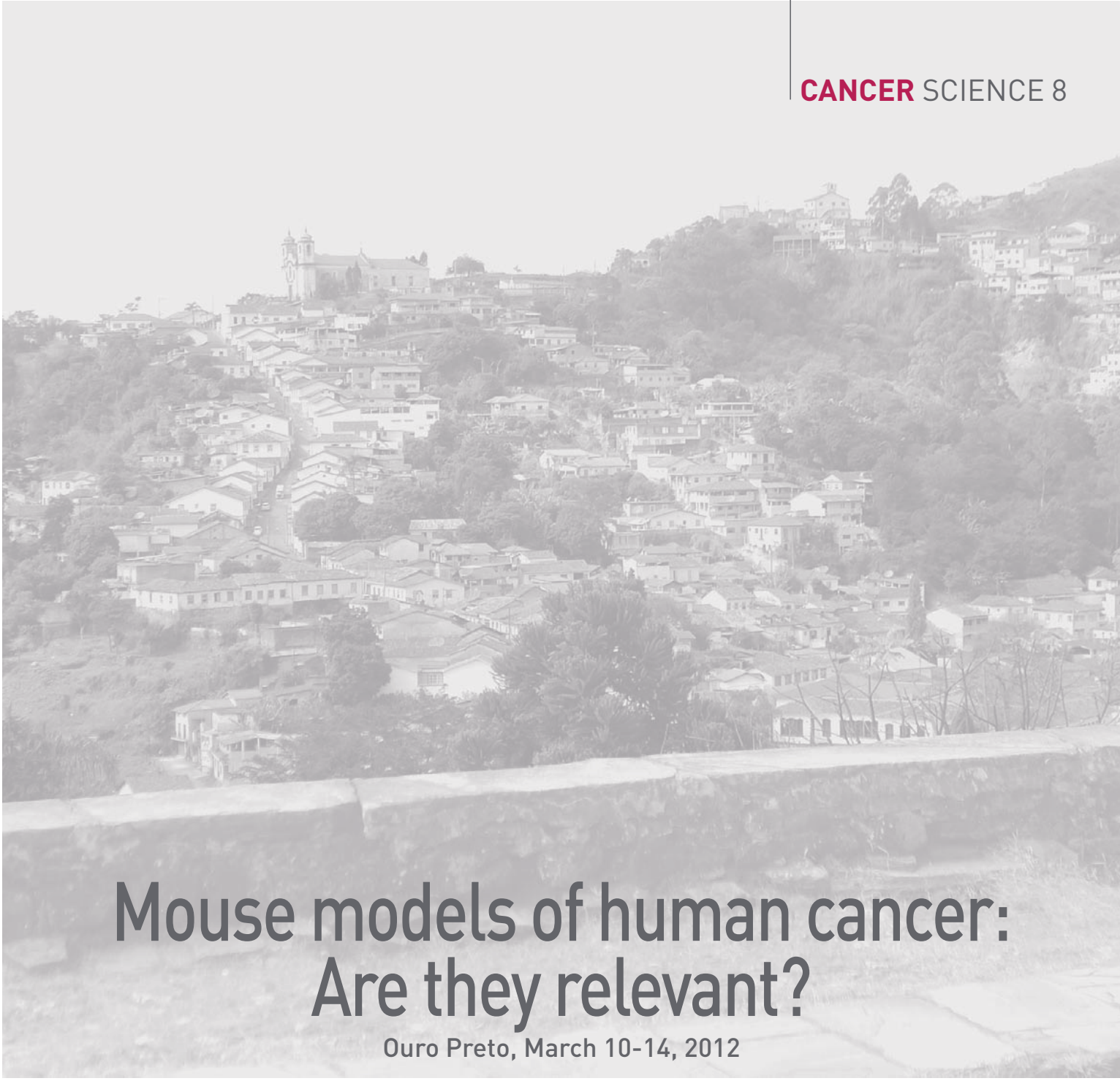

Mouse models of human cancer: Are they relevant?

Ouro Preto, March 10-14, 2012

COLLOQUES MÉDECINE ET RECHERCHE

FONDATION IPSEN

Cancer Science 8 – Mouse models of human cancer: Are they relevant?

Fondation Ipsen

SCIENTIFIC REPORT BY APOORVA MANDAVILLI

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FOREWORD by INDER M. VERMA

"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."
Robert Weinberg, *Forbes*, March 22, 2004

There are primarily four types of models to investigate hypotheses in cancer: cell culture, xenografts, genetically engineered mouse models, and tumor tissue taken from patients. Mouse models are invaluable for understanding the function of genes in mammalian development and disease.

The availability of a large diversity of strains with different susceptibilities to diseases, and the possibility of introducing defined mutations into the mouse germline, have boosted the understanding of how genes act in complex organisms. Yet, many times, mouse or other animal models do not mimic the subtleties of human cancer and are poor models for predicting the outcome or survival from disease.

The 8th *Fondation IPSEN* Cancer series meeting was devoted to discussing the utility — and limitations — of mouse models in cancer research in the beautiful old colonial town of Ouro Preto in the state of Minas Gerias, Brazil.

The speakers recognized the contributions of mouse models, pointing out the advances we have made in understanding pancreatic ductal adenocarcinomas (PDAC), for example.

Anton Berns opened the meeting describing various advances that were made possible by mouse models. He also discussed the promise of somatic gene transfer methods, as well as next generation fast-track autochthonous mouse model generation.

As efforts to sequence thousands of human cancer genomes gain momentum, the challenge of distinguishing driver cancer genes from passengers, and determining how these genes contribute to tumorigenesis, remains a major challenge. **David J. Adams** presented new oncogenes and pathways that have been identified using mouse models, transposon-mediated mutagenesis, and exome and whole-genome sequencing.

Scott W. Lowe described a new approach that is revealing unexpected principles about the nature and organization of cancer genes. For example, shRNA pools targeting genes deleted in human B cell lymphoma in the E-μ-MYC model have identified about 10 tumor suppressors, including those targeting the polyamine pathway.

Mouse models typically rely on introducing oncogenes into large numbers of cells, which doesn't recapitulate the true nature of oncogenesis. Lentiviral vectors can be used to introduce oncogenes into a small number of cells and generate mouse models of human cancer in a cell-specific manner. **I** described how this approach has been used to study glioblastoma multiforme, a lethal brain cancer with a high recurrence rate.

Mouse models of PDAC have arguably been among the most successful in elucidating the mechanisms underlying tumorigenesis. Tumors in these models are initiated through the somatic activation of oncogenic KRAS, either through a spontaneous recombination event or via Cre-mediated recombination.

Expression of oncogenic KRAS in the absence of p53 leads to the development of distant metastases

after a long latency. **Tyler Jacks** presented results from ongoing studies that are providing an increasingly complete view of tumor evolution in this model system.

KRAS oncogenes are implicated in about one-fourth of all human cancers, including non-small cell lung cancer (NSCLC) and PDAC. However, acinar cells of the pancreas are highly resistant to oncogenic insults, and are not transformed by a KRAS oncogene even in combination with loss of p53, unless the mice suffer from mild pancreatitis. Based on results from mouse models, **Mariano Barbacid** suggested that anti-inflammatory treatment for pancreatitis may reduce the risk of PDAC.

p53 is a transcriptional activator that can induce numerous target genes, but is also important for other biochemical activities. **Laura Attardi** addressed the importance of mitigating the deleterious p53-dependent side effects of DNA-damaging radiation and chemotherapies, while preserving p53 tumor suppressor function.

The progression of primary carcinomas to stages of invasion and metastasis involves the acquisition of many phenotypes associated with a cell-biological program termed the epithelial-mesenchymal transition. **Robert A. Weinberg** suggested that transit-amplifying/progenitor cells are the direct targets of the mutations that drive multi-step tumor progression.

Michael Karin suggested that, in addition to unraveling these sorts of basic mechanisms underlying tumorigenesis, mouse models are important for developing therapeutic and preventive strategies. He described models that have elucidated a major role in colon cancer for the pro-oncogenic transcription factors NF-κB and STAT3 and for inflammatory cytokines, such as interleukin-6 and tumor necrosis factor, that control their activity.

In another example, acute promyelocytic leukemia is characterized by a specific t(15;17) translocation, generating a PML/RARA fusion protein. Preclinical studies in several mouse models of this disease have shown that retinoic acid and arsenic trioxide dramatically synergize for APL clearance although they antagonize for differentiation. **Hugues de Thé** suggested that oncogene degradation as a therapeutic strategy may be effective in some other cancers.

PDAC is the most lethal common malignancy, with little improvement in patient outcomes over the past 40 years. A transposon-based genetic screen for genes that cooperate with a sensitizing mutation identified the deubiquitinase USP9X. **David Tuveson** argued that although PDAC's unique microenvironment participates in its resiliency to therapeutics, it also provides vulnerabilities to exploit for clinical benefit.

In conditional mouse models of KRAS-driven NSCLC and PDAC, deletion of one or both alleles of BRAF significantly enhances lung tumor burden and disease progression, leading to decreased overall survival. **Leisa Johnson** described the preclinical interrogation of genetically engineered mouse models and orthotopic models to address some of the contentious work on anti-angiogenic inhibitors.

Mouse models of glioma with conditional inactivation of three of the five most frequently mutated genes in glioma, p53, NF1, and PTEN, develop tumors that histologically and molecularly resemble human astrocytomas with 100% penetrance. **Luis Parada** reported that an unbiased, large-scale chemical compound screen has identified several compounds that can specifically block proliferation of tumor-derived cells.

Mouse models also provide powerful tools to study drug resistance mechanisms in a realistic *in vivo* setting. *In vitro* functional genetic screens and *in vivo* genotype-phenotype correlations show that therapy response and resistance is affected by several factors. **Jos Jonkers** reported that a cell-based screening approach has found that bifunctional alkylators such as nimustine may cause remission of BRCA1-deficient mouse mammary tumors.

Invasive tumors release large numbers of cancer cells into the circulation, but only a small proportion of these cells survive in, and ultimately overtake, distant organs. Based on research with mammary and other tumors, **Joan Massagué** argued that therapies that target the stromal signals in a primary tumor could specifically eradicate micrometastatic seeds disseminated by the tumor and thus prevent metastasis.

Mario Capecchi shared his experiences with modeling human synovial sarcoma in the mouse, beginning with the genetic and molecular characterization all the way up to clinical trials of promising therapeutics. And **Reuben Shaw** discussed the potential for using metabolic drugs such as metformin and phenformin as anti-cancer agents.

However, some researchers contend that there is far too much reliance on genetically engineered mice. At the meeting, **Neal Rosen** repeatedly pointed out that, over the past decade or so, few therapies have resulted from work on mouse models. He argued that, to be useful, genetically engineered mouse models must be used concordantly with patient biopsies, in order to prove that the models mimic what happens in patients.

As in the past there were lively discussions of the nature of the cancer stem cell, heterogeneity of tumors, barriers in drug delivery and relevance to human tumors and patients. Once again **Jacqueline Mervaillie** and **Yves Christen** managed to find a gem of a town, and a lovely period hotel in the middle of the city. **Sonia Le Cornec**, as cheerful as ever, made sure that the all the transportation and audiovisual needs of the participants were met. Although **Apoorva Mandavilli** was not present, **Virginia Hughes** was a wonderful and very well informed scribe. I am sure the current Cancer Series 8 monograph will be very exciting to read.

Inder M. Verma

PART I: Mouse models for genetic discovery

Mouse models for cancer, what are they good for?

A report on a lecture by
Anton Berns
The Netherlands Cancer Institute, Amsterdam, The Netherlands

Mouse models are invaluable for understanding the function of genes in mammalian development and disease. The availability of a large diversity of strains with different susceptibilities to diseases, and the possibility of introducing defined mutations into the mouse germline have boosted the understanding of how genes act in complex organisms. In cancer research, large-scale insertional mutagenesis strategies have identified many genes that can contribute to cancer. The resulting large database of genes permits cross-validation of genes found through sequencing and other approaches. The studies also pinpoint combinations of mutations that are likely to be critical for tumor phenotype and response to therapy. They have also uncovered targets for intervention that would not have been identified easily by other approaches. Mouse models help explore strategies for harnessing the immune system to act against tumor cells and help study the roles of tumor heterogeneity, cells-of-origin and cancer-initiating cells, among others. They can also serve as experimental systems for testing new drug regimens. Although precise dose and schemes cannot be directly extrapolated from mouse models, the models provide conceptual insights relevant for clinical application. ***Anton Berns*** *described the promise of somatic gene transfer methods, as well as next-generation fast-track autochthonous mouse models.*

Genetically engineered mouse models (GEMMs) can be used for several purposes: as the source of new cancer genes, to study how the immune system can be instructed to eliminate tumors, to identify and test relevant therapeutic targets, and to examine relapses and resistance.

Most importantly, GEMMs are useful for simply understanding how tumors arise, and which factors are crucial for tumor development and treatment. They have helped identify targets that would have been missed by genomic sequencing. For example, PIM kinases and polycomb group proteins are targets, but are not mutated in human cancer.

Mutagenesis screens in mice can reveal both co-occurring and mutually exclusive lesions, which is important. Finally, they can help cross-validate genes that are mutated in human cancers.

Insertional mutagenesis screens in mice have recently become popular but began in the early 1980s. In this approach, newborns are infected with the murine leukemia virus, which induces mostly T-cell lymphomas by inserting the provirus at a particular location that confers selective advantage.

Because the integration of transposons is relatively random, if transposons are found in the same segment of chromosomal DNA in independent tumors, the insertion is likely to be causative in tumorigenesis.

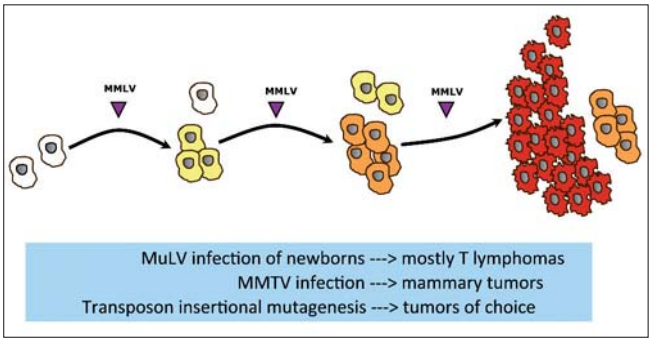

Figure 1
Insertional mutagenesis: identifying oncogenic networks.

This provides a system for finding interesting genes. Other viruses or transposons of different specificities and idiosyncrasies can activate different genes, stabilize messenger RNA, truncate or inactivate proteins, or disrupt control elements.

Using this system in wild type mice, it is possible to find oncogenes and tumor suppressor genes, or in tumor suppressor or oncogene transgenic mice to identify collaborating oncogenes. Proto-oncogene knockout mice can help identify parallel and downstream pathways that are relevant, and grafting tumors can lead to the identification of genes involved in tumor progression or drug resistance[1].

In one example, knocking out a gene frequently found to be collaborating with the MYC oncogene identified PIM1 and PIM2 kinases. If PIM1 is knocked out, PIM2 becomes activated in nearly 100% of the cases, indicating how important that pathway apparently is. If both genes are knocked out, the third member of the family is forced to become activated, as well as a set of other genes that might identify pathways that are alternatives for PIM.

More recently, the method has been applied at a much larger scale, in different predisposed backgrounds, with the cloning of insertion sites in roughly 1,000 tumors. This pinpointed nearly 600 common insertion sites (CIS), or genes that might be relevant for tumorigenesis. Some occur frequently, others are rarer. Interestingly, the CISs that are found correlate with many features, such as genotype specificity, gender, age and cell type.

Whole spectrum:

Analyzing all the different genes identified reveals a whole spectrum that points to specific interactions between genes. It indicates preferred combinations, co-occurring mutations and mutually exclusive mutations, making it possible to create relation maps and identify specific roles.

Even in a highly defined genetic system, such as inbred mice with a predisposed lesion, this method finds hundreds of new oncogenes, tumor suppressors and microRNAs. This suggests that many different combinations of lesions can give rise to tumors, even though the tumors themselves are almost indistinguishable.

These insertions can reveal underlying mechanisms of oncogenicity because they sometimes disrupt protein domains or activate regions of the protein. Genotype specificity and distinct co-occurring CIS mark cooperating genes, or may point to synthetic lethal interactions.

In general, stem cell modules and PI3 kinase and RAS pathway components are over-represented among these targets. Importantly, there is an incremental value in combining data with other datasets, permitting reciprocal validation of relevant cancer genes.

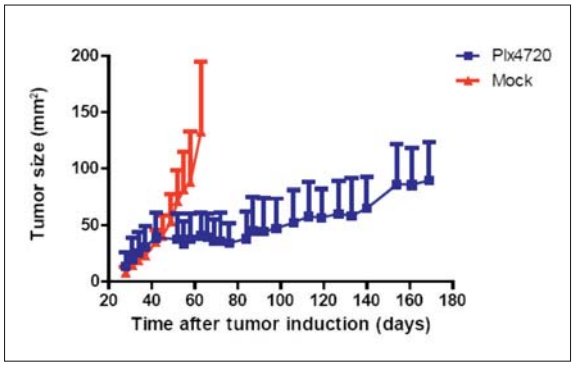

Figure 2
Vemurafenib (PLX4720) in melanoma model.

This approach is being applied to many different tumor types, in order to target lesions to specific subset of cells, address the role of tumor heterogeneity — by single-cell sequencing, for example — and to integrate data with all other datasets that are available.

Immunotherapy is a promise dating back more than 30 years, but it has been difficult to achieve its promise because the complexity of the immune system has consistently been underestimated. There is increasing interest given new options to prime the immune system

and modulate the immune response, particularly for treating cancer. Clearly, mouse models have been instrumental for these strategies.

Combination therapies are obviously of great interest, for example to treat melanoma. When the BFafV600E/PTEN -/- mice, which develop melanomas in the C57BL/6 background, are treated with vemurafenib, there is significant delay of tumor outgrowth, but no tumor regression is observed. This may be because of the PTEN-deficiency in the tumors.

Analysis of this mouse model shows that when vemurafenib inhibits the BRAF pathway, it depletes the whole system of T and B cells. This explains why adding immunotherapy to the drug has no effect, indicating that timing and order of treatment modules are likely to be critical.

Another aspect in which mice might be useful is to assess targets for which no drugs are yet available. For example, in the case of MYC, OmoMYC, which inhibits the transactivation functions of MYC, can have a remarkable effect, ablating even tumors that do not over-express MYC. In this regard, Aurora-B inhibition is also of interest as it has been shown to act in a synthetic lethal fashion with MYC over-expression. Those are activities primarily identified in model systems. The same is true for RAS, and might also hold for some chromatin modifiers such as BMI1 and EZH2.

Clonal effects:

The current view of cancer development is clonal selection, in which the tumor begins with a cancer-initiating cell but, subsequently, further mutations give rise to sub-clones, some of which might expand, whereas others die. In effect, there is clonal co-existence, or a sequential clonal existence. Some of these effects are being studied in mouse models of lung cancer.

In these models, RAS activation results in non-small cell lung cancer (NSCLC) and p53/RB deletion in small cell lung cancer (SCLC). These models are being used to address whether distinct subsets of tumor cells can be identified within the tumor mass and, if so, whether that heterogeneity has a function[2]. This is particularly relevant because transitions from non-small cells to small cell tumors have been observed in a sizable fraction of the tumors after treatment with EGFR inhibitors.

Single-cell cloning of SCLC tumors has shown the presence of different subsets of cells: neuroendocrine (NE) and non-neuroendocrine (non-NE) cells. Both subsets of cells can have a clonal origin because they carry the same rearrangements as detected by CGH analysis.

