suggest that PTEN has an important role in the regulation of stem cells. PTEN deletion from mouse embryonic stem (ES) cells shows that in those cells, PTEN negatively regulates cell proliferation and survival. Even if all the growth factors are withdrawn, these ES cells can still proliferate and survive, at least for a short period of time.

Because conventional deletion of PTEN causes embryonic lethality, most of the knowledge about its function, especially in tumorigenesis, comes from conditional deletions. When a PTEN conditional allele is created in neural stem cells, PTEN is found to negatively control stem cell self-renewal both in the embryonic stage and in the adults. Deleting PTEN in these stem cells results in extensive self-renewal, enhanced proliferation and decreased apoptosis.

The possible mechanism of PTEN control in neural stem cells is similar to that seen in the p21 knockout (see Scadden, page 37). PTEN modulates G0-G1 cell cycle entry. PTEN deletion makes stem cells extremely sensitive to growth factors, so that they need only a hundredth of a growth factor such as fibroblast growth factor in order to form neurospheres.

Many other labs have also used PTEN conditional deletions in primordial germ cells, either the

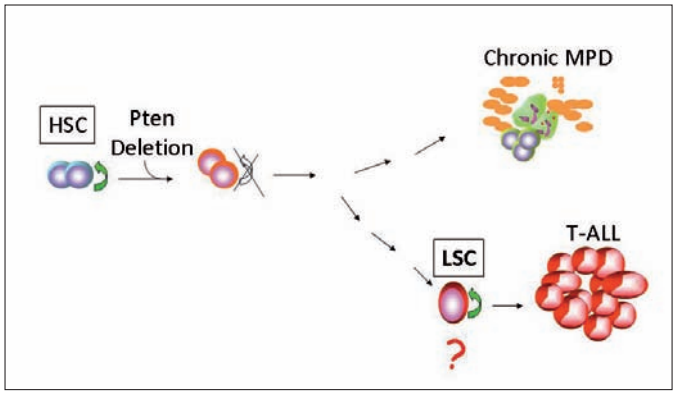

Figure 1
Mechanisms of leukemia stem cell self-renewal.

sperm or the oocyte. In both cases, PTEN negatively regulates germ cell proliferation and self-renewal.

A large array of PTEN-deleted animal models also suggest that PTEN may have role in regulating stem cells, which may link to its function in controlling tumorigenesis[1]. In organs in which there is no evidence of stem cell effect — such as cardiac muscle, skeletal muscle, insulin-secreting cell beta cells, or insulin-responsive cells, including adipocytes — PTEN deletion reveals its most conserved function, which is response to insulin signal and the control of cell metabolism. Interestingly, several of these PTEN conditional deletion models are hyper-sensitive to insulin and resistant to chemical-induced diabetes[2].

When PTEN is deleted either in the stem cells or in the progenitor population, it causes either pre-cancer, or a cancer phenotype, except in the brain. This suggests that stem or progenitor cells may be more effective targets for oncogenic events.

Multiple molecular and genetic events are known to contribute to tumorigenesis, but how these events lead to CSC transformation is not entirely clear. The PTEN-null acute T cell lymphoblastic leukemia (T-ALL) model has helped to understand cancer evaluation and CSC evolution.

In a Mx-1-Cre inducible system — in which PTEN is deleted in nearly 100% adult hematopoietic stem cells (HSC) — PTEN deletion leads to HSC exhaustion, then myeloproliferative disorder (MPD), followed by the dramatic development of acute leukemia, including acute myelogenous leukemia (AML) and acute lymphoblastic leukemia (ALL). However, two issues remain to be addressed. One is the identity of leukemia stem cells (LSC). Second, because the disease is so fast, it's difficult to sort out the molecular and genetic events involved in LSC formation.

Using another experimental system with conditional PTEN deletion in about 40% of fetal liver HSC, the impact of PTEN deletion in the adult is similar to that seen in the Mx-1-Cre inducible system, in which there is a transient increase of HSC and the G0-G1 cell-cycle transition.

The first phenotype observed in this alternative model is an increase of neutrophils. Later, at around 2 months, there is an increase in white blood cells. The spleen is enlarged and there are more MPO+ myeloid cells but few B cells. After the transition from chronic or pre-leukemia phenotype, all the animal dies within 3-4 months of acute leukemia.

A unique blast population can be identified and characterized by CD45 side scatter analysis using fluorescence activated cell sorting (FACS). Interestingly, in this model, only T-ALL-like phenotypes are observed, but never any AML.

To identify an LSC population and the events involved in their transformation, Rosa26-LacZ PTEN-deleted cells are used. Three cell populations, CD3-, c-Kit-CD3+ or c-Kit^{mid}CD3+, which comprise the majority of the blast population, were subjected to serial dilutions and bone marrow transplantation — using cells from the same animal so that everything is comparable.

In the blast population, roughly about 100,000 cells are required to generate leukemia. However, in the c-Kit^{mid}CD3+ minor population, about 5% to 7% of the total blast population, just 100 to 500 cells are needed. The observations suggest that at least in the PTEN null T-ALL model, leukemia-initiating cells are associated with the minor population.

When the self-renewal capacity of this minor population is further tested by serial transplantation of the same minor population, the latency of leukemia development is shortened significantly, as seen in human AML. What causes T-progenitor cells that do not have self-renewal capability to become self-renewable LSC?

Chromosomal translocation

One candidate for allowing LSC cells to self-renew is activated β-catenin. An antibody that detects dephosphorylated/activated β-catenin shows a low signal in the wild type T cell lineage. HSC and progenitor cells show a slight increase of this unphosphorylated β-catenin, but there is a significant increase in the enriched population of LSC. And in the leukemia blasts, almost every single cell has a very high level of expression of β-catenin activation, which can be quantified with intracellular FACS analysis.

SKY analysis identifies another event, a recurring chromosome translocation involving chromosome 14 and 15. FISH analysis identified the TCRα/δ-c-MYC translocation. Interestingly, a similar translocation of t(8;14) is known to be associated with a subset of human T-ALL, indicating that the animal model mimics the genetic event in human pediatric leukemia[3].

If this is true, a similar translocation should exist in leukemic transplants. And c-MYC should be over-expressed only in the T cell lineage because of the translocation. When the initial primary T-ALL samples are compared with bone marrow-transplanted recipients or spleen cell transplants or enriched LSC transplants, the same translocation can be detected, suggesting that this cellular genetic event follows leukemia transplantation. FACS analysis confirms that only CD3+ cells have a high level of c-MYC expression.

Together, these observations suggest that deleting PTEN in stem cells results in leukemia-initiating cells somewhere downstream in the T-cell lineage. There are two additional molecular genetic events, β-catenin activation and TCRα/δ-c-MYC translocation.

Figure 2
SKY analysis identified a reoccurring T(14;15) translocation.

There have been many publications indicating that, at least in pediatric T-ALL, Notch pathway mutation and PTEN mutations can classify the disease into three categories: those with mutations in both Notch and PTEN, those with mutations in Notch and FBXW7, a downstream transcription factor, and those with mutations in PTEN alone.

Sequencing analysis in this model reveals that there are no Notch pathway mutations, and the model is characterized by PTEN deletions alone. Signaling work has shown that constitutive activation of the Notch signaling pathway down-regulates PTEN expression, suggesting that PTEN and its controlled PI3K/AKT/mTOR pathway are critical for the etiology of human T-ALL. Clinical studies also suggest that PTEN deletion appears to correlate with poor response to chemotherapy, especially resistance to pharmacological inhibition of Notch.

This indicates that there are two categories of T-ALL, one driven by Notch constitutive mutation, and the other driven by PTEN deficiency. But the net result of PTEN deletion or Notch activation is probably similar. The perturbation on the signaling pathway is more upstream with one, and more downstream with the other, but the mechanism of leukemia formation has shared characteristics.

Understanding the molecular and genetic events linked to transformation may be able to help eradicate tumors initiated by LSC. The strategy may involve targeting either LSC cell-surface proteins (see Weissman, page 65) or the key signaling pathways.

When the PTEN conditional knockout is crossed with the β-catenin conditional deletion, the homozygous null mutant dies during embryogenesis. The heterozygous condition, which is haploinsufficient for β-catenin, generates chronic disease. Comparing PTEN deletion versus PTEN deletion with haploinsufficiency of β-catenin shows that removing one allele of β-catenin either significantly delays or stops leukemia development. This result suggests that β-catenin activation is involved in leukemia formation.

Early treatment

TCRα/δ-c-MYC translocation probably happens in the early stage of T cell development, which is involved in TCR rearrangement. During T cell development, a key enzyme called RAG is known to control the β-selection checkpoint. Blocking RAG1 activity by crossing with RAG-deficient mice blocks the translocation and T-ALL development, and shows only chronic MPD phenotype.

Interestingly, these mice do not develop AML either, even 7 months after bone marrow transplantation. This result suggests that the TCRα/δ-c-MYC translocation is essential for T-ALL development in the PTEN leukemia model, and any drug that can block the translocation prevents LSC formation.

T cell development and differentiation in RAG-/- mice are blocked at the DN3 stage. However, in PTEN/RAG double mutants, PTEN deficiency rescues cells from DN3 population so that cells can differentiate to the DN4 stage. On the other hand, rapamycin restores the β-selection checkpoint in these double mutant mice.

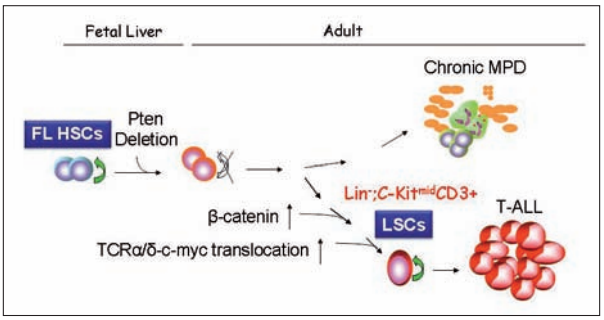

Figure 3
PTEN null T-ALL model.

When PTEN-null mice are treated with rapamycin in the pre-leukemia stage, after about 4 months of treatment, even sensitive FACS analysis cannot detect a leukemia blast population. However, this is not a cure, because rapamycin cannot eradicate pre-existing LSC. When the drug was withdrawn, 5 of 7 animals in the initial test died. Treatment began much earlier in the other 2 animals, suggesting that very early treatment can prevent LSC formation.

Tumors can be resistant to drugs because of mutations that confer resistance, or because the drug does not reach the right target. In the case of PTEN null T-ALL, blast cells and LSC respond differently to rapamycin. If mice are treated with rapamycin in the short term and then cells sorted into different populations, phospho-S6 — used as a surrogate marker to measure treatment response — in blast cells shifts almost to wild type level. In the LSC-enriched population, however, phospho-S6 levels don't change, suggesting that even when treatment shows significant clinical response, it doesn't affect LSC.

A study last year in which researchers deleted PTEN and induced PDGF to create a brain tumor model showed that the PTEN/AKT pathway regulates side population phenotype and ABCG2 activity[4]. ABCG2 is one of the transporters that leads to multi-drug resistance in brain tumor stem-like cells. Experiments are under way to test whether ABCG2 or any of the family of transporters is expressed differentially in LSC versus blast cells.

To summarize, understanding the multiple genetic events linked to tumor initiation could help target LSC, and CSC in general. Treating early enough may be able to prevent the last stage, which is LSC transformation. When LSC form, the translocation has already happened. Rapamycin can efficiently kill blast cells but cannot reach LSC. There is some evidence to suggest that blast cells have a feedback effect on LSC, which may be able to account for the increase in absolute number of LSC after short-term treatment.

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PART IV: Novel approaches

Mechanisms of pluripotency and reprogramming

A report on a lecture by
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Success with in vitro reprogramming using transduction of four transcription factors has shown that the state of differentiation is not fixed, but can be changed experimentally. Human embryonic stem cells and induced pluripotent stem cells are unlike their mouse counterparts in growth characteristics and in differentiation potential. Reprogramming is a continuous stochastic process, in which almost all donor cells eventually give rise to iPSC. Inhibition of the p53/p21 pathway or over-expression of Lin28 increases the cell division rate and results in accelerated kinetics of iPSC formation, suggesting that the number of cell divisions is a key parameter driving epigenetic reprogramming to pluripotency. In contrast, Nanog over-expression accelerates reprogramming in a proliferation rate-independent manner. Genetically identical pairs of human ESC lines isolated from the same embryo show that isolation and culture at physiological oxygen concentrations permits maintenance of the cells in a developmentally immature state. A major impediment in realizing the potential of ESC and iPSC to study human diseases is the inefficiency of gene targeting. Zinc finger nucleases efficiently target expressed and silent genes in human ESC and iPSC. ***Rudolf Jaenisch*** *discussed novel approaches for the genetic manipulation of human ESC and iPSC.*

The genome has been sequenced, but genetic sequences often doesn't provide much information about the function of genes. It is the epigenome that distinguishes one cell from another — for example, a pluripotent cell from a differentiated cell — and this knowledge may be harnessed to convert one cell type to another. Epigenetic programming and reprogramming are also highly relevant for cancer.

Development is unidirectional, but the cloning of Dolly in 1996 proved that it can be reversed. The nuclei of differentiated cells maintain the potential to generate an entire organism. Nuclear transfer and *in vitro* reprogramming seem to be the ultimate test for epigenetic regulation.

In the famous approach to making induced pluripotent stem cells (iPSC) by reprogramming somatic cells, a single cell in a culture dish is transduced with four transcription factors — SOX2, OCT4, c-MYC and KLF4. This single cell then organizes into to a complex animal generated by tetraploid complementation, without the help of cells derived from the host blastocyst[1].

It has been known for several years that three transcription factors, SOX2, OCT4 and Nanog, either activate genes expressed in stem cells, or silence genes that are induced upon differentiation. These three genes form an auto-regulatory loop that is important for pluripotency, and Wnt signaling appears to integrate environmental signals into this core circuitry.

In fibroblasts infected with vectors, the viruses are highly expressed, and the endogenous genes, OCT4, SOX2 and Nanog, are silenced. In the iPSC, in contrast, the endogenous genes are highly expressed and the virus is silenced, suggesting that maintenance of the pluripotent

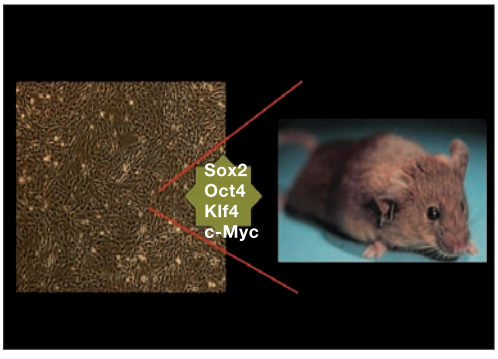


Figure 1
Reprogramming of somatic cells to pluripotency in the test tube.

state depends on the activation of endogenous pathways, not on the viral transduced genes.

There are two technical problems in studying the mechanisms of *in vitro* reprogramming. One is a lack of temporal factor expression control: the virus is inactivated by a self-inactivating system. Second, the retrovirus-mediated factor transduction is inefficient, and selects for a small fraction of cells carrying the 'right' stoichiometry of factors.

The key experiments would therefore not depend on a virus. If Rosa26-M2rtTA carrying green fluorescent protein (GFP) in the endogenous Nanog locus is used, the cells are initially GFP-negative and Nanog is silenced. But after doxycycline-inducible lentiviruses are injected, primary iPSC are selected, and injected in the absence of doxycycline (DOX) into a blastocyst to generate a primary iPSC-derived chimera. These chimeric cells are clonally derived from one of these colonies.

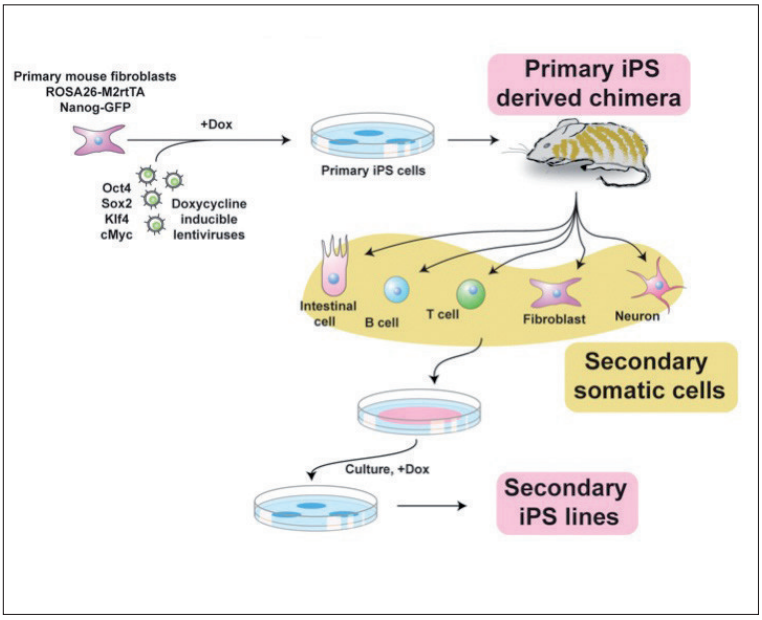
The chimeras can generate any cell type of interest, including intestinal cells, T cells or neurons. Explanting them and then giving DOX to activate the virus generates secondary iPSC lines. This approach doesn't require a new viral infection because the right combination is pre-selected, and the efficiency of reprogramming in many different cell types is 10- to 100-fold higher than in *de novo* infection.

Stochastic model

There are two general models for the mechanism of reprogramming: the deterministic model and the stochastic model.

In the deterministic model, all cells have a fixed probability to generate iPSC, so either any cell can do it or only a subset of elite cells — the somatic stem cells in the population — can. In this case, there would be a fixed time for iPSC to appear, and the factors would act like a switch to turn a somatic cell into a pluripotent cell.