When NE and non-NE cells are mixed together and grafted, they show accelerated growth *in vitro*, but *in vivo*, there's no difference. However, even though the primary tumor doesn't grow differently, the combination gives rise to metastases whereas the single tumor does not. The subcutaneous grafting of the combination potentiates NE cells in that tumor to metastasize to the liver.

SCLC in mice often consists of clonally related cell populations with either of these two marker profiles. Both are capable of inducing tumors locally, but the non-NE cells secrete factors that endow the NE cells with metastatic potential. That requires MAPK pathway activation.

PEA3 is one of the induced components, and is required for metastasis, although it is not sufficient for full metastatic potential. Interestingly, even metastasized NE cells do not have autonomous metastatic potential. They retain their dependency on non-NE cells through this paracrine mechanism.

So, there is significant genetic drift that might select for sub-clones that might jointly promote tumor viability and are therefore retained in the tumor. Although it is unclear what the selective advantage in the primary tumor is, it definitely has an effect on metastatic potential.

Source code:

To explore whether the cell-of-origin affects tumor characteristics, mutation spectrum or tumor eradication, insertional mutagenesis was once again used. Acute lymphoblastic leukemia was induced using different Cres in the same system: either a VAV-Cre that would target the stem cell, or LCK-Cre or CD4-Cre.

Interestingly, the spectrum of genes that is activated in each case shows only partial overlap. This suggests that tumors can originate from any cells provided the right genes are mutated. There are always a few genes in common, however, such as MYC.

To determine whether there are distinct cells-of-origin for SCLC and NSCLC, sporadic mutations were targeted to distinct cell types in the lung, and tumor development was followed over time.

The number of cell types that might serve as cells-of-origin for these tumors is substantial: Clara cells, basal cells, NE cells, alveolar type II cells, and specific cells in the duct junction.

A series of adenoviruses was equipped with specific promoters, in the hope that they would give rise to a specific switching in a subset of cells that express these promoters.

In the p53/RB model, AdenoCre generates SCLC tumors with a high efficiency. The CGRP virus, which limits expression of Cre recombinase to the NE cells, is a bit delayed, but still very efficient, even though the number of cells infected is 100- to 1000-fold less. Interestingly, the SPC virus, which targets alveolar epithelial type II cells, also generates tumors, some of which are in the peripheral area of the lung, where NE cells are typically not seen. This may be from hitting a progenitor cell that could give rise to NE cells, for example[3].

In the RAS model, the SPC virus is the most efficient. CC10, which targets Clara cells, does give rise to adenomas, but initially, to papillary lesions in the terminal duct region. CGRP generates very little. Remarkably, if this model is combined with p53, all three give rise to adenocarcinomas, with the CC10 and CGRP perhaps even more malignant than the SPC.

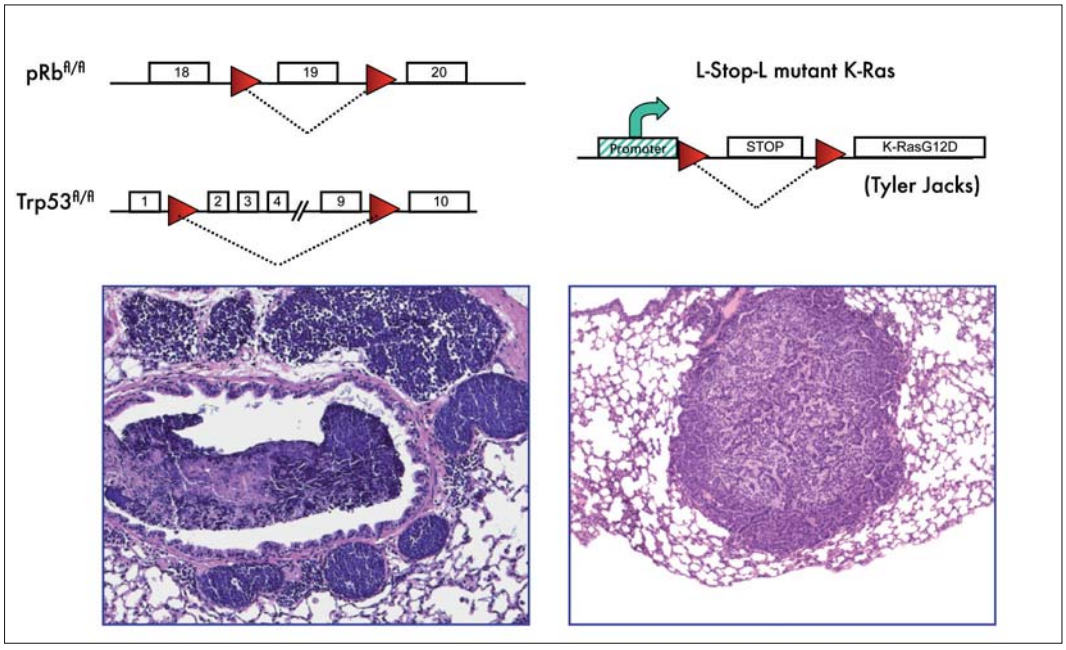

Figure 3
Ad5-CMV-Cre induces different tumors in lung, depending on the conditional alleles present.

These results overall suggest that the capacity of a cell to serve as the cell-of-origin depends on the mutations and the context, and both factors determine the tumor characteristics. As a result, tumors show substantial plasticity and can undergo profound phenotypic changes, which is likely to have major implications for therapy.

Rapid results:

Mouse models are useful for showing promise of new combination therapies, using either drugs or genetic techniques. They can also be used to identify resistance mechanisms by studying tumor remnants and applying genetic strategies, and to study how drug effectiveness is influenced by the tumor microenvironment.

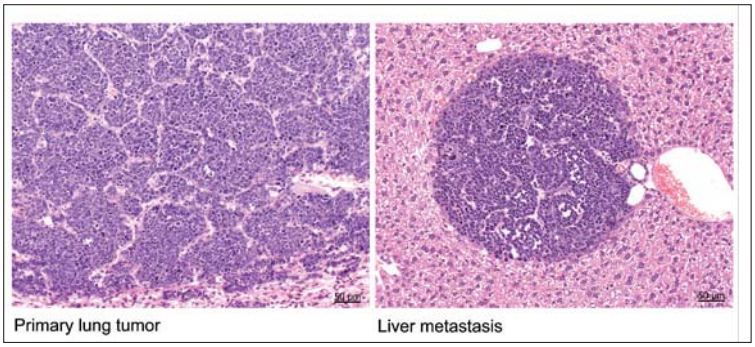

Figure 4
Histopathology of small cell lung cancer in chimeric mice.

But these models also have a number of shortcomings. First, mice are not small humans, so there are differences in responses. Xenopatients with fresh human tumor grafts have their own specific drawbacks, lacking an immune system, and a microenvironment that might be different.

It takes too long to generate models for specific combinations of lesions, and a higher throughput is needed. One way to improve the versatility of current models is to use somatic gene transfer in existing GEMMs and tissue stem cell chimeras, which can be very effective.

It's also possible to re-derive embryonic stem (ES) cells from established mouse models, to equip them with a recombination-mediated exchange cassette, or RMCE, that permits the swift introduction of cDNAs or shRNAs encoding the DNAs of choice, and to use these to produce ES cell chimeras. Experiments can be directly performed with these chimeras or their offspring when backcrossed to the ES cell founder strain. Whereas conventional crossing takes 2 years, the re-derivation and modification of ES cells and generation of mice from these ES cells can be done in half a year[4].

The plan is to establish repositories of these genetically modified ES stem cells equipped with cassettes and create a whole set of reagents that can be easily used.

This means that studies can shift from large breeding colonies to more focused experimental cohorts, and more experiments can be done with fewer mice. This is also likely to be less expensive, and can make GEMMs more widely accessible to the scientific community.

Complex GEMMs can also be modified and analyzed in a short time frame. Tumor phenotypes would be less likely to be misinterpreted because of the identical genetic background, and no unnoticed modifier loci are introduced via intercrosses. However, one has to watch for unwanted mutations that are introduced during ES cell modification.

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Integrative approaches to cancer gene discovery and target validation

A report on a lecture by
Scott W. Lowe
Memorial Sloan-Kettering Cancer Center, New York, USA

In a 'mosaic' approach for studying cancer, genes of interest are transduced into stem and progenitor cells derived from different tissues or mouse strains. The modified cells are then transplanted orthotopically into syngeneic recipients. ES cell lines can also be rederived from mice harboring multiple oncogenes and other useful genetic elements, such as TET-transactivator transgenes, and a homing cassette that helps target an inducible shRNA into a defined genomic locus. These methods can be used to generate mouse models capable of reversibly suppressing tumor suppressors, such as APC and PTEN. Stable expression of the shRNAs that target these genes trigger tumors that are similar to those in mice harboring conditional gene knockouts. Transducing tissue stem and progenitor cells with shRNA pools can also help identify genes that promote tumorigenesis. For example, shRNA pools targeting genes deleted in human B cell lymphoma in the E-μ-MYC model has identified about 10 tumor suppressors, including those targeting the polyamine pathway. This method identified multiple enzymes involved in the biogenesis of hypusine, a unique amino acid, in a novel tumor suppressor network. ***Scott W. Lowe*** *described a new approach that is revealing unexpected principles about the nature and organization of cancer genes.*

To keep up with the pace of information generated by genomic approaches and sequencing efforts, cancer biologists can, to some extent, rely on mouse models. One advantage of the models is that there are many technologies that can be used to manipulate the mouse genome and characterize the resulting phenotypes.

Mice also have limitations: Simply put, they are not humans. And producing genetically engineered mice is a painfully slow and expensive process.

One approach to cancer research is to use so-called mosaic models. These are a type of orthotopic transplantation model, in which cells are isolated from either embryonic or adult stem and progenitor populations, manipulated *in vitro* and then put into a recipient animal. This process circumvents much of the crossing and inter-crossing that is involved in the use of germline transgenic knockout mice.

RNA interference in animal models can help explore loss-of-function genetics, specifically to investigate tumor suppressor genes whose inactivation promotes cancer, and for studying genes whose inhibition in an established tumor leads to its regression or elimination.

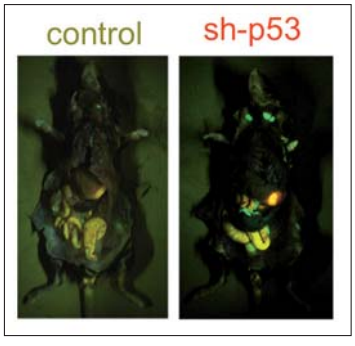

Figure 1
Mimicking tumor suppressor loss using RNAi.

RNAi takes advantage of a conserved machinery that down-regulates gene expression, and provides a rapid way to identify loss-of-function phenotypes. This technique knocks down genes, rather than knocks them out, but eliminates protein. Still, it's very efficient because one RNAi trigger can silence the expression of two alleles in trans. Because it works in trans, it's reversible, which is a powerful attribute. It's also easily scalable[1].

The long-term goal is to use RNAi as a complement to conditional

knockout methods. For example, it might be possible to trigger RNAi-mediated knockdown of gene expression in any tissue, and using conditional systems, for any length of time, as a way to switch endogenous genes on and off *in vivo*[2].

If publicly available comparative genomic hybridization data corresponding to human diffuse large B-cell lymphoma and other lymphomas is plotted across human chromosomes, it reveals various regions of gain and loss that are relevant.

For example, in chromosome 8, there are gains that encompass the MYC oncogene, which is important in human lymphoma. In a subset of tumors, there are losses in chromosome 17, which contains the p53 tumor suppressor that is inactivated in particularly aggressive B-cell lymphomas.

This sort of data can be used to simplify the genome and identify candidate driver alterations. Mouse models can then be used as a filter to identify the most relevant ones.

Mosaic models:

For example, a mosaic model can be made from the so-called E-μ-MYC transgenic mouse model of B-cell lymphoma, either by isolating stem and progenitor cells, or by culturing fetal livers. When a p53 shRNA is now introduced using a retroviral vector as proof of principle, it confirms that MYC cooperates with p53 inactivation to dramatically accelerate the disease.

Multiple shRNAs can similarly be introduced into these stem and progenitor cell populations to repopulate the hematopoietic system of a lethally irradiated mouse, and ask whether there are any combinatorial effects on the development of a lymphoma. Each of the vectors has a fluorescent readout linked to the shRNA that helps track the transduced cells.

Genomic deletions that occur in human tumors are likely to be enriched for tumor suppressor genes. Once the mouse orthologs for these genes are identified, an shRNA library can be generated with multiple shRNAs per gene, and these shRNAs then assembled into pools[3].

For example, 100% of transplanted mice with a particular shRNA pool rapidly develop lymphomas, almost as fast as with the p53 shRNA. In this example, 100% percent of the reads target a gene called AMD1. Knocking down this gene promotes lymphoma formation in this system, validating the hit.

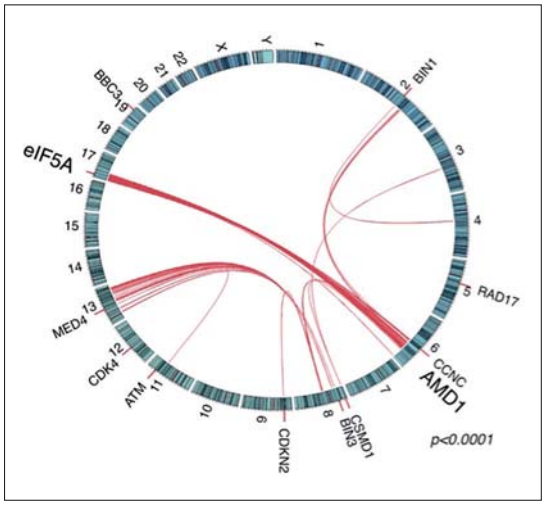

Figure 2
AMD1 and eIF5A are often co-deleted in lymphoma.

The approach identified about 10 other genes, including the ARF tumor suppressor located on human chromosome 9. This gene is well known as a tumor suppressor in the lymphoma model.

It also pinpointed mediator 4 and cyclin C, two components of the mediator complex. Knocking down either one of these promotes lymphomas. Other components of the mediator complex are known to be oncogenic in an ovarian cancer model, suggesting that, depending on the context, mediator can function as either an oncogene or a tumor suppressor.

There are three regions, located on chromosomes 8, 6 and 17, that show multiple hits. Many human tumors contain deletions that encompass all of these genes. There is rarely just one hit per deletion,

suggesting that genomic deletions in human cancers probably target more than one activity.

Two of the hits are linked in the literature to polyamine metabolism, which produces three major polyamines: putrescine, spermidine and spermine. Surprisingly, high levels of polyamines have been linked to cancer, so there is a bit of a paradox. One of the two hits, AMD1, is a rate-limiting step in the production of spermidine. The other, called eIF5A, is an offshoot of polyamine metabolism.

Spermidine creates a unique amino acid called hypusine through two enzymatic steps. Hypusine arises from modification of a lysine on eIF5A1 and may be the only molecule with this modification. The two enzymes conserved from yeast to man that carry out this modification are essential: When they are knocked out, yeast don't grow well, so it is surprising that they might be tumor suppressors.

Secondary screen:

The literature suggests these genes might be involved in translation initiation or elongation, although the precise mechanism is much debated. A secondary screen that targets every single enzyme in this network found only two additional enzymes that when knocked down would cooperate with MYC in lymphoma development. These two enzymes make a line all the way from AMD1 to eIF5A.

This provides strong genetic data that this pathway, which really had not been recognized before, is a relevant tumor suppressor network. Biologically, in the lymphoma system, this module regulates apoptosis. If any one of these genes is knocked down, apoptosis is attenuated.

If either wild type eIF5A or a mutant eIF5A that cannot be hypusinated is introduced into lymphomas with knocked down eIF5A, those that have the wild type version inhibit proliferation of these cells but those that have the mutant do not. That suggests that this is the critical endpoint of this new tumor suppressor network[4].

If AMD1 and eIF5A function in the same pathway, you would expect to see exclusion of mutations in cancers. Instead, deletions in the region that encompasses EIF5A often co-occur with those that encompass AMD1. This could be accounted for by the fact that these deletions contain additional genes. eIF5A is located five genes from p53, which is known to be relevant.

Or, it could be that there is biological relevance, which is plausible as these are essential genes. To test this hypothesis, GFP-linked AMD1 shRNA and cherry-linked eIF5A shRNA were transduced into the mosaic model so that each cell only got one, and a very small fraction got both. If there is an advantage to knocking down both genes, the tumors would be double positive, whereas most of the other cells would be single positive.

When both genes are knocked down, there is an acceleration of tumorigenesis. This is statistically significant, but what is remarkable is that every single one of the tumors that arises in these doubly transduced cells is double positive, strongly suggesting that there's an advantage to knocking down both genes.

By two-dimensional gel electrophoresis and

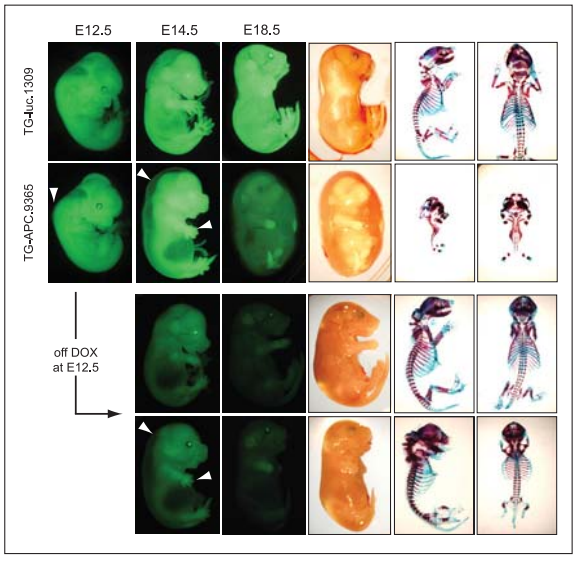

Figure 3
Role of APC in specific developmental windows.

immunoblotting, the combined effect of knocking down AMD1 with eIF5A seems to reduce the amount of hypusinated eIF5A. This suggests that by combining haploinsufficiencies, one offshoot of a metabolic pathway that's otherwise essential is being disabled[5].

Scalable method:

A new method aims to manipulate gene expression in mice in a more comprehensive way, by creating mice that have an inducible shRNA that can be turned on or off. This technology is a complement to conditional knockouts, and has some advantages as well as some disadvantages.

For the experiments, a TET-inducible shRNA is linked to a GFP reporter. Cells are electroporated in the presence of FLP recombinase to get recombination of high frequency in this genomic locus. Because the cells already have a TET transactivator, simply adding doxycycline to the embryonic stem (ES) cells turns them green. Mice can then be made using standard approaches, or tetraploid embryo complementation.

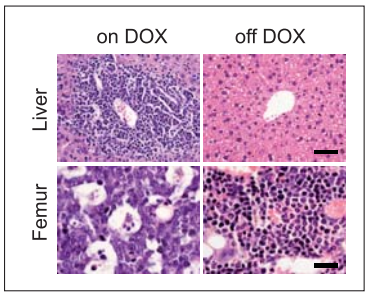

Figure 4
APC suppression leads to T cell leukemia/lymphoma.

Importantly, this is scalable, because these constructs are easy to make and don't involve homologous recombination to integrate. So far, 1,000 ES cell lines with different elements at this genomic locus have been made. It is also reversible: Taking away doxycycline restores expression of the targeted gene.