Figure 2
A "reprogrammable" mouse.



In contrast, stochastic models argue that every somatic cell has the potential to generate iPSC, or that only some elite cells have the potential to generate an iPSC, but with different latencies. In this case, there's a variable or unpredictable time before iPSC appear. The factors would initiate a long process, rather than act as a switch.

In most studies, people have defined the efficiency of reprogramming as a fraction of the donor somatic cells that generate iPSC. Instead, efficiency can be defined as the potential of a somatic cell to generate an iPSC daughter at some point.

Single secondary pro-B cells, which are genetically homogenous donor cells, can be used to assess reprogramming in clonal populations from single cells. The difference between MEFs, which most people use, and pro-B cells is that pro-B cells have genetic marks that make it possible to retrospectively show them as the starting point.

Pro-B cells also have a cloning efficiency of more than 90%, compared with less than 10% for MEFs, and they don't require immortalization, whereas MEFs do.

Primary iPSC, which carry the Nanog-GFP marker, are injected into Rag2-/- host blastocysts so that the only B cells produced would be derived from the donor. The transgenic pro-B cells are sorted into single cells and grown in a 96-well plate. Every week for several months, some of the cells are sorted by fluorescence-activated cell sorting for GFP appearance, and the rest are cultured in another dish. Whenever a well turns up green cells, it is declared a GFP+ iPS daughter cell.

Over 5 months, 92% of the pro-B cells generate iPSC, with 50% reprogrammed at 8 weeks. Macrophages can also be cloned at high efficiency.

The conclusion is clear: most if not all pro-B cells have the ability to generate iPSC at 8 weeks. The results immediately eliminate three of the models and suggests a stochastic model, in which every somatic cell has the potential to generate iPSC, but at what point it does so is not predictable[2].

Overall, direct reprogramming seems to involve reestablishing the core auto-regulatory loop initiated by the four transcription factors. It involves multiple cell divisions and extensive epigenetic resetting.

Manipulating the process can reveal further mechanistic details. For example, cell proliferation can be studied using p53 inhibition, which influences the doubling time of embryonic stem cells (ESC).

Several reports have demonstrated that p53 inhibition enhances reprogramming efficiency, anywhere between 4- and 100-fold, by an uncharacterized mechanism. If p53 is knocked down, it does not increase the fraction of B cells generating iPSC, but accelerates the kinetics of reprogramming.

In the wild type cells, the cell cycle time is about 20 hours. In contrast, in the p53 knockdown cells, it's about 10 hours, meaning that the cells divide twice as fast. They have an identical proliferation rate at different times of DOX, indicating that the kinetics of iPSC formation is a function of the number of cell divisions, not the absolute timing of DOX.

This suggests that a single cell generates an iPSC after a certain unpredictable number of cell divisions. In a p53 knockdown, each cell division is faster, but the number of cell divisions remains the same. Other genetic perturbations, such as inhibition of p21 or over-expression of Lin28, also increase the rate of proliferation.

Over-expressing Nanog increases the efficiency by reducing the number of cell divisions required. This makes sense, particularly because Nanog is one of the genes that has to be activated late in the reprogramming process. In nuclear transfer, OCT4 is activated at the two-cell stage of the clone, so it's much faster. Clearly, that uses a very different mechanism of reprogramming than in *in vitro* reprogramming.

X marks the spot

Even with the developing iPSC technology, ESC are still required, in large part because the epigenetic "ground state" of pluripotency still needs to be defined.

Different ESC lines have different biological characteristics, such as differentiation and growth properties. This has been difficult to sort out because cells are isolated and cultured under different conditions. To understand these differences, genetically identical human ESC are isolated from the same embryo and cultured under different environmental conditions.

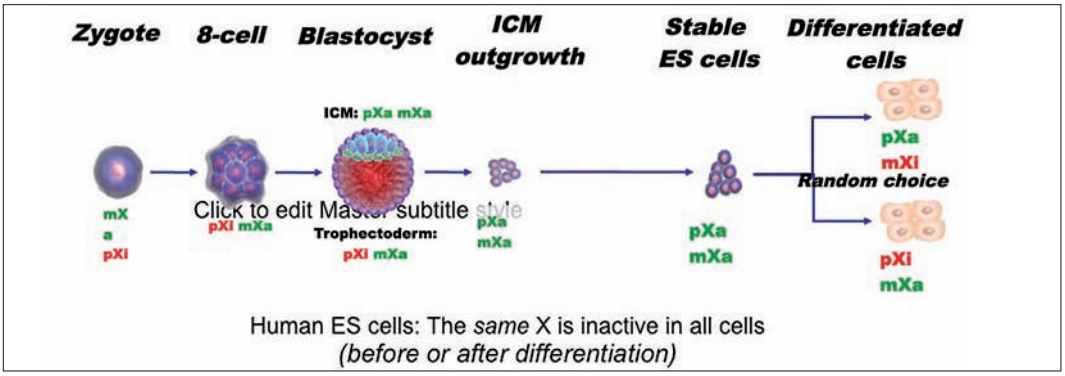
To first compare mouse and human stem cells, the origin of mouse ESC and iPSC is the same as in humans, but the morphology of the cells is very different. Mouse cells have small, compact colonies, whereas human cells have flat colonies. Their growth requirements are also different: LIF/Stat3 signaling is important for mice, whereas bFGF – Activin/Nodal is important for humans. Clonal growth is very efficient in mouse, but much slower in humans, which makes human cells difficult to manipulate.

When it comes to X inactivation, mouse ESC are pre X-inactivation, with both X chromosomes active, and when the cells differentiate, there is random inactivation of one of the X chromosomes. In human ESC, one X is already inactive and, curiously enough, it's the same X that's inactivated in all the cells, regardless of whether they are differentiated.

To assess whether these differences are a result of inherent differences in the system or because of how the cells are isolated and cultured, human embryos are isolated and grown in low oxygen. At the first passage, the cells are split, with one set cultured for one month in 5% oxygen, and the other at 20% oxygen. This essentially derives genetically identical cells, but with two epigenetic states. After many months, some of the cultures were acutely shifted either from 20% down to 5%, or *vice versa*.

The different cell lines cultured at 5% or 20% oxygen have all the markers for ESC, are pluripotent and make teratomas. There are no significant differences in terms of gene expression, or in epigenetic modifications, such as chromatin marks, K4 methylation or K27 methylation.

Figure 3
X chromosome inactivation in mouse embryos and embryonic stem cells.



However, looking at X inactivation, at 5% there is no XIST clone, but at 20% there is, just as is seen in human ESC. Upon differentiation, XIST is activated at 5%, but at 20%, there's no change. On the active X, the XIST gene is methylated because it's inactive, and on the inactive X, it's not methylated because it's active.

In the male line, most of the CpGs are methylated whether at 5% or 20% because males have only one active X. In the female, however, all the CpGs are methylated at 5%, but only about half are methylated at 20%. Human ES cells isolated at 5% may have two active X chromosomes, as measured by XIST expression or by methylation analysis.

Looking throughout the X chromosome at polymorphic markers between the two alleles, at 20%, only one allele, allele A, is expressed for most of the genes. At 5%, there's biallelic expression throughout the X chromosome, with allele A and B equally expressed. When cells cultured at 5% differentiate, they remain bilallelic because there is random inactivation, and the population as a whole is biallelic. When cells shift from 20% oxygen to 5% oxygen, there's no change, suggesting that oxygen-induced X-inactivation is irreversible.

At 5%, cells normally have no XIST expression, but if they are treated with stress-inducing conditions, such as HSP90, γ-glutamylcysteine synthetase inhibition, peroxide treatment and some others, they induce XIST expression. Inhibition of oxidative stress protects the cells against precocious X inactivation.

Although human ESC already have one inactive X, with monoallelic expression of X-linked genes before and after differentiation, it is unclear whether X inactivation has already taken place in the human blastocyst. These results suggest that there must be cells which are pre-X inactivation in the ICM.

Overall, the epigenetic state of ESC is highly affected by derivation and culture conditions, so the ground state of pluripotency in human ESC and iPSC remains to be defined.

Modeling disease

iPSC technology is very attractive for studying disease mechanisms and the effect of drugs and cell therapy in a culture dish. However, there are a number of technical issues that must be resolved to realize the potential of this approach.

The first is that gene targeting in ESC and iPSC cells is very inefficient, which has impeded the generation of mutants, the identification of markers for differentiation and inducible transgene expression.

One alternative approach is to use zinc finger nucleases to target genes in ESC. Zinc fingers can recognize specific sequences. They are fused to a FOK1 nuclease, which binds, dimerizes and introduces a double-strand break, and allows mutations to occur when the break is repaired. This approach may provide the means to genetically manipulate human ESC and iPSC.

The three gene candidates are OCT4, which is expressed in ESC; AAVS1, the AAV integration locus that is ubiquitously expressed in ESC and somatic cells; and PITX-3, which is not expressed in ESC, but is activated upon neural differentiation in the dopaminergic lineage.

For the OCT4 locus, three different pairs of zinc fingers can be used. The vector includes a spliced-in GFP under control of the OCT4 promoter. The targeting frequency is more than 95%. The cells are pluripotent and express GFP when they are undifferentiated, but not when they are differentiated.

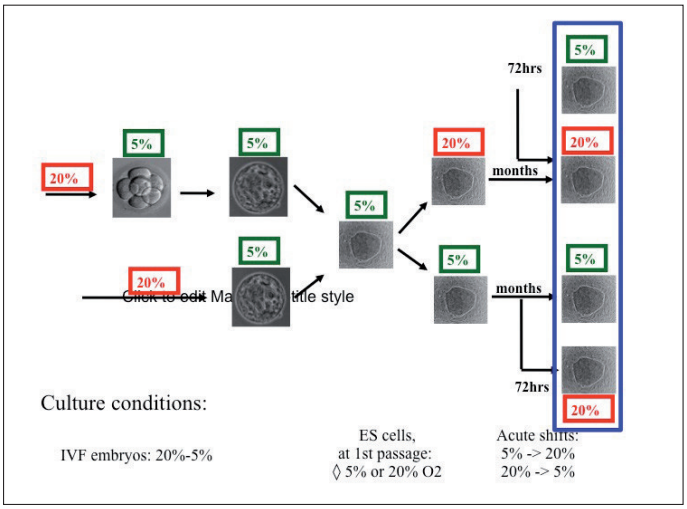


Figure 4
The epigenetic state of embryonic stem cells is highly affected by derivation and culture conditions.

With DOX-inducible transgenes in ESC/iPSC, the goal is to have predictable expression and efficient integration of the transgene into a "safe harbor locus". The AAVS1 locus can be used to over-express genes in human ESC. Using the AAVS1 integration site, under high DOX, the GFP is highly expressed and under low DOX, GFP shows low expression.

The zinc finger approach is efficient for these three genes, OCT4, AAVS1 and PITX-3, with a high targeting frequency, and there are short arms of homology, so there's no need for isogenic vectors[3]. The technical issues that create inefficiency in deriving genetically unchanged iPSC may soon be resolved.

However, there are still several technical challenges in using iPSC technology for disease-specific compounds[4]. For example, in Parkinson's disease, the phenotype comes up in old age. In the iPSC approach, *in vitro* stress over days or months may not be enough to generate the relevant phenotypes of aging. The iPSC approach may therefore be more useful for some diseases than for others. Disease-specific approach would also require large-scale production of iPSC, efficient differentiation and determining the right cell for transplantation and delivery.

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Stem cells and glioblastomas

A report on a lecture by
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The complete genomic sequencing of more than 70 human glioblastomas has revealed that many of them harbor somatic mutations in the core components of the RB, p53, and RTK/RAS PI3 signaling pathways. Mouse models have provided important lessons for understanding gliomas, and can validate genes and pathways. Lentiviral vectors can transduce a wide variety of dividing and non-dividing cells and can be directly introduced into tissues, organs and embryos. Transduction by these vectors is rapid, efficient and convenient, and can be used to make mouse cancer models that closely mimic the initiation and progression of human cancers. Directly injecting Cre-loxP controlled lentiviral vectors expressing oncogenes into GFAP cells in the brain of p53 heterozygote generates glioblastoma-like tumors. These tumors contain both CD133+ and CD133-cells, form tumorspheres and can differentiate into neurons, oligodendrocytes and astrocytes, suggesting that a vast majority of the tumor cells are equivalent to cancer stem cells. There is also evidence that some of the tumor cells transdifferentiate to form endothelial cells that do not express vascular endothelial growth factor, providing a possible explanation for the ineffectiveness of drugs such as Avastin in treating glioblastomas. ***Inder Verma*** *suggested that the use of Cre-loxP controlled lentiviral vectors is a novel way to generate mouse models for glioblastoma and other cancers.*

"A fundamental problem which remains to be solved in the whole cancer research effort... is that the preclinical models of human cancer, in large part, stink."

This quote from Robert Weinberg (see page 47) in the 22 March 2004 issue of *Forbes*, makes several interesting points.

For instance, xenotransplantation is a widely used approach in cancer biology, but it has limitations. In this approach, researchers take about 100,000 cells, introduce them into NOD/SCID mice, which have no immune system, and generate tumors. This is a useful tool and has worked extremely well, but it's not ideal.

The textbook paradigm of cancer holds that cancer begins with a single cell, which acquires a series of mutations, and leads to a full-grown tumor. Transgenic mice, which have also been very useful, introduce a mutation in a gene like RAS into the whole animal. But this is not how most tumors originate. Similarly, knockout mice are valuable, but there again, the gene of interest is deleted from every cell in every tissue in the body.

A better mouse model system might be one in which viral technologies introduce the oncogene into a single cell, or perhaps into a few cells, and then activate second gene and third genes. Generating a tumor in these circumstances is likely to recapitulate how a cancer typically arises.

Cancer genome sequences, specifically in the case of glioblastoma multiforme (GBM), reveal

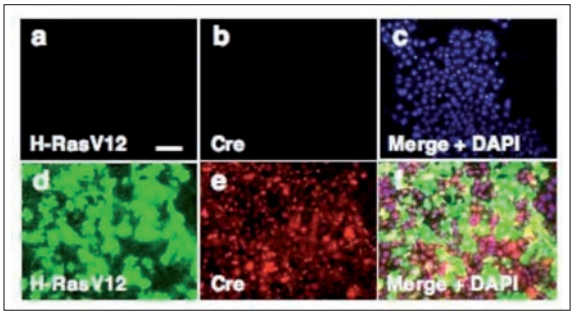


Figure 1
Cre-inducible lentiviral vector infection.

that there are many genes and pathways that are involved[1]. It is impractical to make knockouts or transgenic animals for each of these candidates. But viral vectors can examine the role of these genes much faster.

The Cancer Genome Atlas Research Network showed that 74% of GBM sequences harbor aberrations in three core pathways: the CDK/cyclin/CDK inhibitor/RB pathway, which is involved in the regulation of cell division; the p53 pathway, which is involved in response to DNA damage and cell death; and the RTK/RAS/PI3K pathway, which is involved in the regulation of growth factor signals. The results confirm that alterations in these three pathways are a core requirement of GBM formation[2].

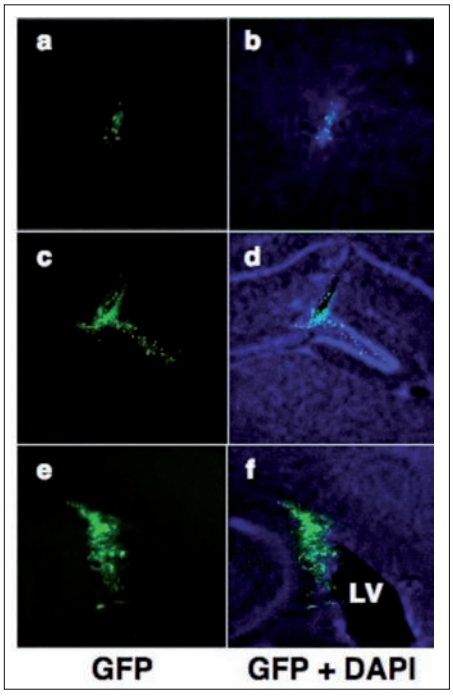
In the new viral vector approach, a lentiviral vector carries the gene of interest surrounded by loxP, green fluorescent protein (GFP) and RAS as a marker gene. The presence of Cre recombinase then selectively turns on the genes. This vector can stereotactically introduce the gene into selective cells in the brain[3]. For example, if this pTomoRasV12 lentivirus is injected into the hippocampus of a wild type mouse, RAS is not expressed. But in a mouse with a background of GFAP-Cre, the hippocampus cells express Flag RasV12, indicating that the Cre-inducible lentivirus is functional.

For proof of principle, H-RAS activation can be used as a surrogate for EGF-R activation, which is common in GBM. The model can also use PTEN inactivation and AKT activation, and p53 mutation, which is also very common.

Versatile tumors

The adult brain contains different types of cells in different regions. For example neural progenitor cells are known to exist mainly in the sub-ventricular zone or hippocampal subgranular zone. To induce oncogenes in a region-specific manner, the viruses are injected into three locations, the cortex, the sub-ventricular zone and the hippocampus. The efficiency of transduction in the sub-ventricular zone is much less than that of the cortex or the hippocampus.

Figure 2
Lentiviral Injections into three different sites.



In the case of the hippocampus, once the vector inserts the genes, there is activation of RAS and myristoylated AKT, and the cells form tumors that are largely GFP+. The mice have an enlarged head, and the gross appearance of the brain shows a massive lesion.

H-E staining of the tumor shows high cellular density, necrosis, perivascular invasion of the tumor cells and pseudo-palisading, which is one of the characteristics of GBM. Confocal images reveal that the border of the tumor is relatively clear, but the tumor cells sometimes infiltrate normal tissues.