For example, APC is a negative regulator of Wnt signaling, which is important in hair follicle proliferation and stem cell maintenance. One of the first effects after adding doxycycline is the proliferation of hair follicles, causing the mice to quickly become very hairy in a matter of days.

However, when treatment is continued, the hair turns gray and falls out prematurely. In this case again, the effect is reversible. If doxycycline is taken away, the hair grows back in a month.

In the intestine, APC inactivation occurs in almost 100% of colon cancer cases. When APC is knocked down with a potent shRNA, in a matter of 10 days, proliferation extends from the crypt throughout the villi, indicating the cells are aberrantly in cycle. If the mice are kept on doxycycline, they develop polyps not only in the intestine, as is seen in the APC^{min} mouse model, but also in the colon. If APC expression is reestablished, the polyps disappear from both the colon and the intestine.

In the presence of two different shRNAs that target APC, the mice also develop a T-cell lymphoma leukemia. These cells are malignant: When they are transplanted into recipient mice, they form a disseminated leukemia.

Once again, when APC expression is restored, within a week, the disseminated disease is gone. These leukemias do come back, but when they do, they have high levels of APC protein, suggesting that it isn't a defect in the system. Rather, the cells become resistant to APC suppression. This sort of system can be used to validate the Wnt pathway as a therapeutic target, but also to anticipate mechanisms of resistance.

In summary, APC inactivation contributes to tumor maintenance. Conditional RNAi enables analysis of gene function at different stages of tumorigenesis. The system can be used for target validation, and is reversible and scalable.

This approach is likely to be powerful for studying more than one target or more than one gene, and will speed up the rate at which a cancer can be understood.

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Large-scale screens for cancer genes in the mouse

A report on a lecture by
David J. Adams
Wellcome Trust Sanger Institute, Cambridge, UK

As efforts to sequence thousands of human cancer genomes gain momentum, the challenge of distinguishing driver cancer genes from passengers, and determining how these genes contribute to tumorigenesis, remains a major challenge. Mouse models, transposon-mediated mutagenesis, and exome and whole-genome sequencing have identified many new candidates, several of which are disrupted in human tumors and represent potential diagnostic and therapeutic targets. Insertional mutagenesis provides the advantage of using engineered elements to initiate mutagenesis, largely through loss- or gain-of-function, while tagging potential cancer genes. Mice with somatic or germline mutations of APC, together with transposon-mediated mutagenesis, have tagged several hundred candidate driver genes in colorectal cancer, including about a third linked to human colorectal neoplasia. Likewise, an extensive transposon screen in the pancreas has identified USP9X as a key driver of pancreatic tumorigenesis, and several new and known drivers in a mouse model of acute lymphoblastic leukemia. Next-generation sequencing of mouse tumors has found several mutations in B-progenitor acute lymphoblastic leukemia. Importantly, exome sequencing has found mutations in several residues known to be mutated in humans with the disease. ***David J. Adams*** *argued that, together, these results demonstrate that mouse models can help identify new genes and pathways contributing to human tumor development.*

The International Cancer Genome Consortium and other efforts are sequencing DNA from more than 25,000 human cancers, and will unquestionably identify many new cancer genes. The data so far suggest that most tumors have a small number of frequently mutated genes, and a very long tail of candidate drivers that are mutated at a higher frequency than would be expected by chance. It's these genes that may significantly alter the outcome of each patient.

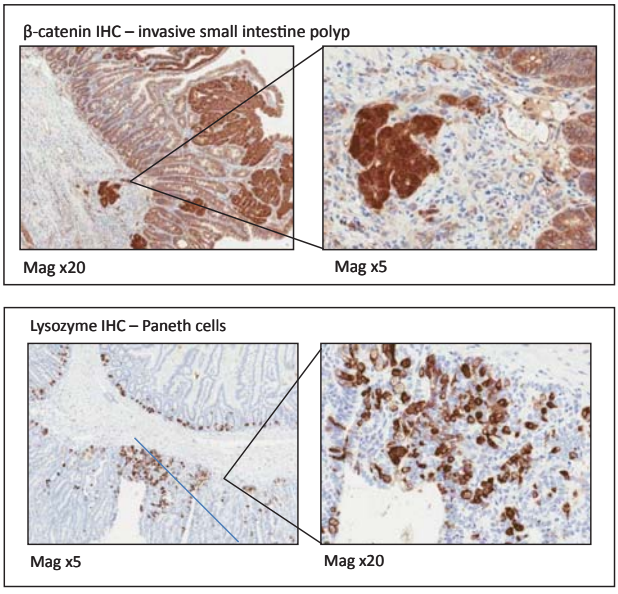

Figure 1
Tumor pathology.

A large proportion of the human cancer genome has regions of copy number gain and copy number loss, and sequence rearrangements. With this sort of complexity, the question is which genes are drivers, contributing causally to driving the development of cancer, and which genes are passengers, rearranged as a result of the underlying mutagenic process.

Several approaches are being used to try to address these questions, and to identify cancer genes using mouse models. Broadly, these approaches have involved

the use of transposon-mediated mutagenesis to identify genes that promote tumor development. More recently, next-generation sequencing of mouse tumors has also been applied[1].

Insertional mutagenesis relies on a transposon called Sleeping Beauty. This is a simple DNA transposon, composed of two repeats called IR/DR, which are about 300 base pairs in length. Different elements such as promoters or stop cassettes can be cloned between these sequences. When a transposase enzyme is expressed, the transposon can move from one site in the genome to another, and as it does so, mutate genes.

Conditional transposase alleles make it possible to express Cre in a tissue of interest, switch on the transposons, and then mobilize mutagenesis specifically in a tissue or cell type of interest. Once tumors are collected, ligation-mediated PCR, coupled with either 454 or Illumina sequencing, profiles all of the mutations found.

A 'background' dataset to find transposon hotspots has found more than 1 million insertions in these tumours[2]. The largest screen performed, in the gastrointestinal tract, began with mice carrying one floxed allele of APC, one wild type allele of APC, transposons, transposase and a Cre driver called Ah-Cre that expresses specifically in the intestine.

Inducing Cre in these animals deletes APC and mobilizes transposons specifically in their intestinal tract. A strong synergy between the loss of APC and mutations generated by the transposons dramatically accelerates tumor formation.

The 467 tumors collected so far show evidence of invasion and other pathological phenotypes — for example, an expanded proportion of Paneth cells, which is a marker of aberrant Wnt signaling.

Long tail:

The mice in these experiments have one floxed allele and one wild type allele of APC. Not surprisingly, the most frequently mutated gene in these experiments is the wild type allele of APC, with multiple independent transposon insertions along the length of the gene.

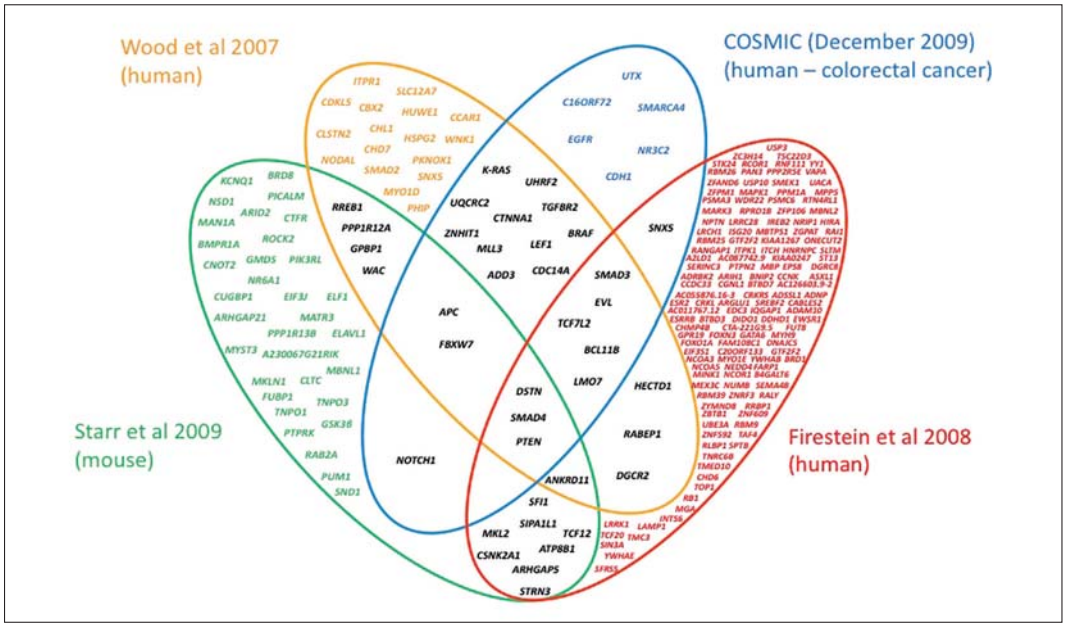

Figure 2
Cross-species oncogenomics.

The analysis also identified 15 genes mutated in greater than 20% of tumors, 140 genes mutated in greater than 10% of tumors, and a long tail of other genes mutated at a frequency higher than that expected by chance. This is a startling result, and it suggests that there are many genes that contribute to tumor development[3].

Even applying rigid statistics with genome-wide cutoffs and low p values yields 200-300 genes. The question is how many of these genes represent cancer drivers.

Compared against various human datasets, there is a significant overlap between transposon data and the datasets. The second bit of evidence that many of the genes in these screens are real comes from functional data.

For example, one of the genes that's a long way down the list is MLL3, a H3K4-methyl transferase that removes repressive marks from the genome, allowing promoters to be expressed. The profile of mutations in these tumors suggests that MLL3 functions as a tumor suppressor. Mice lacking MLL3 spontaneously develop tumors in their intestine.

If these knockout mice are then crossed with APC mice, loss of one allele of MLL3 increases the level of polyposis, and the loss of two alleles increases polyposis even further. Not only do the mice have more polyps, the tumors that form are significantly larger, and show evidence of invasion.

In addition to MLL3, there are a number of other genes on this list that have been validated in experimental crosses with APC and other APC mutant mice. This again suggests that the set of genes discovered from the transposon screen is greatly enriched for driver genes.

Pathway analysis with approaches such as Ingenuity or the DAVD Pathway Tool shows that a statistically significant number of the genes in the list will collapse into known colorectal pathways.

Current research is focused on those genes whose orthologs fall into recurrently altered focally rearranged regions of the human colorectal cancer genome. It is also looking at the overlap between mouse and human expression data, and also outcome data to help drive analyses forward.

Epigenetic silencing:

In collaboration with David Tuveson's group (see Tuveson, page 93), another screen aims to identify genes involved in the development of pancreatic cancer.

In mice carrying PDX1, Cre and KRAS, transposons once again dramatically accelerate the rate of tumor formation. The unambiguous hit in this case is the gene USP9X. About 50% of tumors contain insertions within this gene.

Strikingly, human data, TCGA data and ICGC data don't show any mutations in this gene. However, low levels of expression of USP9X significantly correlate with widely metastatic disease and with significantly reduced survival. There is evidence that USP9X is epigenetically silenced.

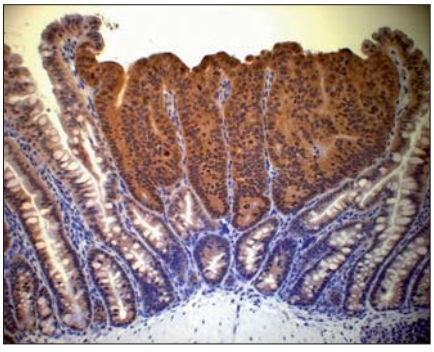

Figure 3
MLL3 knockout animals spontaneously develop adenomas.

This suggests that transposon-based approaches can help identify key driver genes that may not be identified by sequencing analysis alone, such as genes that are disrupted by epigenetic silencing.

Another disease of interest is acute lymphoblastic leukemia (ALL), the most common childhood malignancy and a leading cause of childhood death, because there are few targeted therapies.

A key driver in the disease is the fusion between TEL and AML1 gene. The fusion allele can be made by

knocking the human AML1 cDNA into the TEL locus, together with a Sleeping Beauty transposase.

Mice carrying the TEL-AML1 allele have significantly reduced survival relative to controls, and this is accelerated further when the allele is combined with transposons.

In human tumors carrying the fusion allele, there are mutations in CDKN2a, PAX5, EPOR and IKZF1. However, the mouse tumors only have mutations affecting EPOR and IKZF1, and mutations associated with the genes LEF1, EBF1 and TCF4.

PAX5 is a key driver of B-cell development. Upstream of PAX5 in the pathway is EBF1 and downstream are LEF1 and TCF4. For some reason, the transposon can mutate these particular genes, but cannot seem to mutate PAX5, the key driver.

This suggests that these transposon approaches mutate genes in cancer pathways, but not necessarily the cancer genes themselves. And this may in some respects account for the large number of genes discovered by the screens.

In support of this, the screens performed have found very few big hitters in each malignancy. For example, none of the screens performed have found mutations associated with p53.

Next-gen sequencing has also been used to analyze genomes of mouse cancers. In a pilot study, two tumors representing models of basal-like, lobular and high-grade intraductal carcinoma (see Jonkers, page 99) have been sequenced.

From the structure of the genome, paired-end sequencing data identified rearrangements as being intra- or inter-chromosomal. What's immediately evident when comparing two human basal-like tumors with corresponding mouse tumors of the same genotype is that the mouse tumors contain significantly fewer rearrangements compared with the human tumors.

This is probably because the genomes of these mice have been engineered with strong driving mutations, and don't have the opportunity to acquire the load of mutations that occur in human tumors. However, not all mouse tumor genomes are created equally. There are significant differences in the structural rearrangements that occur between these different genomes.

As in the human breast cancer genomes that have been sequenced previously, these genomes have in-frame fusion genes formed by segmental duplication or deletion. These fusion products are expressed in each of the tumors, but not in the normal samples.

The genomes also contain features such as homozygous deletions. For example, a homozygous deletion within the LRP1B gene creates an in-frame fusion gene. This particular homozygous deletion occurs in about 5% of human cancer cell lines, as identified by CGH analysis by Sanger's cancer genome project.

One particular feature observed in the sequencing of human cancer genomes is a tandem duplication phenotype, which is absent from mouse genomes. It may be that the mouse genome functions differently than that of the human in response to the deletion of these genes. These observations suggest that some aspects of tumorigenesis or the tumor genome are conserved, but not others.

Perfect models:

In ALL, one of the key drivers is PAX5. Disruption of PAX5 causes a block in the normal differentiation of B-cell lineage. Human tumors show deletions of PAX5, point mutations, and also translocations that disrupt the gene.

When PAX5 heterozygote mice are injected with the point mutagen N-ethyl-S-nitrourea (ENU), there is a dramatic acceleration of tumor formation compared with wild type mice given ENU.

The disease that forms in these animals is not T-cell or myeloid in origin, but is a progenitor B-cell disease, as seen by staining with various markers. Exome-sequencing of 39 tumors collected from this study identifies a number of somatic exonic mutations.

Most of the mutations in PAX5 are clustered in the PD domain, found in the N-terminus. All of the mutations discovered affect the ability of the PD domain to grab hold of DNA and function as a transcription factor.

Overall, the screen identified a number of mutations associated with ALL, and several mutated residues in the mouse that had previously been found to be mutated in humans[4]. This suggests that this model exquisitely recapitulates the mutations down to the amino acid level, and mouse data can help discover new human genes.

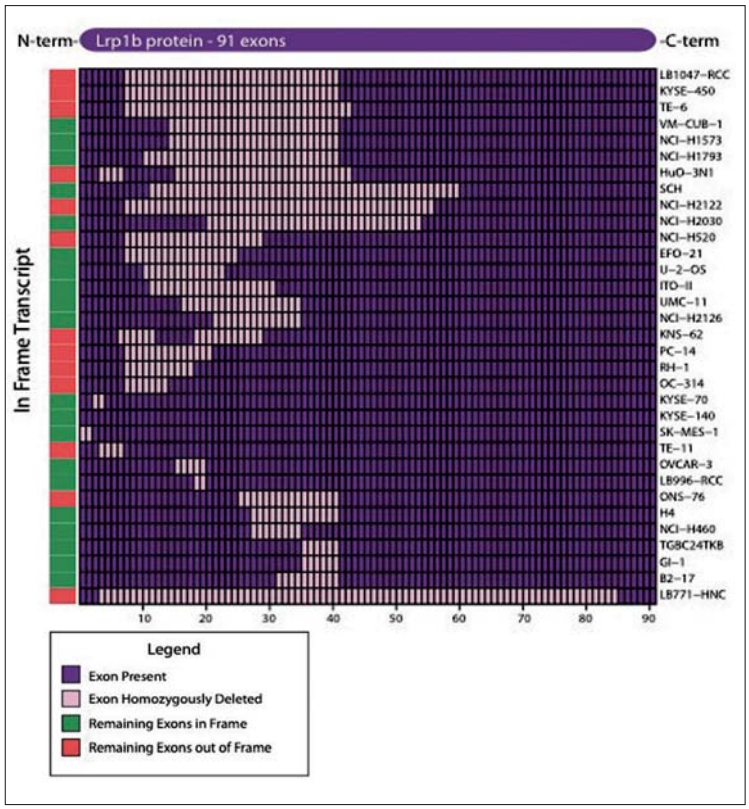

Figure 4
LRP1B is homozygously deleted in both human and mouse tumors.

For example, one of the genes that is recurrently mutated in mouse tumors is SH2B. This gene carries two stop codons and one frameshift mutation in the sequence of mouse B-ALL tumors. In human B-ALL, this gene has been found to be recurrently focally deleted.

Over the past year, several different mouse models, including those for mesothelioma, melanoma and acute myeloid leukemia, have been sequenced to generate an overall profile of the patterns of mutations.

The tumor type with the most sequence data available is osteosarcoma. A direct comparison of the exome sequences of mouse and human osteosarcomas shows that about 10% of genes are recurrently mutated in both mouse and human. The optimists would say this illustrates that the mouse is a powerful biological filter, whereas the pessimists would say this shows that mice are not good models of human disease.

In summary, transposon work generates a long list of genes, and there are interesting questions about whether the transposon tumors form with different kinetics. There is some evidence to suggest that transposon insertions mutate cancer pathways at multiple sites. It's also clear that, although this approach hits many genes known to be cancer drivers, some genes are conspicuously absent.

However, there's strong indication that many genes identified in these screens are important in human tumorigenesis. Mouse models can exquisitely recapitulate human disease, in some cases down to the amino acid level. Careful analysis of the mouse cancer genome is important to fully assess the fidelity of these models as preclinical models for each human disease.

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PART II: Understanding oncogenesis in mouse models

Targeting KRAS oncogene signaling in mouse models of cancer

A report on a lecture by
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Centro Nacional de Investigaciones Oncologicas, Madrid, Spain

KRAS oncogenes are implicated in about one-fourth of all human cancers, including non-small cell lung cancer (NSCLC) and pancreatic ductal adenocarcinoma (PDAC). Unlike lung epithelial cells, acinar cells of the pancreas are highly resistant to oncogenic insults, and are not transformed by a KRAS oncogene even in combination with loss of p53 or p16INK4a/P19ARF tumor suppressors. But KRAS oncogenes can transform adult acinar cells if the mice suffer from mild pancreatitis, which induces tissue injury followed by innate and adaptive immune responses. Attenuation of this inflammation thwarts PanIN expansion, but does not prevent tumor development. The results suggest that anti-inflammatory treatment for pancreatitis may reduce the risk of PDAC. A mouse model of PDAC is being used to systematically eliminate components of the immune system and identify the primary cells responsible for the pro-tumorigenic effect. Mouse models have also been used to validate targeted therapies using genetic approaches. For example, they have uncovered a synthetic lethal interaction between KRAS oncogenic signaling and lack of CDK4 expression that leads to the immediate onset of senescence. Ablation of the BRAF kinase has no significant effect on tumor development in KRAS oncogene-expressing lung cells, but CRAF expression is essential for the onset of NSCLC. ***Mariano Barbacid*** *suggested that the information derived from these studies can help initiate drug discovery programs to treat KRAS oncogene-driven cancers.*

The lung is a simple organ, divided into bronchioli, bronchialveolar duct junction (BADJ) and the alveoli. It has five types of cells: type I and type II alveolar cells, clara cells and variant clara cells, and bronchialveolar stem cells.