However, these tumors with RAS and AKT don't have nuclear pleomorphism, which is a common component of GBM, and they don't have mitotic activity, which requires elimination of p53.

If the mice are crossed with GFAP heterozygotyes to generate GFAP-Cre-p53 heterozygotes, and then the vector is injected directly into the hippocampus of the sub-ventricular zone, histological analyses of the resulting tumor shows all the characteristics of GBM, including nuclear pleomorphism and high mitotic activity. This suggests that combined activation of RAS and AKT on the p53 heterozygous background in GFAP+ cells in the hippocampus causes GBM.

These tumors from p53 heterozygotes have high levels of hemorrhage, necrosis and giant-cell formation. They're Nestin+, one of the markers for neural progenitor cells. They also express GFAP, the markers for glial cells, Tuj1, a marker for neurons, and MBP, which characterizes oligodendrocytes.

This suggests that although it began with GFAP-Cre, the tumor has somehow recapitulated the ability to make cells of all three lineages. The same experiments repeated in the sub-ventricular zone also create tumors.

Efficient method

p53 heterozygotes take a couple of months before they become homozygous. A vector carrying an siRNA to p53 is faster, although it is not a perfect recapitulation because other mutations occur in the transition to the homozygotes. With the siRNA, tumors originate in 4 weeks. Even with just RAS and p53 and without AKT activation, they show high cellular density and nuclear pleomorphism, hemorrhage and vascularity, perivascular infiltration, pseudo-palisading and infiltrative characteristics.

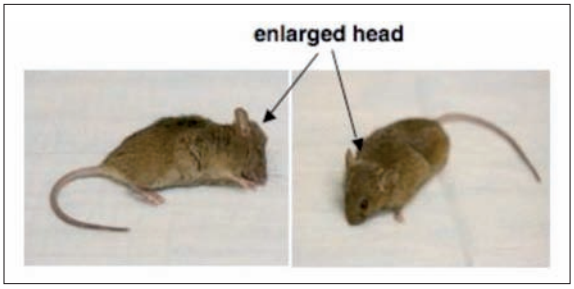


Figure 3
H-RAS and AKT induced tumor.

RAS-sip53 can also be injected into Nestin-Cre mice instead of GFAP-Cre mice. This is a little more complicated because Nestin isn't expressed everywhere, but it still creates tumors.

These vectors have the great advantage that it is possible to add or subtract genes, activate or suppress them, by direct injection into very specific regions of the brain. For example, adding an siRNA to PTEN, which is a common component of GBM, quickly generates tumors.

The approach is also efficient, and guarantees a tumor almost every time, even with just 10 cells introduced into NOD/SCID mice. At least in these cases, every cell in the tumors that have GBM-like structures has the ability to initiate tumors.

The cells make tumors whether they are CD133+ or CD133-. The secondary tumors again have about 50% segregation for CD133 (see Morrison, page 23). Each of these cells has the formal capability to make neurospheres and then differentiate into all three major lineages or maybe more.

Ideally, it would be useful to be able to look in the hippocampus every day and track the growth of these GFP+ tumors. A high-resolution large-scale mosaic image of RAS-sip53 tumor 2 weeks post-injection shows that the tumor has grown from about 55 cells to about 200 cells.

Interestingly, if the tumor is followed at 1, 2, 5 and 8 weeks, what emerges is that although the cells are initially both GFP+ and GFAP+, by 8 weeks of time, there is much less GFAP and there are more Nestin+ cells.

This suggests that some proportion of the glial cells have been reprogrammed into Nestin+ cells, which by many criteria are neural progenitors, and these neural progenitor cells would have no trouble making cells of different lineages. It is possible that it doesn't really matter which cell type is used initially and that the addition of these oncogenes — the very oncogenes that have the ability to make induced pluripotent stem cells — changes the cell into a neural progenitor cell.

The hippocampus can have both dividing and non-dividing cells. Retroviruses only transduce dividing cells, whereas lentiviruses transduce both dividing and non-dividing cells. When the experiments are repeated with a retrovirus, there is some gene expression, but it does not result in a tumor, suggesting that the cells of origin are non-dividing terminally differentiated cells.

Alternate route

GBM tumors are highly vascularized. The tumor cells produce vascular endothelial growth factor (VEGF), and a high concentration of circulating VEGF is related to poor prognosis. In GBM, but not in anaplastic glioma, vascular endothelial proliferation and mitotic figures are seen.

In patients with GBM, bevacizumab (Avastin) shows a response rate of 60%. The remaining 40% don't respond to the drug. What's more, even though the tumors show some decline, the effects of the drugs are transient in most patients.

The GFP+ tumors have many blood vessels, which are lined with endothelial cells, as seen by the presence of von Willebrand Factor (vWF), a marker for endothelial cells. There is typically no superimposition between these two markers, GFP and vWF.

However, a number of the GFP+ blood vessels also express vWF, CD31 or CD34, suggesting that there is transdifferentiation of a GFP+ tumor cell into an endothelial cell. If some of the endothelial cells lining the blood vessels derive from a tumor and not from normal angiogenic processes, it might explain why bevacizumab is not more effective.

These transdifferentiated blood vessels from tumor cells are mostly located in the deep part of the big tumors, where there is the least amount of oxygen, and only a few are present at the surface of the tumor. A hydroxyprobe (see Wicha, page 71) shows that this deep part of the tumor is hypoxic.

Regular endothelial cells, generated by normal angiogenic processes, express both vWF and VEGF. However, the tumor-derived, GFP+ and vWF+ endothelial cells do not express VEGF. This again might explain why bevacizumab, which is a VEGF inhibitor, is ineffective against the tumors.

There are several possible explanations, apart from transdifferentiation, for these endothelial cells. For example, the vector may have transduced an endothelial precursor. Second, the transdifferentiation events could be the result of fusion between tumor cells and endothelial cells. It could also be the result of *in vitro* induction of differentiation of the cells into endothelial cells, or it could be vasculogenic mimicry, in which tumors form small channels that blood passes through. Finally, the endothelial cells could be an artifact, and not functional.

To address whether the vector transduced an endothelial precursor, the tumor can be disseminated into 15 or 20 lines and used to make single-cell clones. If these clones are injected separately, in one clone, for instance, about 15-30% of the cells express both vWF and GFP. It can't be that every one of these clones hits an endothelial precursor.

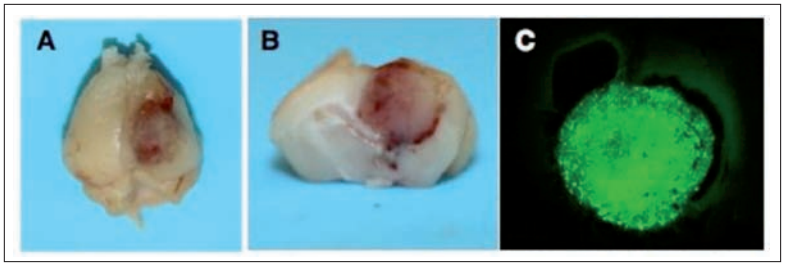


Figure 4
SVZ tumor caused by RAS and AKT in GFAP-Cre/p53+/- mice.

In a separate experiment, if GFP+ tumors are transplanted into a DsRed nude mouse, there is no superimposition between the two markers, even in endothelial cells, which rules out the possibility of cell fusion.

An *in vivo* lectin-binding assay can determine whether the blood vessels are functional. Biotinylated lectin binds to endothelial cells of the vessels when blood is flowing, and can be detected by streptavidin. Streptavidin staining of these vessel walls then indicates that the vessels are functional.

Together, these experiments indicate that the endothelial cells are transdifferentiated, non-fused and biologically functional.

Oxygen's role

In normoxia or 20% oxygen, these endothelial cells make tubes, which look like blood vessels, only in the presence of desferrioxamine (DFO). But in 2% hypoxia, they make the same blood vessel, independent of DFO. This tube formation is also independent of VEGF, which is unsurprising because the cells originate from transdifferentiation of a tumor cell, and not from normal angiogenic processes.

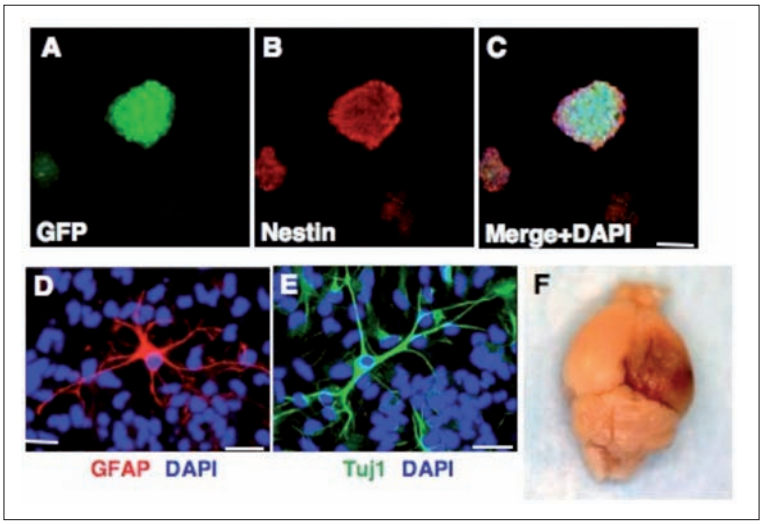


Figure 5
Characteristics of the brain tumor initiating cells.