To an existing mouse model of lung cancer (see Jacks, page 73), another mutation was introduced in order to eliminate the normal allele and speed up oncogenesis. If tumors get to a certain point in 6 months with wild type KRAS, tumors without wild type KRAS take only 4 months to get there.

The other change for experimental reasons is to introduce a color marker in a bicistronic fashion. When 4-hydroxy tamoxifen is added, it turns on Cre, the oncoprotein is expressed, the normal protein is lost, and the color marker is expressed.

At the same time, giving only a little bit of tamoxifen ensures that single cells can be identified in individual regions, without being masked by an overwhelming number of KRAS-expressing cells.

As measured by the surrogate marker, a single injection expresses

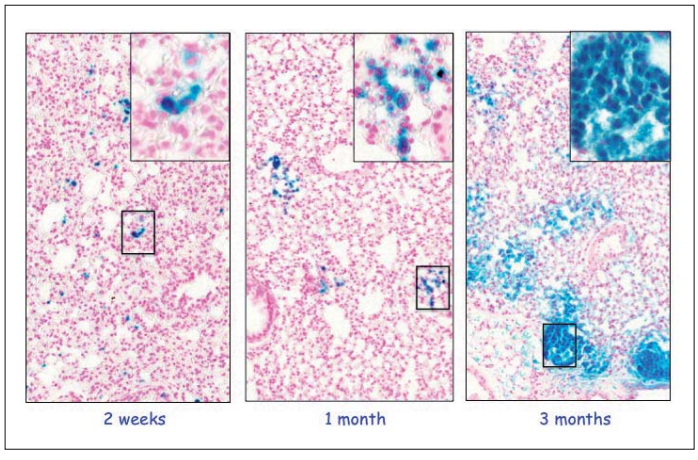

Figure 1
KRAS-expressing cells in the alveolar region are able to proliferate in a sustained manner.

the oncogene in about 0.2% of cells of the entire lung. The mice are then sacrificed 1, 2 or 4 weeks later. At 4 weeks, about 2% of cells have undergone at least 6 cycles of cell division.

KRAS-expressing cells are generally uniformly distributed in the main areas of the lung, including the alveoli, BADJ and bronquioles. However, only those KRAS-expressing cells located in the alveolar region are able to proliferate in a sustained manner. These proliferating cells have been identified as type II alveolar cells based on the fact that they express the SPC marker.

By loose quantitative analysis, at 2 weeks, there are about 4 cells in the alveolar region. At 1 month, the cells have proliferated to about 20. Then at 3 months, there are hyperplastic areas throughout the alveoli, but there is no senescence or cell death.

At 2 weeks in the BADJ, the numbers are similar to those in the alveoli. But at a month, they remain at these low numbers, rather than proliferate actively as they do in the alveoli. The same is true for clara cells.

In a small area of maybe 20-30 cells, staining for β-galactosidase, which is the surrogate for the KRAS oncogene, corresponds with surfactant protein C, which stains clara cells. However, there are some SPC-positive cells that do not express KRAS.

Among type II cells, there is a perfect correspondence with oncogene-expressing cells, suggesting that only type II alveolar cells are capable of proliferating as a consequence of KRAS.

Clear differences:

These proliferating cells may be mature type II alveolar cells, as the numbers are probably too high for them to be stem/progenitor cells. Because the number of clusters that become adenomas or adenocarcinomas is

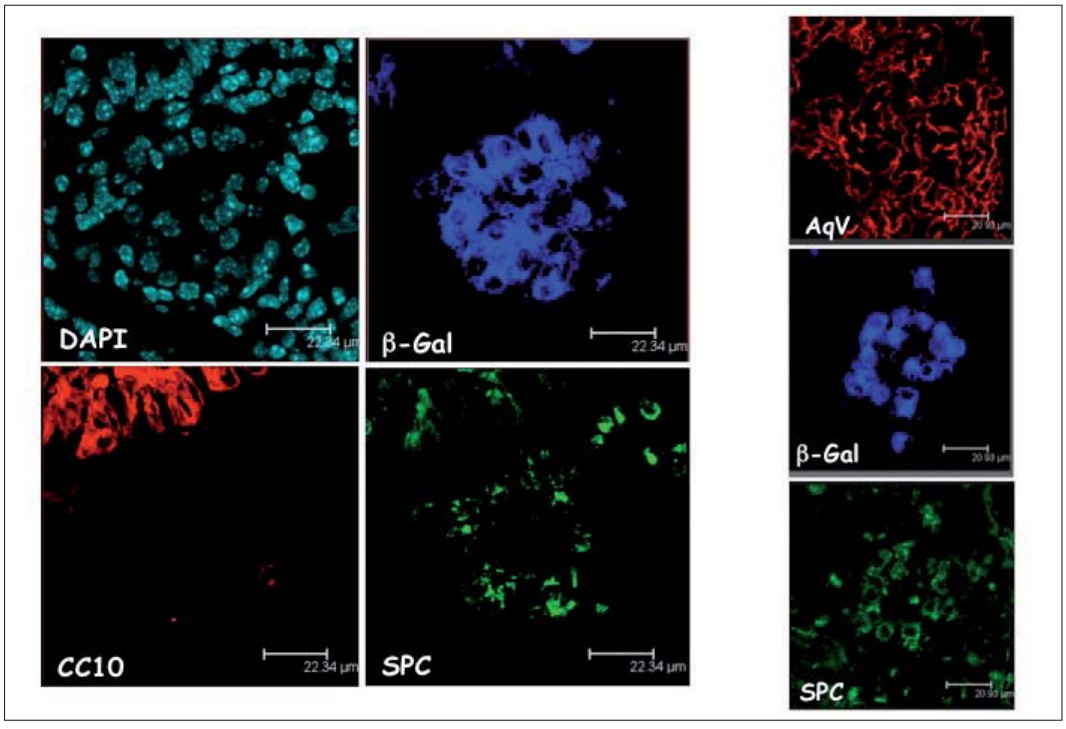

Figure 2
Expression of the KRAS oncogene corresponds with surfactant protein C.

small, it is also possible that only 'clusters' derived from stem cells progress to form tumors. Alternatively, tumor formation may be a stochastic process that occurs by accumulation of mutations or errors in any cluster.

An expression profile analysis on the hyperplastic areas helps explore these possibilities. In this type of analyses, there are clear differences between hyperplastic and normal areas. Of 7 samples, 4 show a near-perfect correlation to previously published results by others. However, the remaining 3 have a pattern that is similar to that of normal areas.

At the top of the ladder are two genes, DDR1, a tyrosine kinase receptor also called PTK3, among other names, and serpin B5 (SPB5), a serpin protease inhibitor. This particular member of the family doesn't have enzymatic activity, but is a tumor suppressor.

Antibodies against DDR1 and SPB5 show that areas that are positive for DDR1 are negative for SPB5 and *vice versa*. It is possible that there are two classes of alveolar type II cells, and the expression of the KRAS gene is one set leads to DDR1 and in the other to SPB5.

In these mice, turning on the oncogene results in big tumors 10 months later. The tumors can be divided into two classes based on SPB5 expression. It is not clear whether the SPB5-negative ones are always DDR1-positive. The difference in SPB5 expression could be a result of clonal expansion or could be a mix of cells grown in the same area.

Likewise, in a set of 84 non-small cell lung carcinomas, the majority express one or the other marker. Adenocarcinomas all express DDRI, whereas squamous and large cells express SPB5. The rest of the tumors are double positive, and there is one double negative.

Target validation:

There is a surprising synthetic lethal interaction between expression of KRAS and loss of CDK4 that leads to an immediate senescence and prevents cell proliferation[1]. This event is unique to lung cells.

Eliminating CDK4 alleles, but not CDK2 or CDK6 alleles, from advanced tumors detectable by computed tomography induces senescence and prevents tumor progression. But one has to be careful not to over-interpret the results, as this is stable disease at best. If anything, CDK4 inhibitors may someday be used in combination with other drugs because by themselves they do not do much.

A similar analysis of the kinases directly downstream of RAF shows that KRAS signals through CRAF but not through BRAF. When BRAF is eliminated, tumor initiation is unaffected, but when CRAF is deleted, there is no tumor initiation. The few tumors that develop all retain CRAF expression, suggesting that they escaped[2].

Ablation of both MEK kinases or both ERK kinases also completely prevents tumor development. This indicates that the bottleneck of signaling is not at the level of RAF, but at the level of MEK.

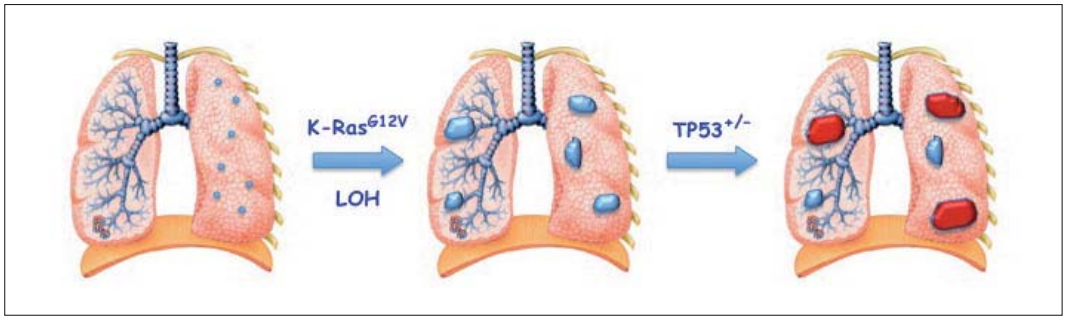

Figure 3
KRAS RERT non-small cell lung cancer model.

Unfortunately, complete elimination of MEK or ERK kinases in about 50% of the cells leads to the rapid death of mouse. Mice expressing one MEK allele, either MEK1 or MEK2, survive for at least 9 months, although there are some deaths.

However, the goal is to validate CRAF in tumors that are already formed, not just in tumor initiation.

A modified form of the pancreatic cancer model includes frt-stop-frt instead of the usual lox-stop-lox strategy. In this model, a flippase can induce the tumor, and once the tumor is positive by CT or PET, tamoxifen is added and the response assessed.

The results are encouraging. The mice survive when CRAF is eliminated but elimination of BRAF has only a tiny effect. With CRAF alone, there is some tumor regression, but it is a small effect and unlikely to lead to a cure. It may help to identify targets for inhibitors, however.

There are experiments under way in which the p53 allele is also being introduced at the same time. Kinase-dead alleles are also being generated, as it is not the same to eliminate the protein as it is to make it inactive. Kinase-dead alleles for CDK4 and CRAF have already been made. With the CDK4 kinase dead allele, there is not much difference, and in fact, there is less effect than when the protein is eliminated.

A mouse clinical trial platform is also being established to test combinations of genetic and selective inhibitors. The premise is to take a strain in which the tumor is already compromised — for example, by eliminating CRAF and expressing kinase-dead CDK4 — and then hit it with whatever is in the clinic.

To combat tough tumors, it may be necessary to give patients combinations of as many as 6 drugs at once that are selective for the main pathways including MET, EGFR, PI3K, Notch and Hedgehog.

This is difficult to do without too much toxicity but at the same time, based on sequencing data, hitting just one point in a pathway is unlikely to be effective. All of the targets have to be validated in more aggressive NSCLC models carrying mutations in key tumor suppressors such as p53 null alleles.

Poor patterns:

Sequencing studies show that, disappointingly, there is no pattern to the mutations. Early lesions in more aggressive and metastatic tumors will also be sequenced, but in 2 mice with adenocarcinomas, mutations in one mouse are totally different from mutations in the other.

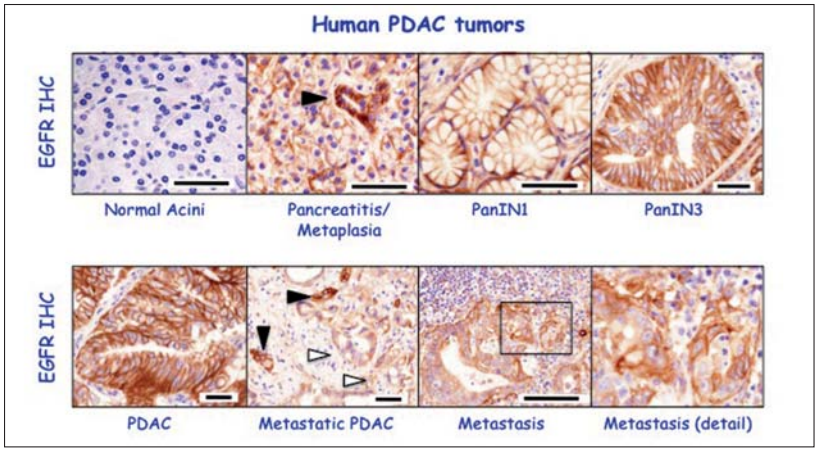

Figure 4
EGFR and KRAS oncogene-driven pancreatic ductal adenocarcinomas.

In a pancreatic cancer model, KRAS is activated at a late point in embryonic development, and using the Tet-O system, Cre can be activated only in acinar cells. This is a very clean system, and generates tumors in 20% of mice by one year and high-grade PanINs in 100% of mice.

K-RAS$^{+/LSLG12Vgeo}$ *Elas*TA *Tet-O*-Cre mice

are even better because by giving doxycycline in the drinking water to the pregnant mothers, expression of the oncogene can be delayed until the animals are adults. Because pancreatic cancer is a disease of adults, this model is more appropriate.

However, this model does not develop any tumors. The adult acinar cells are resistant to KRAS, and the mice only develop tumors when the pancreas is damaged by pancreatitis[3].

In general, blocking the EGFR/RAS/PI3K oncogenic cascade is a bad idea, particularly because of feedback loops. Normal acini do not express EGFR. However, even the earliest lesions, called metaplasia, already expressing EGFR, and all PanINs, whether low-grade or high-grade or tumor, also express EGFR.

In the mice that are given pancreatitis, acinar cells in even relatively normal looking pancreatic areas express EGFR. The same is true in human cells. Normal acini in human samples do not have any EGFR. But in biopsies from people with pancreatitis, the so-called normal acinar cells already express high levels of EGFR.

Also, cells in different stages of tumor development, from metaplasia all the way through to metastasis, all express EGFR. In the lung, EGFR mutations and KRAS mutations are mutually exclusive. And in the colon, patients with KRAS mutations are not given cetuximab.

Mice with oncogenic KRAS alone survive for a long time. At 1 year of age, only about 10% of the mice are dead. When the double tumor suppressor p16INK4a/p19ARF$^{lox/lox}$ is introduced, the tumors are very aggressive[4].

However, if EGFR is eliminated, not only do the mice survive, there are no tumors, no lesions, and not even early-grade PanINs. The few lesions that do develop express EGFR. When EGFR is eliminated in the lung or in the intestine, however, there is no increase in survival.

EGFR is essential for tumor initiation or even PanIN initiation. In mice that are wild type for p53 and EGFR, if p53 is eliminated, the mice die in 20 weeks. If EGFR is eliminated, once again the mice die, but this time, there is 83% increased survival. Loss of p53 somehow activates oncogenic pathways independent of EGFR signaling to promote pancreatic cancer.

All this is in tumor initiation. To repeat experiments in tumor progression, tumors were cultured and the experiments conducted in early explants. In this system, eliminating EGFR by shRNA has a big effect on representative tumors with only oncogenic KRAS, with KRAS and loss of p16 and p19, or KRAS and loss of p53. But if these explants are maintained, the cells will grow, suggesting that the patients develop tumors despite the therapeutic effect.

The goal now is to identify those pathways that the p53 knockout activates that cannot be turned off in the absence of EGFR. Two pathways were chosen to begin with: STAT3 and a PI3K inhibitor. Neither of them has much effect, but when combined, the effect is dramatic. This is preliminary data, but it creates a base to test other inhibitors and see which ones might have an effect.

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BRAF deletion enhances mutant KRAS-driven tumorigenesis *in vivo*

A report on a lecture by
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KRAS and BRAF both frequently acquire gain-of-function mutations in human tumors, but the mutations are mutually exclusive, suggesting that the two genes have overlapping, redundant functions. In conditional mouse models of KRAS^{G12D}-driven non-small cell lung cancer and pancreatic ductal adenocarcinoma, deletion of one or both alleles of BRAF significantly enhances lung tumor burden and disease progression, leading to decreased overall survival. These findings are in contrast to others who have reported a unique requirement for CRAF in these models. By immunohistochemical staining, a fraction of tumors in the double heterozygote mice show complete loss of BRAF expression, suggesting that BRAF functions as a suppressor of oncogenic KRAS signaling. Various experiments also suggest that although BRAF may be dispensable for MAPK pathway activation, it serves a critical role in suppressing oncogenic KRAS-induced transformation by regulating CRAF activity and establishing negative feedback regulation of constitutive mutant KRAS signaling. ***Leisa Johnson*** *described these experiments, as well as preclinical interrogation of genetically engineered mouse models and orthotropic models to address some of the contentious work on anti-angiogenic inhibitors.*

There have been many knockout mouse models that have tried to address the role of effectors downstream of oncogenic RAS and their role in tumorigenesis. Knocking down most of these effectors inhibits oncogenesis.

Similar experiments were done to determine whether effectors in the RAF/MEK/ERK signaling cascade would also inhibit tumorigenesis.

These experiments relied on BRAF rather than on CRAF. ARAF, BRAF and CRAF are highly homologous, but have different signaling epitopes and roles in the process. CRAF is more tightly regulated, but BRAF appears to be the primary kinase, coupling RAS with MEK/ERK signaling in the cell. BRAF is also most frequently mutated in human cancer.

Knocking out CRAF has shown that it is required for KRAS^{G12D}-driven mutagenesis in the lung[1,2]. By contrast, others have reported that knocking out BRAF has little impact and, if anything, may even delay tumorigenesis and increase overall survival.

However, the following experiments have found the opposite result: In both non-small cell lung cancer (NSCLC) and pancreatic ductal adenocarcinoma (PDAC), BRAF deletion decreases overall survival in KRAS^{G12D}-driven carcinomas.

With both heterozygous and homozygous loss of BRAF, a time course analysis using microCT found an increase in overall tumor burden, driven by an increase in tumor number.

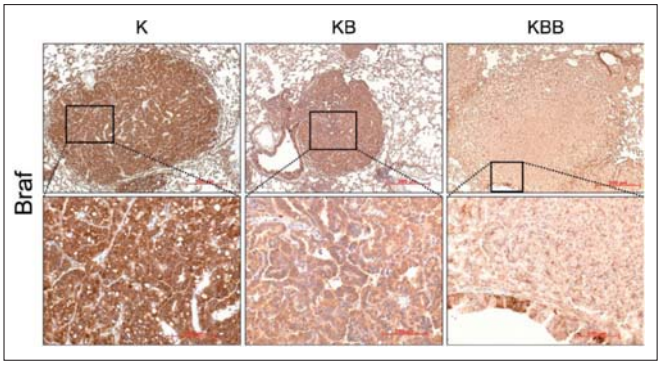

Figure 1
BRAF expression in KRAS^{G12D}-driven tumors.

These experiments used BRAF immunohistochemical staining (IHC) as a marker to determine the popout rates in the tumors. BRAF IHC is sensitive to copy number, and detects full expression, heterozygote expression or loss of expression. In some of the heterozygote mice, there is a complete loss of the other copy of BRAF, and an indication of inefficient popout in the BB mice.

By laser capture microdissection of the tumors also, heterozygote BRAF mice appear to be losing the other wild type copy, suggesting that BRAF may act as a tumor suppressor.

To analyze this further, more than 1,000 lesions were counted for each genotype, and the time frames broken down into more or less than 20 weeks after induction. The tumors were scored for atypical alveolar hyperplasia, the earliest stage of disease, benign adenomas, and low- and high-grade carcinomas.

In mice both less and more than 20 weeks after induction, there was a distribution change in the frequency of late-stage disease, with a decline in the earliest stage lesions and a compensatory increase in adenomas and adenocarcinomas. The data suggest that loss of BRAF accelerates $Kras^{G12D}$-driven tumor progression.

However, analysis of the Ki67 index in these tumors shows no difference between the two groups of mice. There are also no discernible differences in the Ki67 index between the earliest benign lesions and later-stage disease, suggesting that loss of BRAF does not significantly alter overall tumor proliferation.

BRAF loss also does not affect levels of phospho-ERK. However, there is a trend towards increased pERK in KB and KBB animals, which is accounted for by an increase in adenocarcinomas.

Signaling effects:

These findings have been difficult to parse *in vivo*. Mouse embryonic fibroblast lines have been generated to try and assess the events better *in vitro*. Two different clones have been made for each of these lines, using lenti-Cre-loxp self-excising virus so that there is no long-term Cre expression.

Once the entire population has fully deleted both copies of BRAF, the mice consistently have a higher proliferation rate than either the controls or the heterozygotes. Mirroring the overall survival patterns, the heterozygotes also have a proliferative advantage.

Biochemically, in both the heterozygote and homozygote mice, loss of BRAF induces and augments CRAF activation and downstream effector signaling.

For example, there is an increase in the activation of pCRAF at serine 338 following epidermal growth factor (EGF) induction. There is also augmented expression of pMEK and pERK, but importantly, the pathway gets down-regulated. pAKT is also elevated for a more sustained time but down-regulated following EGF induction.

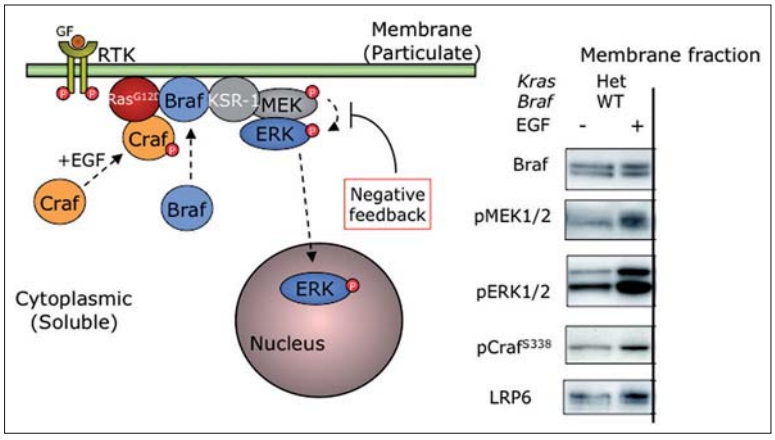

Figure 2
BRAF deletion increases CRAF membrane localization and decreases negative feedback.

In KRAS heterozygotes with wild type BRAF, there is always some basal amount of signaling in this pathway under starvation conditions. EGF induction typically

increases downstream signaling and increases phospho-serine 338 levels at the membrane.

However, when BRAF is deleted, CRAF may automatically translocate to the membrane, resulting in at least a two- to three-fold increase in pCRAF, and in pERK and pMEK signaling. When the pathway is now induced with EGF, it starts translocating faster into the nucleus and starts dampening down.

The loss of BRAF also increases colony number. Unsurprisingly, this growth is more dependent on CRAF than on ARAF[3]. When a BRAF inhibitor is used, it binds to BRAF and dimerizes with CRAF. Homodimers of CRAF activate this pathway, and there is still some negative feedback. In the case of BRAF null, CRAF is up-regulated at the plasma membrane, and there is enhanced signaling in response to growth factors or even at the basal state.

To summarize, BRAF is a unique effector that suppresses $KRAS^{G12D}$-driven tumorigenesis via tighter negative feedback regulation. BRAF loss increases tumor number and burden, decreases overall survival in mouse models of NSCLC and PDAC, accelerates tumor progression and increases proliferation *in vitro*, and results in enhanced pCRAF membrane localization and downstream signaling.

Exploring angiogenesis:

Angiogenic inhibitors are currently controversial in the field. Genentech's Avastin or bevacizumab has been shown to have significant efficacy in different tumor types. The extent of that benefit depends on tumor type, and it is approved for NSCLC, metastatic colon and renal cancers, and recurrent glioblastoma multiforme (GBM).

A significant issue with the drug is that there is no predictive biomarker that can help select patients most likely to respond to therapy. However, recent work suggests a promising signature that is translating well into the clinic in retrospective analyses.

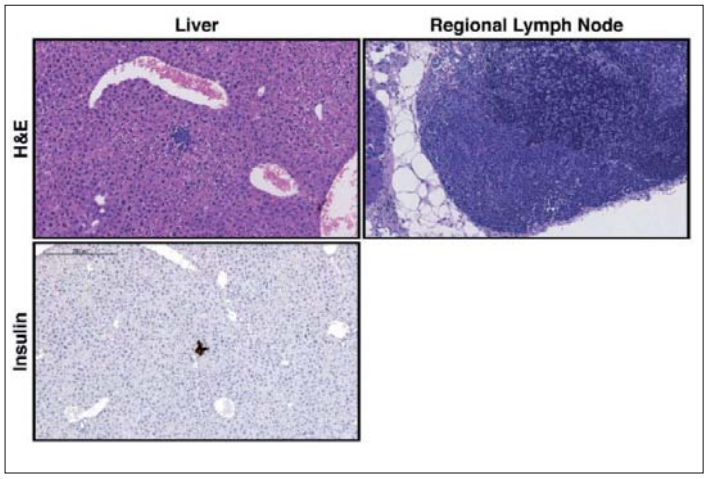

Figure 3
Short-term α-VEGF treatment does not accelerate the onset of metastases in PNETs, but sunitinib does.

The clinical evidence to date suggests that tumors will eventually escape therapy targeting the vascular endothelial growth factor (VEGF). A major confounder is that once a patient experiences an adverse event, they discontinue the drug, making it difficult to interpret the observations. A recent ovarian cancer trial, which failed in phase III, suggests that prolonged treatment achieves better results.

It's important to establish optimal duration and combination for dosing with anti-VEGF as well as other anti-angiogenic drugs. It's also critical to elucidate mechanisms of inherent refractoriness and resistance in response to this therapy.

Does the choice of molecular inhibitor influence effects on tumor invasiveness and metastatic potential? And is this tumor type- and model-specific, which the clinic is tending to indicate?

Experiments to address these questions relied on several different inhibitors. Genetech has a monoclonal

antibody called B20-4.1.1, the Avastin equivalent for mouse studies. The goat anti-mouse VEGF antibody is likely to have problems in an immunocompetent mouse. DC101 is a monoclonal antibody that binds to VEGFR2 and blocks VEGF binding and signaling. Finally, sunitinib is a small molecule inhibitor that binds to all three VEGF receptors as well as several other RTKs.

The experiments used four different genetically engineered mouse models. RIP-Tag2 is a neural endocrine model that uses SV40 T-antigen to functionally inactivate the RB and p53 tumor suppressor genes. Because of oncomouse patent issues, an in-house version of RIP-Tag2, dubbed RIP-Tβ Antigen, was developed.

Another model also functionally inactivates RB and p53 through the expression of adenovirus Cre into the lung following intranasal administration. Two KRAS-driven tumors, one of the lung and one of the pancreas, were also used.

In the RIP-Tβ Ag, overall tumor burden decreases with both anti-VEGF and sunitinib, but only anti-VEGF achieves statistical significance. Importantly, sunitinib treatment increases the number of tumors. Both treatments significantly decrease microvascular density by about 30-50%, and both increase overall survival.

In the neuroendocrine SCLC model, by contrast, treatment lowers vascular density. The mice are not responsive to single agents but they are highly responsive to the chemotherapeutic doublet of carboplatin and CPT-11 that is commonly used in the clinic.

When an anti-VEGF agent is added to that combination, there is a slight further reduction in overall tumor burden over 6 weeks. That correlates ultimately to a quadrupling in the median overall survival which, in this model, is highly significant.

Quantitative analysis:

Quantitative analysis reveals that sunitinib treatment significantly increases the tumor number for both invasive carcinomas 1 (IC1s) and IC2s. Single agent anti-VEGF also shows a statistically significant increase in the invasive borders, but only in the IC2s. So, the two drugs differ in their effects on tumor morphology.

Sunitinib also shifts the tumor distribution, with a decrease in the islet adenomas, and a concomitant increase in IC2s. Single agent anti-VEGF also shows a significant increase in IC2s, but not much of an impact on either islet adenomas.

However, even though there is an increase in invasive borders, it does not have a clear effect on metastasis. Sunitinib results in significant increases in both lymph node metastases and liver metastases, the two most common sites, and an overall rate that is significantly higher than that of controls or anti-VEGF.

The results are similar in the SCLC model. Analysis of up to 140 lesions shows no significant increase in late-stage disease. Importantly, this was done at 14 days, but even long-term, in mice that have been on the drug for up to 7 months, there is no change in the overall distribution.

In the short term, there is also no significant change in metastases, and longer term, the combination of anti-VEGF with chemotherapy extends the time it takes to see local metastases at lymph nodes and distant metastases in the liver.

If a drug affects the invasiveness and metastatic potential of tumors, withdrawing it would be expected to accelerate tumorigenesis. This phenomenon is known as rebound.

In the RIP-Tβ Ag mice or the SCLC mice, discontinuing anti-VEGF doesn't increase microvascular density or metastases. Overall, there is no evidence for rebound following discontinuation of anti-VEGF[4].

When data from five different trials — two colorectal cancer trials and one each of renal, pancreas and breast

cancers — were analyzed retrospectively, those results also showed no evidence of rebound. In fact, one trial showed a significant improvement in time to progressive disease. These trials also did not show any difference in the spectrum or incidence of metastasis.

Giving anti-VEGF continuously from an early stage of the disease delays progression. When the NSCLC model mice are treated with anti-VEGF for 14 weeks, after 2 weeks of induction, they show mostly atypical alveolar hyperplasias, significant decreases in tumor burden and a delay in progression to advanced disease.

At the end of 14 weeks, there is no increase in metastases in mice treated with anti-VEGF relative to controls, either alone or in combination with chemotherapy. So, at least in these four GEMMs, there is no difference in metastasis rates.

When BALB/c mice are pretreated with sunitinib, serafinib or gleevec for 7 days and then inoculated with 66c14, a murine breast cell line, all three inhibitors increase the number of mets that can seed and outgrow in the lung. However, anti-VEGF, anti-PIGF, DC101 or soluble FLT1 do not show that effect.

Using *Ricinus* lectin shows that 7 days of treatment with sunitinib induces leaky vasculature within the normal lung niche, increasing the extravasation of tumor cells. By contrast, anti-VEGF does not increase *Ricinus* staining.

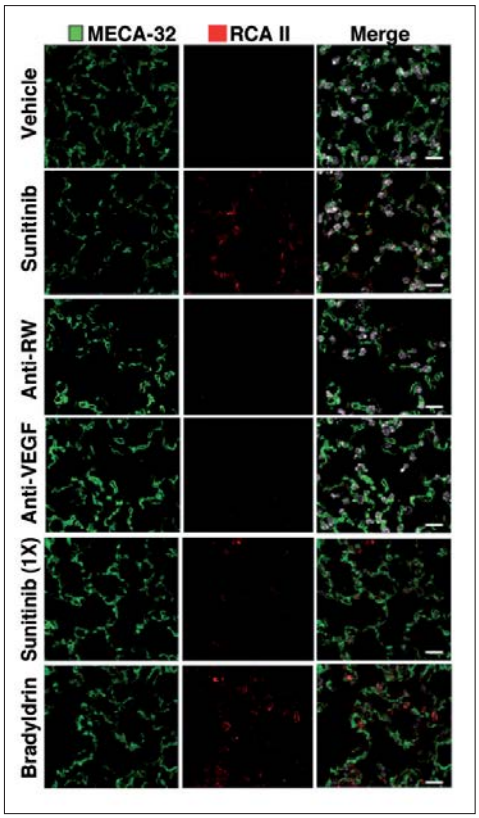

Figure 4
Elevated dosage of sunitinib compromises barrier function in the lung microvasculature.

This observation supports a model in which disruption of the microvascular barrier is the primary injury event following treatment with elevated sunitinib, which may subsequently elicit the observed downstream, pro-inflammatory effects.

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Deconstructing p53 pathways *in vivo* using mouse models

A report on a lecture by
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The tumor suppressor p53 is a transcriptional activator that can induce numerous target genes, but it also displays other biochemical activities. Analysis of p53 knock-in mouse strains expressing mutants altered in the first, second or both transcriptional activation domains (TAD) reveals that the TAD1 mutant p53^{25,26} is compromised for transactivation of most known p53 targets, but retains the ability to activate a few primarily novel target genes. By contrast, the TAD2 mutant p53^{53,54} retains wild type transactivation potential, and the quadruple mutant p53^{25,26,53,54} lacks transactivation activity altogether. Although p53^{25,26} cannot induce growth arrest or apoptosis in response to acute genotoxic stress, it suppresses the development of tumors in mouse models of non-small cell lung cancer, B and T-cell lymphomas and pancreatic cancer, indicating the clear distinction in p53 responses to acute DNA damage and oncogenic signaling. Comparing the gene expression profiles of cells expressing the different p53 variants generated a list of mostly novel p53-dependent genes that are likely to be important for tumor suppression. Most of these genes are direct p53 targets and encode proteins with tumor suppressor activity. ***Laura Attardi*** *addressed the importance of mitigating the deleterious p53-dependent side effects of DNA-damaging radiation and chemotherapies while preserving p53 tumor suppressor function.*

Data from human both sporadic and hereditary cancers have shown that p53 is a critical tumor suppressor. Mice lacking p53 develop cancer at 100% frequency. What's more, when p53 deficiency is in the background of other tumor models, it virtually always accelerates or enhances tumorigenesis. Mice are therefore useful models to understand the mechanism by which p53 functions.

p53 is also a cellular stress sensor. It responds to a plethora of diverse stress signals, including DNA damage, hypoxia, oncogene expression and nutrient starvation. These stresses cause p53 to activate any of several cell fates, such as a temporary cell cycle arrest, senescence or apoptosis. And all of these responses are thought to contribute to p53's activity as tumor suppressor.

p53 also has numerous physiological and pathological functions. For example, it plays roles in fertility and in skin pigmentation and tanning. In addition, it can have deleterious effects in an organism. For instance, it promotes some of the side effects of cancer therapies, and causes cell death associated with neurodegenerative disease and stroke. It's important to find ways to inhibit these pathological effects of p53, without compromising its beneficial ones.

In response to acute DNA damage, p53 drives cell cycle arrest and apoptosis. This is thought to be one of the ancestral functions of p53, as it's conserved down to lower eukaryotes such as worms and flies.

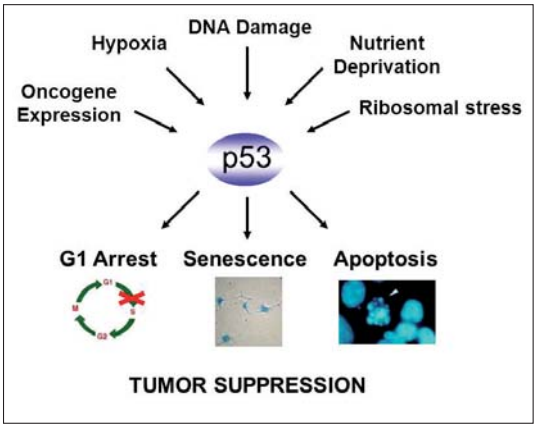

Figure 1
p53 Is a general cellular stress sensor.

The protein was also thought to have originally played a role in protecting the germline from genotoxic damage.

Downstream of oncogene expression, p53 promotes senescence and apoptosis as safeguards against tumor development. The mechanics of this activity are poorly understood, but this understanding is important clinically because it would be optimal to find ways to minimize the side effects of cancer therapies without perturbing p53's tumor suppression function.

p53 is a known transcriptional activator and, as such, has a DNA-binding domain, a transactivation domain (TAD) and an oligomerization domain. It binds to consensus sites in a variety of genes, and to mRNAs, lincRNAs and microRNAs to drive their activation.

What's perhaps less known is that p53 has a number of diverse biochemical activities. For example, it plays a role in transcriptional repression and in various other DNA-based processes, including replication, repair and recombination. It also has some cytoplasmic functions, the best characterized of which is its ability to perturb mitochondrial membrane integrity through its interaction with BCL-2 family members.

Independent effects:

A number of target genes downstream of p53 are known to be involved in cell cycle arrest, apoptosis and senescence programs. But strikingly, mice lacking these genes don't show a clear cancer predisposition. For example, mice lacking p21 are defective in the cell cycle arrest response to DNA damage, and those lacking PUMA have a faulty apoptosis response to DNA damage, but even mice lacking both p21 and PUMA do not develop cancer.

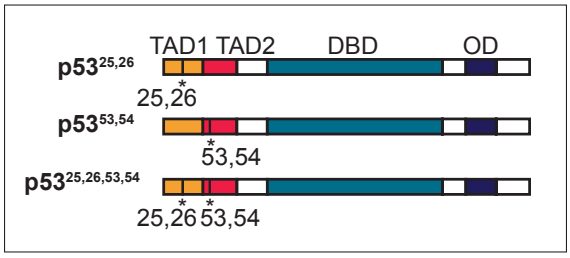

Figure 2
Defining the role of p53 transactivation function.

This begs the question, what is the role of transcriptional activation in tumor suppression? Are there new critical p53 target genes to be identified, or is transcriptional activation not relevant? To parse the target gene activation function of p53 from its transactivation-independent effects, a panel of knock-in mutant mice were made by introducing transcriptionally defective mutants in the p53 locus.

The p53 TAD is bipartite. The knock-in mutant mice have alterations in TAD1, TAD2 or both. Human p53 with mutations in residues 22 and 23 of the first TAD domain (corresponding to mouse residues 25 and 26) has been shown in reporter assays to be severely compromised in transactivation, but retains sequence-specific DNA binding activity. p53 protein with mutations in residues 53 and 54 in TAD2 is partially compromised for transactivation in reporter assays. A quadruple mutant with mutations in residues 25, 26, 53 and 54 is completely defective for transactivation.

These knock-in mutants are conditional, carrying a lox/stop/lox cassette, and adenoviral Cre is used to activate expression of the mutants. This is a tight system, with widespread expression of the p53 mutants.