This cell-fusion independent differentiation of neural stem cells to the endothelial lineage has been shown before in mice[4]. In this case, a glial cell has re-differentiated to become Nestin+, which is one of the markers of a neural progenitor cell, and that then leads to an endothelial cell.

In the case of humans, too, if a human tumor is grown into neurospheres, transduced with lenti-GFP and transplanted into the brain of NOD/SCID mice, about 20-30% are endothelial cells that are GFP+, vWF+ and Nestin+, suggesting that the human GBM cells transdifferentiated into endothelial cells. The tumor cells surrounding the endothelial cells express mutated EGF-R, as do the endothelial cells, suggesting that these endothelial cells are tumor-derived.

To make induced pluripotent stem cells from normal cells, the method typically involves adding c-MYC, Sox2, Oct4 and Klf4 genes. Blocking p53 increases the efficiency.

Similarly, in the case of glial cells, the combination of RAS and AKT or RAS alone, and then sip53, generates malignant gliomas that are positive for SOX2, Nanog and MYC. Because these gliomas have acquired similar, albeit limited, pluripotency powers, they can go on to differentiate into other lineages.

The glial cells are redifferentiated into a neural progenitor cell that can generate all lineages. This methodology is not unique to brain tumors and can be extended to many other types of tumors, for example lung and prostate cancers.

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Mammary epithelial hierarchy and breast cancer

A report on a lecture by
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The epithelium of the mammary gland exists in a highly dynamic state, undergoing dramatic morphogenetic changes during puberty, pregnancy, lactation and regression. The identification of stem and progenitor populations provides evidence that the mammary epithelium is organized in a hierarchical manner. An organ-specific in vivo reconstitution assay has allowed the evaluation of mammary repopulating activity in defined cell subsets when transplanted into cleared fat pads. Mouse mammary stem cells (MaSC) can generate extensive ductal outgrowths upon transplantation and display the defining characteristics of multi-lineage differentiation and self-renewal. A single genetically tagged MaSC can regenerate an entire mammary epithelial tree. In human breast tissue as in mice, there are three epithelial subpopulations: basal stem/progenitor cells, luminal progenitors and mature luminal cells. Investigation of early changes occurring in high-risk breast cancer families reveals an aberrant luminal progenitor cell population in pre-neoplastic tissue from BRCA1 mutation carriers. Gene profiling of precancerous tissue from these individuals demonstrates striking similarities with the gene signatures of luminal progenitor cells and basal breast cancers, suggesting that the luminal-restricted progenitor cell is a key target of transformation in BRCA1 mutation carriers. ***Jane Visvader*** *proposed that the epithelial cell hierarchy can be used to identify potential targets of transformation in breast cancers.*

Like many solid tumors, breast cancer is very heterogeneous, both at the histological and molecular levels. Breast tumors cluster into at least six distinct molecular subtypes.

The pressing question in the field is whether these different subtypes reflect a different cell of origin. To answer that question, it is important to understand the normal epithelial cell types that reside in breast tissue.

There are three epithelial cell types in the mammary gland that fall into two lineages. The luminal epithelial cell lineage comprises both the ductal epithelial and alveolar epithelial cells, which emerge in pregnancy. In addition, there is a single type of myoepithelial cell that forms a sleeve around these other two cell types. These three cell types, together with a remarkable regenerative capacity that is evident on successive pregnancies, point to the existence of mammary stem cells (MaSC).

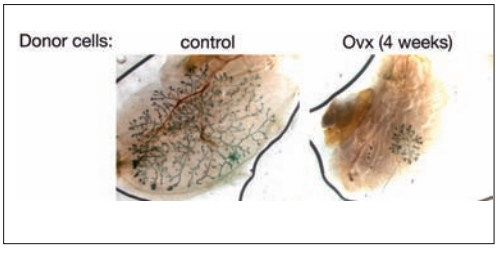

Figure 1
Mammary stem cell behavior is affected by hormone withdrawal.

A combination of CD24 and CD29 resolves four populations, only one of which has reconstituting ability *in vivo*. In fact, a single cell from this double-positive population, when implanted into a cleared mammary fat pad of a 3-week-old female, is capable of regenerating an entire mammary gland that produces milk. So this single cell fulfils the defining characteristics of a stem cell: multilineage differentiation and self-renewal.

Transplanting a small number of $CD29^{hi}$/CD24+ cells into the cleared mammary fat pad of a 3-week-old female mouse gives rise to a mammary outgrowth. When the recipient mouse is taken through pregnancy, the gland can completely differentiate and form alveolar structures necessary for milk production.

In the population of CD29^{hi}/CD24+ cells, 1 in 64 cells can repopulate a mammary fat pad, differentiating into the various lineages of the gland. Serial transplantation also shows the self-renewing capacity of the mammary repopulating cell, suggesting that it is the MaSC.

Using another integrin, the β-3 integrin or CD61, the CD29^{lo} population can be subdivided into luminal progenitors and mature luminal cells. The luminal progenitors can be classified as such because of their high clonogenic activity *in vitro* and on the basis of immunostaining. It's very clear that these cells are exclusively committed to the luminal as opposed to the myoepithelial cell lineage. Using a combination of three markers, three distinct epithelial cell types can be identified.

Interestingly, the MaSC-enriched population is devoid of the steroid hormone receptors, estrogen receptor, progesterone receptor, and ErbB2, also known as HER2 (see Wicha, page 71). In addition, this population expresses high levels of the EGF receptor and the basal markers p63 and K14. All of these are hallmark characteristics of the basal-like subtype of breast cancer.

Hormone effects

In the emerging model of the mammary gland, a single MaSC can give rise to all the mature cell types found within the mammary gland: ductal, alveolar and myoepithelial[1]. The nature of the intermediates is complex and very much a grey area, although there is a defined CD61+ committed luminal progenitor cell.

Given the importance of estrogen and progesterone to normal mammary gland development, as well as to breast cancer, it is important to look at the effects of hormone ablation by ovariectomy on the different sub-populations.

Not surprisingly, ovariectomy leads to a decrease in the size of the luminal or ductal cell population. However, there is no difference in the CD29^{hi} cells, the MaSC-enriched population. Still, steroid hormone deprivation does affect the functional capacity of the MaSC.

MaSC taken from ovariectomized animals have lower activity, and glands from ovariectomized animals have 4-fold fewer MaSC. Interestingly, the MaSC seem to retain a memory of hormone deprivation. When MaSC from ovariectomized animals are implanted into a wild type environment, the stromal hormones and the growth factors in the recipient are insufficient to fully support MaSC activity, and result in tiny outgrowths, compared with extensive ductal outgrowths in control animals.

Neither estrogen alone nor progesterone alone affects the repopulating activity of the MaSC. But a combination of the two results in a large increase in the absolute number of repopulating cells, suggesting that synergy between estrogen and progesterone underlies the effects observed with ovariectomy[2].

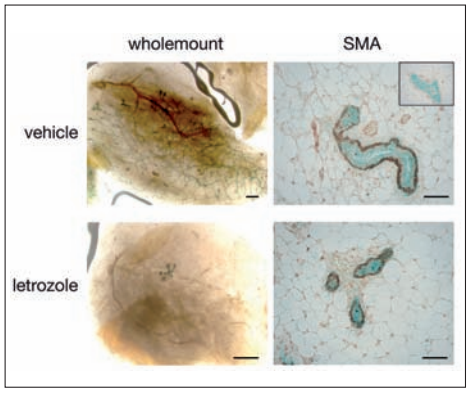

Figure 2
Aromatase inhibition curtails mammary stem cell activity.

There is also evidence of an important role for systemic estrogen in mice, and presumably humans, from two lines of experiments. First, letrozole, an aromatase inhibitor widely used in the clinic, reduces by about 3-fold the absolute number of MaSC. The majority of cases have tiny structures that cannot be defined as outgrowths. Aromatase knockout mice also have rudimentary mammary gland structures, with an 80-fold reduction in the absolute number of MaSC.

In the hormonal environment imposed by pregnancy, there is a transient but profound increase in the number of repopulating cells. In mid-pregnancy, there is an 11-fold increase in the absolute number of MSC, but it drops by late pregnancy.

This increase in MSC activity may account for the short-term increased risk of developing breast cancer during pregnancy and for some years following it.

In summary, despite lacking estrogen and progesterone receptor expression, MaSC are highly responsive to steroid hormones. The data suggest that breast cancer chemoprevention may in part be achieved through the suppression of MaSC function.