When gene expression in p53 wild type cell lines is compared with expression in the mutants, surprisingly, the $p53^{53,54}$ mutant looks like the wild type. The quadruple mutant is reminiscent of a p53 knockout in that it does not transactivate targets. However, this mutant does bind DNA, as shown by chromatin immunoprecipitation. The most interesting and most informative mutant is $p53^{25,26}$, which, at the microarray level, looks intermediate between wild type p53 and p53 null cells[1].

To expand these observations, Northern blot analysis was used to examine several classical p53 target genes, and $p53^{53,54}$ again looks like wild type whereas the quadruple mutant looks like a p53 null. And $p53^{25,26}$ has

an important phenotype in that it is compromised for activation of most p53 target genes, but retains the ability to activate a select few, including BAX[2].

When mice are irradiated, p53[53,54] and wild type both show robust apoptosis in the 'radio-sensitive' organs — spleen, thymus and small intestine — as seen by TUNEL staining. By contrast, mutations in TAD1 abolish the ability of p53 to drive apoptosis, either alone or in combination with TAD2 mutations.

This finding suggests that p53's full transcriptional activation potential is critical for it to drive responses to acute DNA damage. This fits with what is known about some of p53's target genes: p21 is a critical cell cycle mediator of p53's ability to induce G1 arrest in response to DNA damage, whereas NOXA, PERP and PUMA play important roles in apoptosis downstream of DNA damage.

Dispensable function:

The mechanism of p53 action may be different downstream of different oncogenes or in different microenvironments. In the activated KRAS-driven lung tumor model, adenoviral Cre activates KRAS expression and drives the formation of non-small lung cancer. In the backdrop of p53 nullzygosity, there is progression of tumors.

At 12 weeks of age, like wild type p53, the p53[25, 26] TAD1 mutant surprisingly efficiently suppresses tumor development in the lung. So, despite its inability to activate most known p53 target genes, it's completely effective in suppressing cancer. The p53[53,54] mutant is also efficient at tumor suppression, which is perhaps unsurprising given its active transcriptional profile. With the quadruple mutant, there is a dramatic reversal of tumor suppressor activity, and robust tumors are observed in the lung.

These observations suggest that TAD1 and efficient transactivation of most p53 target genes are dispensable for tumor suppression[3]. Transactivation is important for tumor suppression, however, because when both TAD1 and TAD2 are mutated, tumor suppressor activity is largely lost.

p53 null mice also spontaneously develop primarily thymic lymphomas. By immunohistochemical staining, the Rosa 26 Cre-ER allele does not induce p53 in all cells in the thymus. The thymus has a mixed cell population of cells expressing the mutant, as well as some that fail to recombine the stop elements and therefore remain p53 null.

When these cohorts are treated with tamoxifen, p53 wild type mice are alive whereas the p53 null mice die very quickly. p53[25-26] mutant mice and the quadruple mutant mice both look indistinguishable from the p53 null mice, with identical kinetics of tumor formation.

However, when the tumors are analyzed for their p53 status, tumors in the p53[25-26] mutant mice never express p53[25-26], suggesting that tumor formation is incompatible with expression of this mutant. By contrast, the quadruple mutant mice have tumors expressing that mutant of p53.

These observations basically suggest again that, as in the lung cancer model, p53[25-26] is a tumor suppressor. The null cells therefore have a selective advantage and outgrow to form a tumor, whereas the quadruple mutant is inactive and cells derived from these mice can readily form tumors.

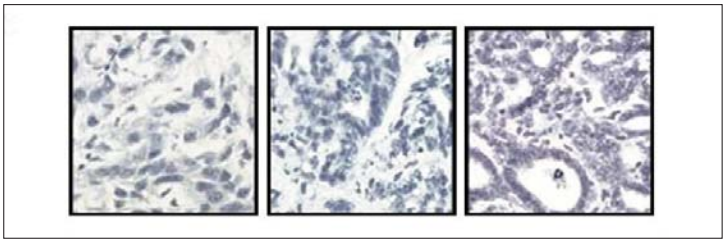

Figure 3
p53[25,26] suppresses tumor development.

The E-μ-MYC lymphoma model and activated KRAS-driven pancreatic cancers both show the same kind of pattern, where tumors form with expression of

the quadruple mutant, but not with expression of the $p53^{25-26}$ mutant. These data suggest that the $p53^{25-26}$ mutant is universally active as a tumor suppressor, whereas the quadruple mutant is not[4].

In summary, complete p53 transactivation, mediated by TAD1, is required for acute DNA damage responses *in vivo*. Complete p53 transactivation is dispensable for senescence and tumor suppression, however. Transactivation is critical for all p53 activity, as the quadruple mutant has loss of all activity.

The findings suggest that mechanisms by which p53 triggers responses to acute DNA damage and to oncogenic signals are different. TAD1 is prominent for DNA damage response, and either TAD1 or TAD2 can work in the setting of oncogenic stress.

Because of its selective ability to activate only a small set of p53 target genes efficiently, $p53^{25-26}$ can be used to identify those genes most important for tumor suppression.

Direct targets:

When wild type and p53 null mouse embryo fibroblasts are compared, more than 1,000 genes are differentially expressed. By defining genes activated efficiently in cells with tumor suppressor active genotypes (wild-type p53, $p53^{25-26}$, $p53^{53,54}$) relative to cells with tumor suppressor inactive genotypes (the quadruple mutant and p53 null) a more limited list of 130 differentially expressed genes can be generated.

By and large, the tumor suppression gene expression signature segregates human breast cancers with wild type p53 from those with p53 mutation pretty efficiently. Similarly, breast cancers that have higher expression of the gene set tend to have better survival than those with lower expression, indicating that this gene set is relevant in human cancer.

As an additional filtering step, the list of 130 genes can be narrowed down to those that are commonly down-regulated in mouse and human cancers, based on the EBI gene atlas. This generates a limited set of 14 genes that are potentially novel tumor suppressor targets of p53.

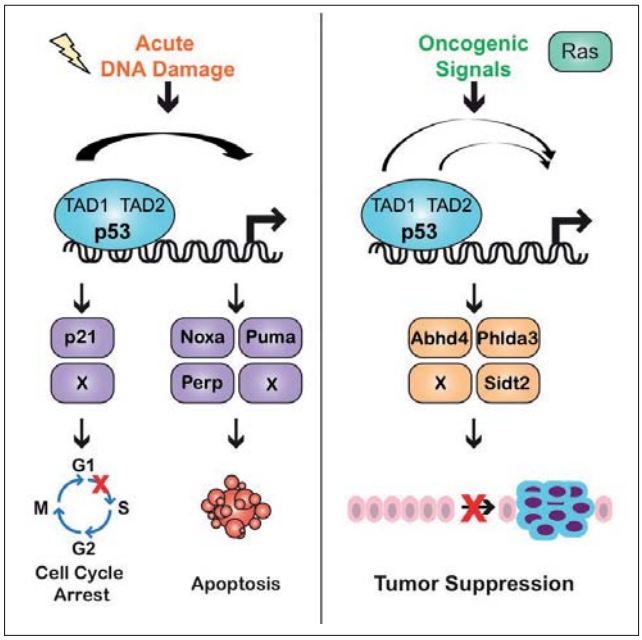

Figure 4
Tumor suppression-associated p53 targets fall into numerous functional categories.

These genes are always more highly expressed in wild type than in p53 null cases. Interestingly, only one of these, PHLDA3, is a known p53 target gene. The genes fall into three major functional categories, encoding proteins involved in regulating actin dynamics, cell signaling, and DNA repair.

All of these genes are p53-dependent, and the $p53^{25-26}$ mutant effectively activates them in MEFs and in lung cell lines. In fact, chromatin immunoprecipitation reveals that nearly all of the genes are direct p53 targets, and are therefore proximal to p53. And virtually all these genes are activated when human p53 is stimulated in fibroblasts.

To examine gene function, the target genes were introduced into HRAS p53-deficient cells. The genes were cloned

into expression vectors carrying an HA tag, and assessed for their ability to arrest the cell cycle, as measured by BrdU incorporation.

Compared with GFP as a negative control, wild type p53 robustly inhibits cell cycle progression. Of the target genes, a couple of genes have no effect on cell cycle progression, but several have a partial effect and inhibit BrdU labeling.

It makes sense that p53 targets might only partially recapitulate p53 function because a network of targets presumably collaborates for full p53 function. The data are encouraging because they suggest that these target genes have activities consistent with tumor suppression.

Knocking down p53 expression causes robust tumor growth when transformed MEFs are implanted into immunodeficient mice. Using shRNAs against the p53 targets shows that they enhance tumor growth to varying extents. None of them is as efficient as p53 knockdown, but again, that makes sense given that p53 activates a whole network of targets to induce tumor suppression.

Going back to the bigger list of 130 differentially expressed genes, 55 are genes activated by p53, and only a small handful are known p53 targets. There are a number of new genes that may be interesting to pursue.

Overall, these new tumor suppression-associated p53 targets fall into numerous functional categories, including classical cell cycle arrest, apoptosis, DNA repair, actin dynamics, cell signaling and metabolism.

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Epithelial stem cells and the epithelial-mesenchymal transition

A report on a lecture by
Robert A. Weinberg
Whitehead Institute for Biomedical Reasearch, MIT, Cambridge, USA

The progression of primary carcinomas to stages of invasion and metastasis involves complex changes whose molecular bases have been elusive. The acquisition of many of these phenotypes is associated with a cell-biological program termed the epithelial-mesenchymal transition (EMT). Once this program is activated, both normal and neoplastic epithelial cells acquire many attributes usually associated with high-grade malignancy, including motility, invasiveness, and a heightened resistance to apoptosis. This program is choreographed by a set of transcription factors normally expressed during embryogenesis and wound healing. Stromal cells release a spectrum of EMT-inducing signals, including TGF-β, canonical and non-canonical Wnts and prostaglandin E2, that act in concert to induce EMT. This initial paracrine induction of the EMT is followed by the expression of the same factors by mesenchymal cells, resulting in autocrine signaling that maintains the mesenchymal state. Forcing mammary epithelial cells through an EMT confers on them the additional trait of stemness. Mammary epithelial cell progenitors can spontaneously enter into the stem cell state without the forced expression of any of the EMT transcription factors, indicating that the interconversion of progenitors into stem cells and the reverse can both occur spontaneously. ***Robert A. Weinberg*** *suggested that transit-amplifying/progenitor cells are the direct targets of the mutations that drive multi-step tumor progression.*

The invasion-metastasis cascade is a complex process, and raises question of how cancer cells acquire the distinct capabilities that enable them to form metastases at distant sites. One possible solution to this is a cell biology program called the epithelial-mesenchymal transition (EMT).

This program enables epithelial carcinoma cells to become mesenchymal, and begin to invade into nearby stroma. Cells that have undergone EMT tend to be on the outer edges of islands of carcinoma cells, and have acquired expression of the mesechymal marker vimentin.

The EMT program is ancient, and was present in the ancestors of all modern metazoans. It can be choreographed by 6 to 8 different transcription factors, each of which is expressed at distinct stages of embryonic morphogenesis, and is also expressed transiently during wound healing.

EMT involves the acquisition of different cell-surface markers and cytoskeletal proteins. Comparing 5 different ways of inducing EMT reveals a common set of 320 genes that are up-regulated, and 304 genes down-regulated.

Figure 1
Twist is essential for 4T1 tumors to metastasize from the mammary gland to the lung.

Implicit in this is that cancer cells opportunistically appropriate this EMT program in order to acquire a whole series of attributes that are advantageous for invasion and metastasis.

Highly metastatic mouse breast cancer cells, if deprived of the expression of one of the EMT transcription factors, Twist, lose about 85% of their ability to metastasize to the lung. The residual metastases all continue to express Twist, indicating that Twist was not successfully shut down in all the primary tumor cells.

A primary cancer cell that has activated the EMT should be able to execute all of the steps involved in tumorigenesis, including extravasation, up to the point of creating micrometastases. However, the subsequent step of adaptation to the microenvironment of the distant tissue is beyond the purview of the EMT.

If true, it suggests that carcinoma cells from a primary tumor are already genetically equipped to metastasize — that they do not require additional mutations beyond those needed to create the primary tumor — and only require environmental signals to do so. This is a speculation at present, but a highly plausible one given the available data.

Stemness markers:

Cancer stem cells are relevant to the phenomenology of metastasis because, defined operationally for their tumor-initiating capability, they should be qualified to seed metastases. Conversely, the other cells in the tumor should not be qualified.

Two cell-surface markers, CD24 and CD44, can be used to isolate experimentally immortalized human breast cancer cells. $CD44^{lo}CD24^{hi}$ cells marks the majority population of non-stem cells, and$CD44^{hi}CD24^{lo}$ marks the minority population of stem cells.

When the EMT-inducing transcription factors Snail or Twist are expressed in the non-stem cells, the cells migrate *en masse* from the non-stem cell to the stem cell position. This is accompanied by a change in their morphology in monolayer culture, from a cobblestone appearance to a more mesenchymal morphology. This suggests that there is a connection between EMT and stemness[1].

By RT-PCR, the putative stem cells express $1/200^{th}$ of the epithelial marker E-cadherin, the keystone of the epithelial state. And they express between 80-120 fold higher levels of the mesenchymal markers N-cadherin, vimentin and fibronectin. They also overexpress the EMT-inducing transcription factors, FOXC2, SIP1, Twist and Snail, by factors of between 20 and 120.

What's more, the $CD44^{hi}CD24^{lo}$ cells can produce mammospheres, surrogate assays *in vitro* for mammary stem cells (MSCs), whereas the $CD44^{lo}CD24^{hi}$ cells look more epithelial in 2D culture and fail to form mammospheres.

There's a close alliance between $CD44^{hi}CD24^{lo}$ cells in normal mammary glands and $CD44^{hi}CD24^{lo}$ cells in breast cancers, suggesting that the stem cell program that operates in the normal mammary gland is similar to the one that operates in breast cancers.

If that's so, EMT is doubly dangerous for cancer patients because it enables the ability to disseminate and self-renew that are essential for seeding metastatic outgrowths.

In the decades-old mammary fat pad implantation assay, as few as a single MSC can be implanted into a cleared mammary stromal fat pad and generate a mammary ductal tree. Because of the availability of this *in vivo* reconstitution assay, the mammary gland is a robust model system for studying epithelial stem cells.

A different set of cell-surface markers shows that the putative stem cells from this compartment indeed make mammary ductal trees upon implantation and look mesenchymal. By contrast, the non-stem cells

fail to engraft when implanted into the mammary fat pad.

Relative to the non-stem cells, the cells in the stem cell fraction have a 36-fold increased expression of Slug, an EMT-inducing transcription factor. Slug is specifically expressed by basal cells, which contain the putative MSCs. In a set of these putative MSCs isolated by fluorescence activated cell sorting (FACS), Slug is overexpressed by a factor of about 120 relative to the non-stem cells.

When the YFP fluorescence marker is knocked into the endogenous Slug locus, those mammary epithelial cells (MECs) that express high levels of YFP have about a 25-fold higher ability to engraft into a mammary stromal fat pad, strengthening the connection between Slug expression and stemness.

Transient exposure:

If MECs that transiently express Slug are mixed with cells that express an empty construct, and then injected into the mammary stromal fat pad, both sets of cells initially form equal numbers of stem cells. However, within a week, the cells that had experienced Slug expression are about twice as abundant. By 6-7 weeks later, those cells are vastly more capable of generating a mammary ductal tree than are the cells with the empty construct.

This suggests that a history of having experienced Slug expression imparts to these MECs the ability to engraft in a mammary stromal fat pad.

If MECs are deprived of Slug, they lose their mammosphere-forming ability and thus, putatively, their stem cell ability. This suggests that Slug is necessary for the maintenance of the MSC state and it's sufficient to induce it from non-stem cells. But in fact this conclusion is wrong.

When the Slug construct is put into basal cells in the MECs, which have their own naturally existing stem cell compartment, there is hardly any increase. But if Slug is introduced into luminal progenitors separated on FACS, they exhibit a large increase in their mammosphere formation.

However, expressing Slug in mature, fully differentiated MECs does not give them the ability to form mammospheres. This indicates that Slug, on its own, is not sufficient to convert fully differentiated luminal cells into stem cells.

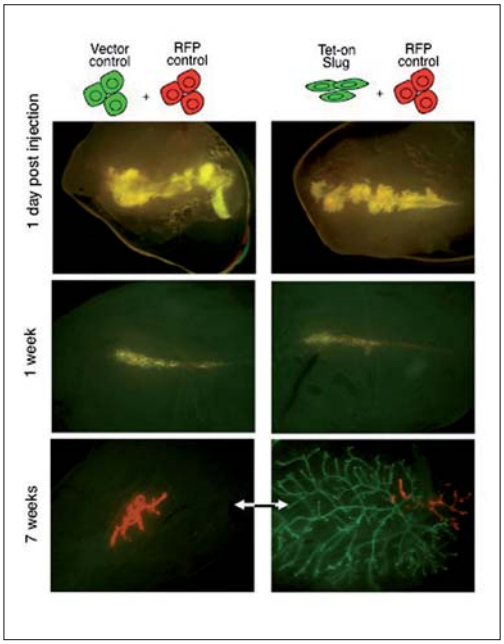

Figure 2
Previous exposure to Slug greatly increases gland-reconstituting activity.

The SOX9 transcription factor, when co-expressed transiently with Slug, induces mammosphere formation. As many as 10,000 control mouse MECs fail to engraft, but 1/100th as many cells exposed transiently to Slug and SOX9 during early implantation generate a mammary ductal tree.

Taken together, these observations suggest that the transient expression of these two transcription factors can force a differentiated MEC all the way up to the stem cell state. Luminal progenitors, which endogenously express their own Slug and SOX9, can be induced into the stem cell state. Myoepithelial progenitors naturally express Slug and only require SOX9 to become stem cells.

If either Slug or SOX9 is knocked down, there is a profound decrease in the tumor-forming ability of MDA-MB-231 cells (see Massagué, page 53), suggesting that lessons learned from the normal mouse mammary gland are transferable to human breast cancer cells.

Spontaneous conversion:

It's possible that the passage through EMT is the main entrance to the epithelial stem cell state. Lessons learned from the mammary gland may be generalizable to non-MECs, albeit with different sets of EMT-inducing transcription factors.

When $CD44^{lo}$ non-stem cells are propagated in culture, over time, they spontaneously generate $CD44^{hi}$ stem-like cells. The outgrowth of these stem-like cells is in contrast to the fact that the mesenchymal cells proliferate more slowly than do the non-stem cells, indicating that this is a conversion rather than an outgrowth of more rapidly growing sub-clones[2].

In fact, if a highly purified fraction of $CD44^{lo}$ cells is put into a mouse, about 20% of the cells are mesenchymal just 2 weeks later. This has implications for the canonical cancer stem cell model, which predicts that the cell-of-origin of a tumor is a normal stem cell.

This canonical scheme cannot be correct, because the sizes of the target cell populations are small, requiring an unrealistically high mutation rate per cell generation. Also, weakly mitotic populations do not readily spawn mutants. Instead, the reality is more likely to be a scheme in which there is a flux between stem- and non stem-like compartments.

The process of multi-step tumorigenesis is instead likely to involve transit amplifying cells, which are more numerous, and have the vast share of mitotic activity.

One other consequence of this is that cancer stem cells have a higher degree of resistance to therapeutic agents, allowing them to survive and repopulate a tumor once therapy has lifted. Epithelial cells are more sensitive to therapy, and are killed at lower concentrations of drugs such as doxorubicin and paclitaxel, than are mesenchymal cells.

However, other compounds such as salinomycin have the opposite effect. If cells are treated with paclitaxel, the proportion of those in the stem cell position jumps from 5% to 70%. Conversely, if they are treated with salinomycin, only 0.2% of cells that survive are in the stem cell position. This suggests that salinomycin selectively kills cancer stem cells[3].

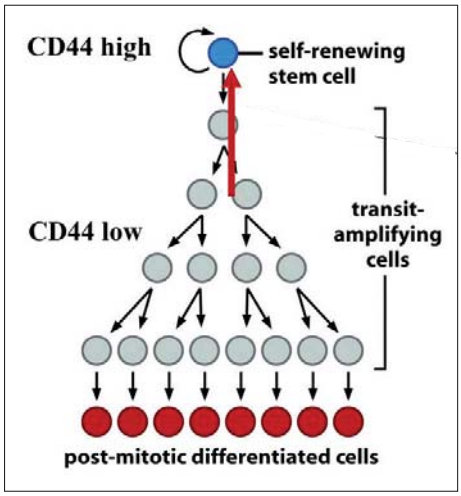

Figure 3
Differentiated epithelial cells can spontaneously dedifferentiate into stem-like cells.

If non-stem cells are capable of spontaneously turning into stem cells, however, both compartments need to be targeted.

Strong signals:

Cells that spontaneously go into the mesenchemyal state without any kind of experimental stimulus maintain their residence in the mesenchymal state via autocrine signaling. The levels of several classes of factors are changed dramatically in the supernatant medium of these mesenchymal cells.

For example, canonical Wnt signals are down, and inhibitors of canonical Wnt signaling, DKK1 and SFRP1, are down enormously. Non-canonical Wnt signals are up. BMPs, which are potent antagonists of TGF-β, are down by more than a factor of two. And Gremlin and chordin, which are potent antagonists of BMPs, in turn are significantly up.

For example, spontaneously mesenchymal cells down-regulate SFRP1, a potent Wnt inhibitor, by more than a factor of 100, and down-regulate DKK1 by a factor of 6.

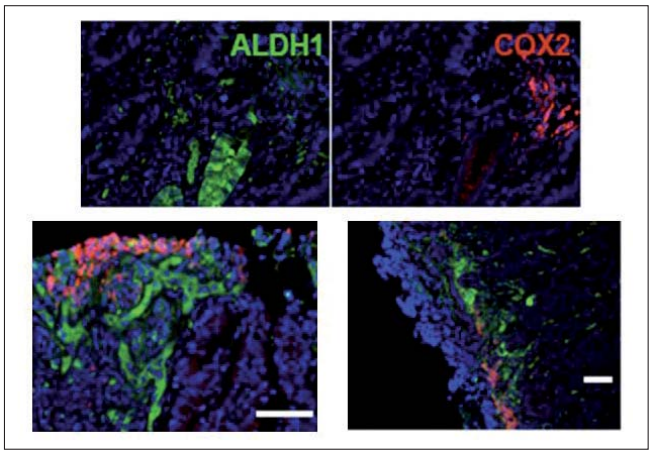

Figure 4
COX2-positive cells are often close to cells that are ALDH1-positive, which is a marker of stemness.

If Twist cells or the spontaneously mesenchymal cells are treated with BMP4, they lose their mammosphere-forming potential. This suggests the importance of ongoing autocrine signaling in maintaining these states.

In summary, residence in the stem cell and mesenchymal state seems to be maintained by a series of autocrine loops, and this may be generalizable to other kinds of epithelial stem cells[4]. It's also the case for cells that express an EMT-inducing transcription factor.

There are different sets of heterotypic signals that can induce the EMT. Some colorectal carcinoma cells, when they're mixed together in culture with mesenchymal stem cells, show an enormous up-regulation of prostaglandin production, but others do not.

One can mimic this induction by, for example, adding recombinant interleukin-1 (IL-1) to mesenchymal stem cells, which are recruited in large numbers to the stroma of carcinomas. When IL-1 is added to mesenchymal stem cells, it induces a 60-fold increase in prostaglandin production by the mesenchymal stem cells.

Cells that fail to release IL-1 on their own fail to elicit prostaglandin E2 production in co-cultured mesenchymal stem cells. Knockdown of cyclooxygenase2 (COX2) in the mesenchymal stem cells reduces prostaglandin E2 production.

The interactions that result in the release of prostaglandin E2 also release four other factors: GRO-α, IL-8, IL-6 and RANTES. Of these, RANTES requires cell-to-cell contact between carcinoma cells and the mesenchymal cells. The other three can be elicited by percolating through transwell assays.

Co-cultured MSCs lead to profound invasiveness on the part of cancer cells, which otherwise have a rather encapsulated appearance. Co-culture also leads to strong EMT, as seen by the profound induction of fibronectin and vimentin, down-regulation of E-cadherin, and up-regulation of Snail. There is also a 2- to 3-fold increase in the number of tumor-initiating cells.

Cells that are COX2-positive are often quite close to cells that are aldehyde1 positive, which is a marker of stemness. Interestingly, the prostaglandin from MSCs activates and converges on the Wnt pathway.

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Metastasis seed pre-selection by the primary tumor stroma

A report on a lecture by
Joan Massagué
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Invasive tumors release large numbers of cancer cells into the circulation, but only a small proportion of these cells survive in, and ultimately overtake, distant organs. The likelihood of metastasis in many types of cancer can be predicted from the overall gene expression profile of primary tumors. The reasons for the presence of such 'metastasis signatures' in a primary tumor have remained a mystery. New work on the biology of disseminated micrometastases posits that, when the stroma of a breast tumor resembles that of a distant organ, the tumor selects for cancer cells that are predisposed to survive in that organ. Heterogeneous mammary tumor populations growing in culture or in vivo in an environment that is rich in cancer-associated fibroblasts (CAFs) become 'pre-selected' — skewed towards a preponderance of clones with a superior ability to survive and proliferate on the CAF-derived factors CXCL12 and IGF1. This ability is supported by SRC, a known enhancer of cancer cell survival in the CXCL12/IGF1-rich microenvironment of the bone marrow. The phenomenon of metastasis seed pre-selection has significant implications for the evolution of metastatic traits and the dynamic interplay between a primary tumor and its distant metastases. Clinically, these insights imply that breast cancer cases with a CAF-rich stroma could benefit from chemokine receptor blockers or SRC inhibitors as adjuvant therapies. ***Joan Massagué*** *argued that therapies that target the stromal signals that prevail in a primary tumor could specifically eradicate micrometastatic seeds disseminated by the tumor and thus prevent metastasis.*

A tumor that will be diagnosed in 6 months has already acquired driver mutations, secondary mutations, alterations and heterogeneity. It has engaged in invasion and angiogenesis, and is releasing cells to the bloodstream, every hour of every day.

A minority of these cells manages to infiltrate distant tissues and take residence there. This is something that can be and is being studied extensively in terms of the steps of metastasis: invasion, cell motility and epithelial-mesenchymal transition (see Weinberg, page 47), among others. These are critical steps in the biology of metastasis.

However, for many patients, this is all part of the past at the moment the tumor is diagnosed and surgically removed. What the patients and their oncologists are left to deal with is disseminated, invisible disease. Even if there is no apparent lymph node involvement, many of these patients will relapse.

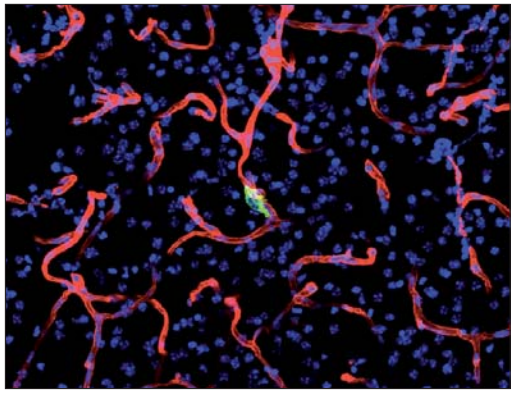


Figure 1
Biology of disseminated disease.

The biology of latent metastasis is practically unknown compared with the level of knowledge about the prior steps (cancer cell dissemination) and final steps of overt metastasis. However, many experimental models and clinical studies show that only a tiny minority of cells that leave the tumor manage to survive and to stay fit after reaching a distant organ.

These cells may therefore have qualities that are relevant for their survival and fitness as latent micrometastatic colonies, even though these qualities may not be so relevant to their survival in a primary tumor or later on, once they form overt metastases.

Understanding how latent metastatic cells deal with these liabilities, and identifying those qualities may create a window of opportunity that has not been explored as a potential means of preventing metastasis.

There are barriers to studying latent metastasis, however. For example, there are misconceptions about the biology of these cells. It is not clear whether they are they dormant, whether they are stem cells, or they engage in the angiogenic process. There is also a lack of models and technologies to interrogate these cells, and a prejudice that trials on metastasis that is latent are impractical.

Analyses of tumors and their metastases have not found mutations that are specifically involved in metastasis. Nonetheless, some digging has begun to reveal biology that's quite provocative. For example, in a brain metastasis model, whether breast to brain or lung to brain, it's clear that the cells that seed the metastasis know certain things. They know to hug the vessel that they just egressed from, and they proliferate around the vessels to initiate micrometastases.

It is also useful to keep in mind that metastasis can be a bidirectional, highly dynamic process, at least experimentally. Cancer cells in the circulation, or even cancer cells that have resided for a while at distant sites and go back as circulating tumor cells (CTC), find their way to the primary tumor if it still exists[1].

Seeder cells:

The seeder cells that tumors scoop out from the circulation are enriched for metastatic ability. These cells that infiltrate tumors are the 'best of the worst'. The seeding doesn't overtake the tumor, but these aggressive clones expand more efficiently in the tumor of origin than at distant, hostile tissues. The seeder cells that the tumors scoop out from the circulation are enriched for metastatic ability.

Thus, the primary tumor may act as an amplifier of the most aggressive clones. Even after therapy that targets the tumor, perhaps even after surgical removal, the inflamed stroma may welcome CTCs, expand them, breed local relapse and perhaps even relaunch to distant sites.

Of six pathways analyzed in primary breast tumors for their link to metastatic relapse, only one, the SRC pathway, appears to prime breast cancer cells for long-term survival as latent metastasis in the bone marrow[2]. Biochemical analysis reveals that SRC strongly buttresses the ability of CXCR4 or EGFR to activate the PI3K-AKT cell survival pathway. In the bone marrow, there is a natural abundance of CXCL12 and of IGFs compared with other tissues.

A cell that arrives with a high level of SRC activity in the bone marrow has a higher chance of survival. This is a quality is not particularly critical in the primary tumor, and will no longer be precious to the cell after it has managed to become an overtly aggressive metastatic entity. But, it is vital during the challenges that cancer cells face after infiltrating the bone marrow.

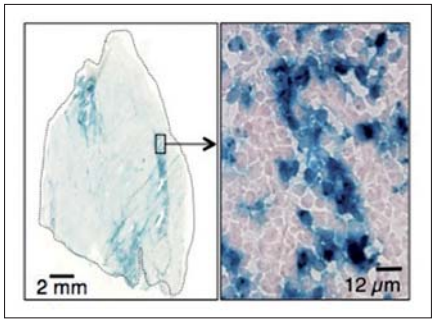


Figure 2
Self-seeding amplifies aggressive clones.

Targeting these cells with inhibitors of SRC activity in mice helps deplete the bone marrow of latent metastasis. For example, if SRC is blocked with an RNAi or treated with an SRC kinase inhibitor, there is a 10-fold drop in the ability to recover micrometastatic cells from the bone marrow of these mice. If cells are allowed to form overt metastases before the initiation of treatment with SRC inhibitor, they are no longer vulnerable to the loss of SRC.

Positive origins:

The cells that are primed to have a chance of being disseminated are somehow selected for in a primary tumor. They have abilities that do not score in primary tumor growth, yet they accumulate there to an extent that can be detected, for example with the presence of a metastasis gene expression signature.

For example, in two of the three major types of breast cancer, the estrogen receptor alpha (ER-α) forms a complex with SRC, and this incites the transcription-independent signaling activity supporting cell survival and proliferation.

Not surprisingly, 90% of ER+ tumors are SRC-positive. HER2/HER3 also interact biochemically with SRC and they help each other achieve high activity. Here again, about 50% of HER2-positive tumors score as SRC positive. About 25% of triple-negative tumors are also SRC positive.

All these SRC-positive tumors have a predilection for metastasis in the bone marrow. SRC confers the cancer cells with a superior ability to survive on CXCL12 and IGF1 that naturally occur in the bone marrow. (CXCL12 is an important factor in hematopoietic stem cell niches).

In gene expression profiles of primary tumors that are SRC-positive, 44 genes are co-expressed with it. Remarkably, these genes include CXCL12 and IGF1. CXCL12 and IGF1 are strongly expressed in CAFs. Furthermore, tumors that have high levels of SRC are rich in CAFs, and so it fits that the tumors are also rich in these cytokines.

In 100 independent clones from patient-derived cell lines, there is a nearly 20-fold dynamic range in the distribution of pY416 SRC, which represents SRC activity. In restricted culture conditions with low serum supplemented with CXCL12 and IGF1, the clones that survive are those with high levels of SRC activity.

When tested for bone metastatic activity, these clones are about one log better at populating the bone marrow than are the control cells. *In vivo*, in this case by placing cancer cells in the mammary fat pad and allowing them to form tumors, admixing them with mesenchymal stem cells skews the population towards a high SRC content and high bone metastatic activity. CXCR4 inhibitors and IGFR inhibitors prevent this enrichment of bone metastatic activity.

The conclusion of this work is that breast tumors with a CAF-rich stroma skew the cancer cell population towards SRC-positive clones, which thrive in CXCL12 and IGF1. As a result, the cancer cell population is also more likely to successfully seed and survive in the bone marrow, owing to the similar presence of CXCL12 and IGF1 in this microenvironment.

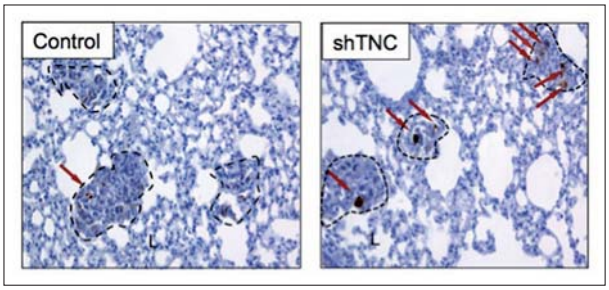


Figure 3
TNC and VCAM-1: fitness of metastasis-initiating cells.

Priming niches:

The alternative approach to studying metastasis is to interrogate end products — that is, the cells that progress to an overt metastatic state — using xenografts, gene expression profile, microRNA expression profile and, given the availability of inexpensive sequencing, mutational analysis.

The aim in this approach is to identify genes and functions associated with the ability of these cells to form overt metastases[3]. For example, in the bone, this led to identification of genes that are crucial for engaging the osteoclasts for osteolytic metastasis. But it may also pinpoint genes important to these cells during the

entire time they have to infiltrate, reside, and stay fit in the organ for years.

Pleural fluid from patients with advanced disease contain discrete, defined metastatic entities, with organ-specific ability, from which it is possible to identify genes and microRNAs.

Focusing on lung metastasis, 54 genes score as being specifically associated with the ability of cells to form metastases. Of these 18 comprise a lung metastasis signature that is associated with a high rate of lung relapse[4]. Among these genes, there are some that are specifically important for passing from the circulation into the distant tissue, in this case lung. Two of those genes are Tenascin-C and VCAM1. Knocking down either of these genes decreases the ability of these cells to enter the bloodstream and seed the lungs by 5- to 10-fold. These genes have nothing to do with the ability to extravasate, only with the fitness of the micrometastases installed in the lungs.

Tenascin-C is an extracellular matrix protein with a remarkable distribution. It's abundant in connective tissue, but not elsewhere, except in stem cell niches and in tumors. It interacts with cell-surface components such as integrins, and with extracellular matrix proteins, including periostin and fibronectin.

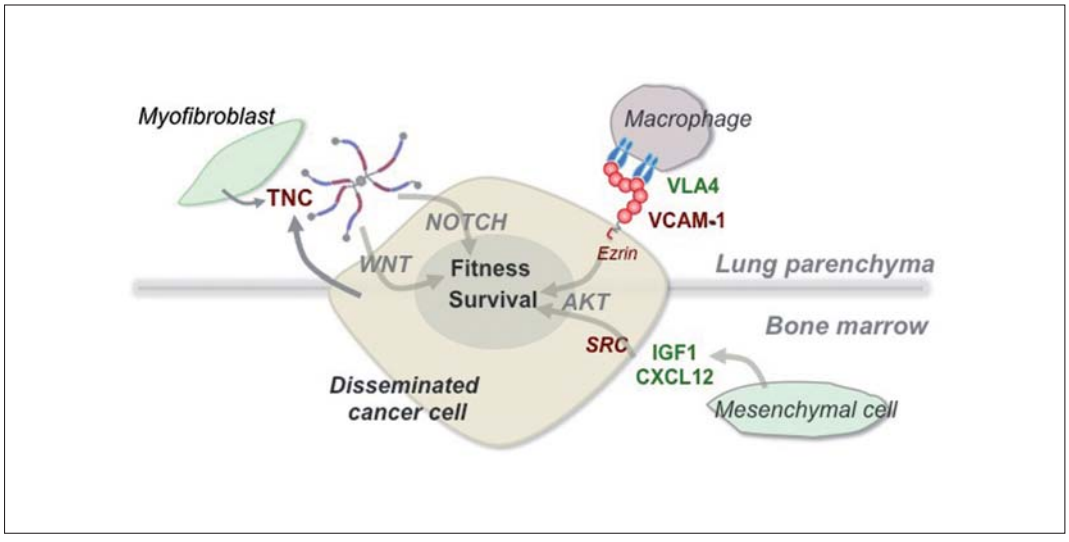
Tenascin-C has a remarkable molecular architecture. It is a hexamer with disulfide bonds at the N-terminus, and looks like it's built to coordinate things between the cell surface and the matrix.

In tumors, Tenascin-C accumulates at the invasive front, where there is profuse angiogenesis as well as residence of presumptive tumor-initiating slices. At the level of micrometastases, the entire small lesion stains with Tenascin-C, but as the lesion grows, the protein is relegated to the invasive front.

Human lung metastases that are rich in Tenascin-C correspond to cases that progress very rapidly. Those that have less of the protein have a more sluggish disease course. Oncospheres of cells derived from the fluids of patients also show an enrichment for Tenascin-C, indicating an association with the tumor-initiating subpopulation in these cells. As a consequence of Tenascin-C knockdown, the growth of primary and secondary oncospheres is severely compromised, as is micrometastatic progression.

Tumor-initiating cells have two classes of markers and mediators of the stem progenitor phenotype: Those that are the real enforcers of the stem cell phenotype, such as Nanog and SOX2, and those that are components of the Wnt and Notch pathways.

Figure 4
At the metastatic niche: cues, readers and pathways.



When Tenascin-C is knocked down, the cell still expresses the enforcers of the stem progenitor state, but loses the expression of components that are important mediators and amplifiers of the Wnt and Notch pathways. This cell remains a tumor-initiating cell by molecular phenotype, but is a doomed one.

In conclusion, metastasis-initiating cells that express Tenascin-C give themselves a jumpstart at forming a niche that they need for the probability of survival. If Tenascin-C is taken away from micrometastases, those cells have a reduced chance of survival.