Regulatory pathways

To identify regulators of lineage commitment and differentiation along the hierarchy, a number of targeted mouse models have been investigated. Another strategy involves culturing stem cells *ex vivo* for a short period of time, retrovirally transducing them to manipulate gene expression, and then transplanting them back into the cleared fat pad to assay the effect of a given gene on mammary development.

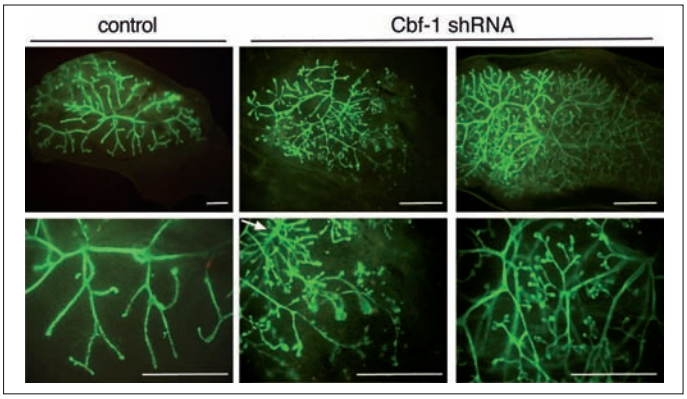

Figure 3
Knockdown of the Notch effector CBF1 increases mammary stem cell numbers.

One of the first pathways examined was Notch. Knock down of the canonical Notch effector CBF1 in the MaSC-enriched population leads to a modest but reproducible increase in the absolute number of MaSC. The outgrowths also contain an expanded basal-cell population.

More strikingly, the outgrowths seen in these mice are disorganized, showing excessive branching and the presence of terminal end buds, which shouldn't be there by week 8. There are also aberrant areas of basal cells in these terminal end buds, which are not present in controls.

Compatible with these *in vivo* findings, there is an expansion of p63+ basal cells *in vitro*, either with γ-secretase inhibitor or the CBF1 shRNA. Conversely, however, over-expression of Notch ICD1 leads to differentiation along the luminal cell lineage, and the disappearance of basal cells. Notch activation appears to promote luminal cell-fate determination[3].

Transducing the MaSC with Notch ICD and then transplanting them into cleared fat pads results in nodules containing exclusively luminal cells, compared with the extensive ductal outgrowths seen with control MaSC. In fact, the results can be phenocopied by transducing the luminal progenitor population with the active form of Notch1.

These luminal nodules eventually go on to form tumors. Luminal progenitors or the mature luminal population never produce outgrowths *in vivo* when they're transplanted into a fat pad.

In vitro, luminal progenitor cells in which the Notch pathway has been constitutively activated undergo detachment, forming luminal spheres. These spheres can be serially passaged and give rise to tumors.

Notch appears to be acting on at least two different levels within the mammary gland. It seems to keep a check on MaSC numbers, so knocking it out increases MaSC activity. Importantly, it's also playing a role in binary cell-fate decisions, which is reminiscent of its role in many other cellular systems.

Notch promotes differentiation along the luminal lineage at the expense of the myoepithelial lineage, so inappropriate Notch signaling leads to increased proliferation and self-renewal of luminal progenitor cells, and presumably underlies the transformation, leading to tumorigenesis, observed in these mice.

Tumor heterogeneity

Examining pre-neoplastic mammary tissue from a number of different mouse models shows that MMTV-Wnt-1 transgenic mice, but not MMTV-Neu/ErbB2 mice, exhibit an expansion of the MaSC population. The luminal progenitor population has no repopulating ability under normal circumstances, but in the case of ectopic Wnt-1 expression, hyperplastic structures emanate from luminal progenitor cells, suggesting that they are endowed with self-renewing capacity.

Wnt-1 therefore appears to target two different cell types along the hierarchy. The luminal progenitor could be de-differentiating into a stem-like cell based on the expression of basal cytokeratins in this cell type from Wnt-1 glands.

There are two primary models to explain tumor heterogeneity: the hierarchical cancer stem cell (CSC) model and the non-hierarchical clonal evolution model. CD61 is useful for identifying CSC in the Wnt-1 transgenic model. The CD61+ population confers a 20-fold enrichment for tumor-forming cells *in vivo*. Only CD61+ cells are capable of giving rise to tumors on serial transplantation and at a comparable frequency, thus fulfilling the criteria for a CSC[4]. CD61 regulates breast tumor cell metastasis and is a prognostic indicator in breast cancer.

In contrast, no CSCs have been identified in MMTV-Neu/ErbB2 tumors using multiple markers including CD61. Indeed, these tumors homogeneously express the luminal progenitor marker. The data suggest that this tumor model may not follow a CSC paradigm, and may instead use the clonal evolution model of tumorigenesis.

There are remarkable parallels between the human and the mouse systems, but there are two primary differences. One is the presence of the terminal ductal lobulo-alveolar unit in human breast tissue, which is not found in mouse, and the other is the predominance of fibroblasts in human stroma.

Using the humanized fat pad system, epithelial subsets are co-injected with fibroblasts. EpCAM and CD49f, or α-6 integrin together delineate four discrete sub-populations. Again, only one, CD49f^{hi}EpCAM^{-}, has regenerative capacity *in vivo*. The frequency is much lower than that seen with mouse, presumably reflecting an inadequate microenvironment.

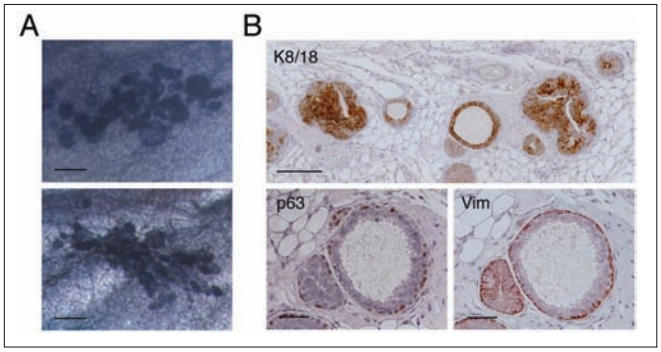

Figure 4
CD49f^{hi}EpCAM– are enriched for mammary repopulating capacity.

Still, there are extensive outgrowths, many of which show signs of branching, and others that appear to be more lobular. The primary outgrowths are of human origin and they comprise all the epithelial cells found in normal human breast tissue. They are also capable of self-renewal, albeit limited.

In vitro, these four sub-populations exhibit very different characteristics. CD49f-EpCAM+ is the mature luminal sub-population and has no clonogenic activity. CD49f-EpCAM- corresponds to the stroma. Only these two populations, CD49f^{hi}EpCAM^{-} and CD49f+EpCAM+ have colony-forming ability. The MaSC-enriched population, CD49f^{hi}EpCAM^{-}, gives rise to heterogeneous structures and occasional tubular structures, whereas the CD49f+EpCAM+ luminal progenitor cells give rise to homogeneous acinar structures that only express markers of luminal cells.

Thus, there are functional similarities emerging between human and mouse, such as three different epithelial cell types. Most importantly, the mouse and human MaSC is negative for expression of estrogen and progesterone receptors and ErbB2. This is a hallmark feature of triple-negative or basal-like breast cancers.

Transformation targets

The epithelial cell hierarchy can be used to identify potential targets of transformation in breast cancers. Breast tumors in individuals that carry the BRCA1 mutations are often basal-like. This is reminiscent of the MaSC phenotype, and has led to the hypothesis in the field that the MaSC is the cell of origin for BRCA1-associated cancers.

In precancerous tissue from BRCA1 mutation carriers, however, the luminal progenitor cell population is expanded whereas the MaSC population is concomitantly diminished. What's more, it is the luminal cell population, not the MaSC population, that displays aberrant growth properties in the matrigel assay.

The addition of B27 as a growth supplement increases clonogenic activity from this luminal progenitor population in the BRCA1 individuals compared with normals. Only the luminal progenitor population from the BRCA1 mutation carriers displays this characteristic.

This result is paralleled in pre-neoplastic tissue taken from BRCA1-deficient mice. It is only the CD61+ luminal progenitor cells, not MaSC, that are capable of growing in the absence of B27. Intriguingly, there is a 4-fold drop in the absolute number of MaSC in precancerous tissue taken from BRCA1-deficient mice.

Taken together, aberrant luminal progenitors seem a likely target population for basal tumor development in BRCA1 mutation carriers. To explore this further, it is necessary to go back to the different molecular subtypes of breast cancer. They are luminal-like A (ER^{++}), luminal-like B (ER^{+}), 'normal' breast tissue-like, basal ($ER^{-}PR^{-}HER2^{-}$), ErbB2/HER2 over-expressing and claudin-low. To interrogate relationships between the normal epithelial subtypes and these breast cancer subtypes, specific gene signatures were derived from normal epithelial cells and compared with the expression profiles of the subtypes.

Surprisingly, the gene signature of the luminal progenitor is most similar to that of the basal subtype. In contrast, the MaSC-enriched signature is most concordant with that of the claudin-low and normal breast-like subgroups. When the gene signature of luminal progenitor cells is compared with that of pre-neoplastic tissue from BRCA1 mutation carriers or non-BRCA1 carriers, again the luminal progenitor signature is found to be most closely associated with the BRCA1 group.

In summary, the claudin-low and normal-like cancers may emanate from the MaSC, whereas the luminal-A has a very close signature to that of the mature ductal cell. Most strikingly, the signature of the basal subtype of breast cancer closely resembles that of the luminal progenitor cell.

One of the defining markers of the luminal progenitor population in both human and mouse is the c-KIT tyrosine kinase. Interestingly, expression of c-KIT is up-regulated in the luminal progenitor population of precancerous tissue taken from BRCA1 carriers compared with normal women. c-KIT is also over-expressed in 11 of 21 breast tumors that arise in BRCA1 mutation carriers.

In summary, different cells within the hierarchy appear to be targets of oncogenesis in breast cancer. Wnt-1 is likely to increase the self-renewal of stem and luminal progenitor cells, whereas constitutive activation of Notch1 seems to target the luminal progenitor cell. Finally, there is a striking case for the luminal progenitor being a key 'cell of origin' in individuals carrying a BRCA1 mutation.

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Abbreviations and glossary

1. ABBREVIATIONS

ALL	acute lymphoblastic leukemia
ALDH	aldehyde dehydrogenase
AML	acute myelogenous leukemia
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BrdU	5-bromo-2-deoxyuridine
CML	chronic myelogenous leukemia
CSC	cancer stem cells
EMT	epithelial-mesenchymal transition
EPO	erythropoietin
GBM	glioblastoma multiforme
GFP	green fluorescent protein
HDAC	histone deacetylase
HER2	human epidermal growth factor receptor 2
HIF	hypoxia inducible factor
HSC	hematopoietic stem cells
iPSC	induced pluripotent stem cells
LINE	long interspersed nucleotide elements
LSC	leukemia stem cells
MaSC	mammary stem cells
NSC	neural stem cells
PFK	phosphofructokinase
PI3K	phosphoinositide 3-kinase
VEGF	vascular endothelial growth factor

2. GLOSSARY

Terms in *italics* are defined elsewhere in the Glossary.

acetyl-CoA an important molecule in *metabolism*, mainly to convey the carbon atoms within the acetyl group to the citric acid cycle to be oxidized for energy production

adenosine triphosphate (ATP) a multifunctional nucleotide that is most important as a molecular currency of intracellular energy transfer

angiogenesis the formation of new blood vessels

cancer stem cells cells found within *tumors* or haematological cancers that possess characteristics associated with normal *stem cells*, specifically the ability to give rise to all cell types found in a particular cancer sample

glycolysis the sequence of reactions that converts glucose into pyruvate with the concomitant production of a small amount of *ATP*

hematopoiesis formation and development of blood cells

hypoxia a deficiency of oxygen in cells or tissues

Induced pluripotent stem cell commonly abbreviated as iPS cells or iPSCs, a type of pluripotent *stem cell* artificially derived from a non-pluripotent cell, typically an adult somatic cell, by inducing a forced expression of specific genes

mammosphere a clump of mammary gland cells that forms under certain circumstances

mesenchyma the structural tissue of organs; also called *stroma*

metabolism the set of chemical reactions that occur in living organisms in order to maintain life

mutation a change in the DNA sequence of a gene that causes an alteration in the amino-acid sequence of the protein, often affecting its structure and/or function

myelogenous leukemia leukemia resulting from a malignant transformation occurring in the bone marrow

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

neurogenesis the process by which neurons are generated; most active during pre-natal development, it is responsible for populating the growing brain

neurosphere a free-floating structure generated by neural *stem cells* in laboratory settings

neurotransmitter chemicals that transmit signals from a neuron to a target cell across a synapse

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

oncoprotein the protein product of an *oncogene*

oxidative phophorylation a *metabolic* pathway that uses energy released by the oxidation of nutrients to produce *ATP*

parenchyma a longstanding biological concept, and considered to be the functional part of an organ

phosphorylation the addition of PO_4- groups to a protein to activate or de-activate their interactions with other proteins in a signaling cascade

RNA interference technique in which strands of double-stranded RNA are used to silence the expression of a gene

stem cells cells characterized by the ability to renew themselves through mitotic cell division and differentiate into a diverse range of specialized cell types

stroma the structural tissue of organs; also called *mesenchyma*

tumorigenesis the process by which normal cells are transformed into cancer cells

tumorsphere a floating mass of tumor cells

wild type gene of interest with no known *mutations*; animal carrying such a gene; often designated as +/+ if both alleles are wild type, or +/- if one allele is wild type, the other mutated (-/- indicates that both genes are mutated)

xenografts human tumors grafted into immunocompromised mice

Participants in the group picture

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Fondation IPSEN

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 27 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 more recently 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** – Six 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, Metabolism and Cancer, Molecular Targets of Cancer Therapy, Stem Cells and Cancer) have generated outstanding discussions among the participants. The 2011 meeting will deal with the link between epigenetics and cancer.

Partnerships

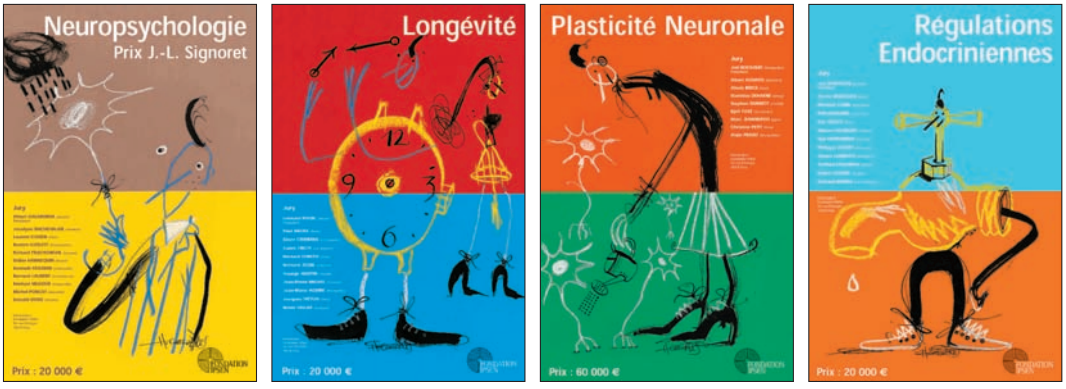
Long ago, the *Fondation IPSEN* has developed partnerships with international institutions and organisations, to encourage cooperation between experts in various disciplines. These partners include: the World Health Organisation (WHO), the *Fondation Nationale de Gérontologie* (FNG) and Harvard University.

Three additional 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": *Transcription diseases (2007), Genes, Circuits and Behavior (2008), Processes of Aging (2009), Sensory Systems : Small Taste, Touch, Hearing and Vision (2010).*
- Second with **Cell Press** and the **Massachusetts General Hospital**. This series, "Exciting Biologies", has been initiated in 2007, with a meeting entitled *Biology in Motion*. The next meetings were: *Biology of Cognition (2008), Biology in Balance (2009) and Biology of Recognition (2010).*
- And finally with **Nature Publishing Group** on the general theme of "Emergence and Convergence", four meetings a year are being organized in various domains. The New York Academy of Sciences hosted the first of the series in October 2007, *Small RNAs in Development, Immunology and Cancer*. Since then, five other meetings have been held: *Genome Evolution and Structural Variation (Seattle), Epigenetics and Behavior (Houston), Multiple Sclerosis: From Pathogenesis to Therapy (Paris), Cell shape and Polarity (Chicago), Mitochondrial Dysfunction in Neurological Disease (Durham), Epigenetic Dynamics of the Immune System (San Antonio).*

Awards to Encourage Research

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



Posters advertising the Fondation Ipsen prizes.

• **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 McMahan; 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; Jean-Pierre Changeux, Peter Kalivas and Eric Nestler; Alim-Louis Benabid, Apostolos P. Georgopoulos, Miguel A. L. Nicolelis; Thomas Insel, Bruce McEwen and Donald Pfaff.

• **The Endocrinology Award**, first given in 2002, has been received by Wylie Vale, Robert Lefkowitz, Pierre Chambon, Tomas Hökfelt, Roger Cone, William Crowley, Ron Evans, Gilbert Vassart and Shlomo Melmed.

• **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, Alvaro Pascual-Leone, Elizabeth Warrington, Pierre Maquet and Giacomo Rizzolatti.

• **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, David Barker, Gerald McClearn, Jacques Vallin and Judith Campisi.

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, 17 titles)

• Research and Perspectives in Longevity (Springer, 5 titles)

• Research and Perspectives in Endocrinology (Springer, 8 titles)

• WHO/Ipsen Foundation series (Springer, 7 titles)

• Brain and Mind Collection

Books and brochures recently published by the *Fondation Ipsen.*

In addition, since 1986 the Fondation IPSEN has published over 210 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 produced teaching films that have received awards from specialized festivals.

FONDATION IPSEN

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