Boosting survival:

VCAM1 is expressed in a number of tissues but most prominently in the vascular endothelium. It allows leukocytes, via VLA4 integrin, to adhere to it for translocation from bloodstream to tissue. It is an important target of therapy for a number of diseases in which there is an excess of infiltration by leukocytes of tissue.

In breast cancer cells, VCAM1 has little to do with translocation, but rather allows the cels to derive a benefit from the presence in the stroma of macrophages and a other leukocytes. On VLA4 binding, the short cytoplasmic tail of VCAM1 associates with the protein ezrin and induces its tyrosine phosphorylation, which makes ezrin a docking site for both PI3K and AKT. This enhances the ability of this AKT pathway to signal, increasing the probability of the cell's survival.

The interactions between VCAM and VLA4 are targetable because there are VLA4 blockers, and preclinical inhibitors of VCAM.

To summarize, a new search for mediators of disseminated cancer cell survival is quickly yielding leads of functional and clinical relevance. Therefore, there is hope that deliberately targeting the mechanisms of latent metastases will open the possibility of better eliminating latent disease in order to prevent overt metastasis.

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PART III: Insights from mouse models of various cancers

Glioblastoma: A novel mouse model

A report on a lecture by
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Salk Institute for Biological Studies, La Jolla, USA

Mouse models have greatly helped advance cancer research, but they typically rely on introducing oncogenes into large numbers of cells, which doesn't recapitulate the true nature of oncogenesis. Lentiviral vectors can be used to introduce oncogenes into a small number of cells and generate mouse models of human cancer in a cell-specific manner. This approach has been used to study glioblastoma multiforme (GBM), a lethal brain cancer with a high recurrence rate. Experiments with GFAP or Nestin-Cre mice generate tumors that are invasive, hypoxic, angiogenic, genotypically heterogeneous and resistant to therapy — all the characteristics of human GBM. These and other results suggest that terminally differentiated cells upon insertion of oncogenes have the ability to either reprogram or de-differentiate to become pluripotent and generate cells of all lineages. The experiments suggest that GBM originates via reprogramming of differentiated cells, which may explain why they're so lethal, and why they rapidly regrow after surgery. Both mouse and human GBM tumor cells can transdifferentiate to functional endothelial cells lining the blood vessels. ***Inder M. Verma*** *described the lentiviral vector approach and suggested that it can be used to make mouse models of other human tumors, including lung, pancreas and prostate.*

Mouse models have been extremely successful in advancing cancer biology. However, human tumors don't start with 100,000 tumor cells injected into them. They presumably start with a single cell, which then acquires more mutations and eventually becomes a tumor cell.

In transgenic mice, the transgene is activated in every cell in that tissue, but this is not what happens in cancer. The same is true for knockout mice: Cancer doesn't begin with a gene knocked out in every cell in the body.

Ideally, an oncogene or a tumor suppressor would be introduced into a single cell. Viral vectors, such as a lentiviral one, can introduce a gene directly into a single cell surrounded by normal cells in an immunocompetent mouse.

This approach has been used in glioblastoma multiforme (GBM), largely because of previous experience with gene therapy in various domains of the brain, and because GBM tumors are almost entirely localized within the brain.

GBM is the most malignant of the

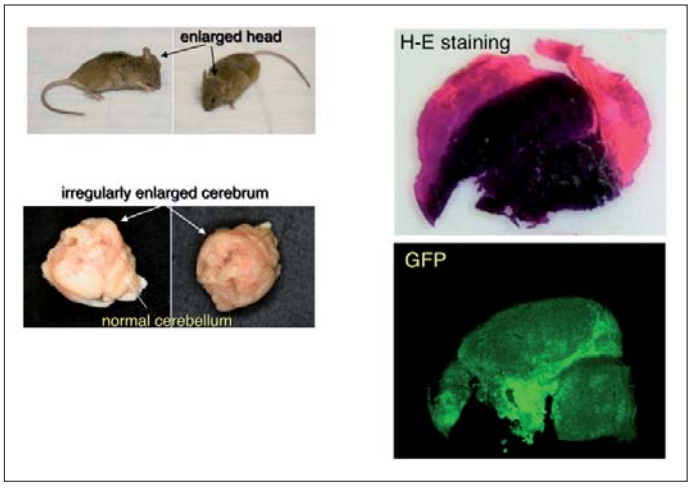

Figure 1
H-RAS and AKT-induced tumor.

primary brain tumors and is almost always fatal. Nearly all patients die within a year, even with full treatments including surgery, radiotherapy and chemotherapy.

The genetics of GBM has been pretty well defined by The Cancer Genome Atlas and others. Although a number of mutations have been found, they can be placed in three pathways: RAS/PI3K pathway, p53 and RB. In fact, nearly 74% of GBMs harbor mutations in all three pathways, confirming that alterations in them are core requirements for GBM formation.

Lentivirus is the vector of choice because the brain has a large number of non-dividing cells, and only lentiviral vectors can transfuse non-dividing cells and stay integrated. By contrast, retroviral vectors require cells to divide. The gene of interest, in this case RAS, is floxed, and is expressed in the presence of Cre[1]. This pTomo RasV12 lentivirus is directly introduced into the mouse brain by stereotactic injection, and linked to GFP so that the infection can be tracked.

This is a pretty tight system, and the gene can be precisely introduced in the location of choice and in the cell type of interest, depending on the expression of the Cre in that cell type. The adult brain contains different types of cells in different regions. For example, neural progenitor cells are known to exist mainly in the subventricular zone (SVZ) or hippocampal subgranular zone. In general, the gene is introduced only in very small number of cells (about 20 to 50 cells).

Clear characteristics:

The mice develop big heads and become lethargic. They also develop GFP-positive tumors that show many of the same histological properties of human GBM: high cellular density, high vascular proliferation, necrosis and pseudopalisading, cells rapidly migrating from one site to others. Confocal images reveal that the borders of the tumor are relatively clear but the tumor cells sometimes infiltrate into normal tissues.

However, the tumors do not have nuclear pleomorphism, which is a characteristic of human GBMs. They also don't have high mitotic activity, unlike human GBMs, which rapidly regrow within a couple of days after surgery.

Human tumors often contain p53 mutations. If RAS and AKT are introduced into GFAP-Cre/p53$^{+/-}$ mice, these mice also develop tumors. However, in this case, the tumors do display the high mitotic activity and nuclear pleomorphism that are characteristic of human GBM.

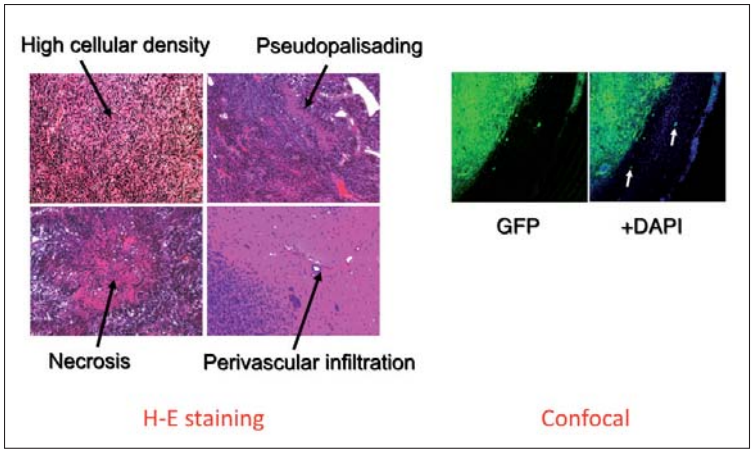

Figure 2
Invasion of the tumor cells into the normal tissues.

Crossing mice takes a lot of time, so an alternative is to use sip53, which generates tumors much faster. These tumors are highly proliferative, highly angiogenic and infiltrative. They also show high cellular density and nuclear pleomorphism.

Viral vectors can be used to look at dozens of genes, and determine which genes are drivers and which passengers. For example,

some vectors can contain up to 4 or 5 transgenes and at least 2 siRNAs.

The early experiments were all done introducing the vector in the hippocampus, but GBM tumors originate in different parts of the brain, including the SVZ. The tumors not only have glial cells marked by GFAP, they have neuronal cells as marked by TUJ1, oligodendrocytes and progenitor cells.

If the vector is injected into Nestin-Cre mice, it generates tumors from progenitor cells rather than glial cells. The tumors that develop have all of the properties of human GBM. They are invasive, hypoxic, angiogenic, genotypically heterogeneous, resistant to therapy, and change over time.

One topic of big discussion in the field is whether the cell-of-origin already exists, and which genes must be introduced to set it on its oncogenic path. For example, if the genes are introduced in the hippocampus, cortex, SVZ or striatum, the mice always develop tumors within 6 to 8 weeks.

Synapsin is expressed in neurons, and CAMPK2 specifically in excitatory neurons, which are clearly differentiated. Transducing neurons with the vector and synapsin-Cre or CAMPK2-Cre generates tumors.

About 20% of all GBMs that have been sequenced have mutations in NF1 and p53. If shNF1-shp53 is introduced in the hippocampus, the tumors originate but they take much longer.

Stem cells:

GBMs typically regrow soon after surgical excision. If just 10 cells of that tumor are taken out and put into the hippocampus of either immunodeficient mice or immune competent mice, 4 of 5 mice develop tumors, whereas control mice injected with normal neural stem cells do not.

When the tumor cells are cultured *in vitro*, GFP-positive tumor cells form neurosphere-like structures that are positive for Nestin and negative for GFAP. In various conditions in culture, the tumor cells differentiate to all lineages — neuronal, glial and oligodendrocytes.

In collaboration with people at the University of California, San Diego, a new technique has been developed to study how these tumors grow, what transitions of genes they go through, what the nature is of tumor cells that are changing, and more importantly, where the tumor cells migrate.

In this approach, GFAP-Cre mice are injected with RAS-shp53 vector and 2 weeks later, the 200 or so cells that are GFP-positive are closely monitored. The cells are s100 and GFAP-positive, which are the markers for glial cells, and negative for Nestin and SOX2, which are markers for stem cells.

By high-resolution large-scale mosaic imaging of the tumor brain and microenvironment, after 2 weeks, there is a GFP-GFAP overlap. By 5 weeks, as things start to go wrong, GFAP starts to disappear and Nestin makes its appearance. This indicates that terminally differentiated glial cells, upon transduction with the oncogene, are transdifferentiating into Nestin-positive stem cells.

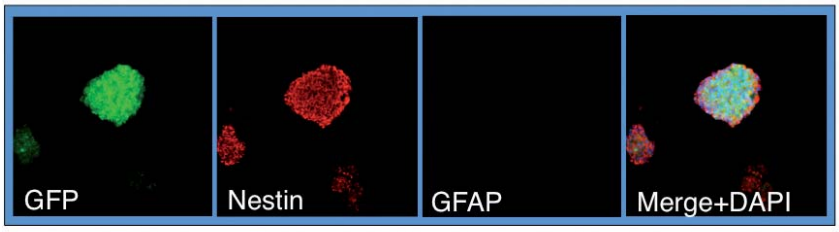

Figure 3
Neurosphere-like structure in EGF+ FGF+ heparin-containing medium.

If the GFAP-positive glial cells are put back into a mouse brain, they generate tumors in NF1 or p53 knockout mice. So these glial cells go on to become progenitors, and then neurons. The same sort of experiment can be done with oligodendrocytes and neurons.

For example, synapsin-Cre mice make tumors that are MAP2-positive, meaning they have neurons, and GFAP-negative. When these are put back into the animals, they make tumors that are GFAP-positive, Nestin-positive, and TUJ1-positive, suggesting that the transduced SynI-Cre derived neurons reprogram to a progenitor or stem cell state.

These cells form primary and secondary neurospheres in culture, which express the progenitor markers Nestin and SOX2. And they give rise to GBM tumors when transplanted into the brain of NOD-SCID mice.

Polycomb group (PcG) genes are epigenetic gene silencers that preserve transcription patterns to maintain cell identity, a function clearly compatible with a role in self-renewal. When the transduced cells revert to the stem cell state, BMI1, a member of the PcG family, becomes an important component.

In fact, in the presence of siRNA to BMI, tumors do not form. BMI1 has a profound effect on neural stem cells, reducing forebrain SVZ neurosphere frequency by 80% at 30 days after birth.

To make induced pluripotent stem cells, certain transcription factors are added to normal cells, such as blood cells or liver cells or fibroblasts. Adding the same factors to glial cells also generates iPS cells. However, *in vivo* in the brain, in the presence of sip53, they make tumors and can only differentiate into neurons.

Together these results suggest that a terminally differentiated cell upon oncogenic insult has the ability to reprogram and de-differentiate to become a stem cell. It can continue to replicate itself and lead to different lineages, each one then going on to become a tumor cell. When a GBM is surgically removed, every cell left behind has the ability to go on and become a new tumor cell because it's already programmed to be a stem cell.

How similar is this to the human situation? That is difficult to say because the experiments are with mouse tumors, and the TCGA data in the literature is from human tumors. There are a lot of sequencing dissimilarities and many genes don't match. Still, most of the GBMs generated in mice are mesenchymal in nature, much like human GBMs[2].

Transdifferentiation:

These tumors are highly vascularized. Tumor cells produce the vascular endothelial growth factor (VEGF), high levels of which are related to poor prognosis. However, targeting GBMs with the anti-angiogenic drug bevacizumab (Avastin) does not have any long-term benefit.

In a phase II trial of patients with recurrent grade III or IV GBM, bevacizumab and irrinotecan therapy showed a response rate of 60%. But this effect is transient in most patients[3].

The tumors have many blood vessels, and these blood vessels are lined with endothelial cells, as seen using immunofluorescence for the von Willebrand Factor (vWF). This suggests that the blood vessels originate independently of tumor cells.

However, about 30% of the tumors transdifferentiate to become endothelial cells that are GFP +ve and positive for the CD31, CD34 and CD144 markers[4].

These cells are not derived from endothelial precursors, there's no fusion between the tumor cells and endothelial cells, they can differentiate *in vitro*, there's no vasculogenic mimicry and, most

importantly, they're functional.

VEGF inhibitors increase the number of these transdifferentiated cells, as the blood vessels are independent of VEGF inhibition. These results are all from mice.

In about 15% of human brain tumors, there is an amplification of the EGF receptor, which can be used as a marker for transdifferentiation. The superimposition of EGFR with vWF suggests that the transdifferentiation is not unique to the mouse model, and also happens in humans.

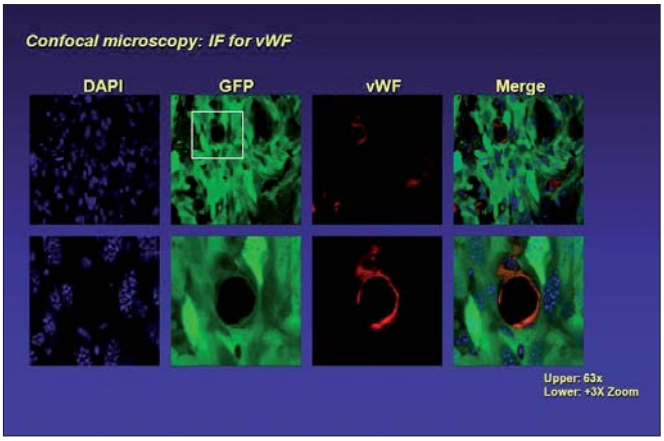

Figure 4
GFP-positive endothelial cells in the tumor.

At the same time, two other teams have also shown that in some cases, 80% of human brain tumor cells transdifferentiate into endothelial cells. Another team has shown cell fusion-independent differentiation of neural stem cells to endothelial lineage. Taken together, these results suggest that the GFAP-positive cells de-differentiate to become neural stem cells and gain the ability to give rise to every cell type, including endothelial cells.

These endothelial cells make tubes *in vitro*, and adding a neutralizing antibody against VEGF doesn't affect tube formation, indicating that it is VEGF-independent. The endothelial cells express high levels of HIF1-α because the highest degree of transdifferentiation occurs in the deep part of the brain, where there is also the highest level of hypoxia. Adding an siRNA to HIF1-α blocks the formation of the tubes.

Although they don't depend on VEGF, the tubes have FGF receptors that are blocked by VEGF inhibitors. A drug called brivanib, which inhibits both VEGF and FGF completely blocks tube formation, meaning these tumors do not make endothelial cells.

In a GBM mouse model, brivanib inhibits tumor growth, but has no effect on survival. In fact, as the tumors shrink, they become more lethal and the mice die faster.

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Genetic mouse models of glioma: translational tools for therapeutic development

A report on a lecture by
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University of Texas Southwestern Medical Center, Dallas, USA

Malignant astrocytomas are incurable, locally infiltrative brain tumors for which conventional anticancer therapy has failed to make significant improvement in prognosis. Although the full spectrum of molecular events that drives tumor initiation and progression has yet to be defined, the cancer genome atlas data has revealed frequent mutation in several tumor-relevant genes. Mouse models of glioma with conditional inactivation of three of the five most frequently mutated genes in glioma, p53, NF1, and PTEN, develop tumors that histologically and molecularly resemble human astrocytomas with 100% penetrance. These tumors arise from a population of neural stem/progenitor cells that can be propagated in culture with high efficiency, and importantly, without evidence of undergoing crisis or massive selection. These tumor-derived cells were used for an unbiased, large-scale chemical compound screen to identify compounds that could block proliferation of these cells. This screen identified a number of interesting hits, many of which have high activity and specificity for the tumor-derived stem cells. ***Luis Parada*** *reported that in-depth characterization of these compound candidates, including target identification, is under way, with priority given to those compounds with the best pharmacokinetic profiles.*

There are many reasons to model cancer in mice, and they're not all necessarily congruent. The stringencies that must be placed on the fidelity of the model depend on the kinds of questions one is trying to ask. Mouse models are particularly useful for studying aspects of cancer that are impossible to examine in patients.

When an individual develops cancer, its natural history is unknown and much of what came before is inferred rather than based on data. At the experimental level, mouse models can help address whether all adult organ cells are equally capable of giving rise to tumors, whether there is a tumor cell hierarchy, and whether cancer stem cells exist. They may also be useful as tools for empirical therapeutic development.

Mice have modeled many aspects of von Recklinghausen's neurofibromatosis, one of the most prevalent neurogenetic diseases in humans. People with the disorder have intellectual deficits and develop a variety of cancers. There is 100% penetrance of dermal neurofibromas, about 25% penetrance of plexiform neurofibromas, and a higher-than-average rate of glioblastoma multiforme (GBM).

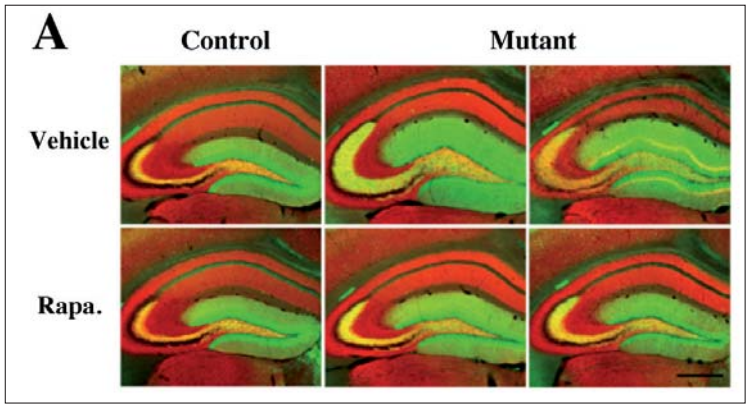

Figure 1
Rapamycin suppresses hypertrophy of the dendritic and massy fiber tract in dentate gyrus granule neurons lacking PTEN.

To illustrate that, in some instances, reversal of tumor suppressor loss is possible, a GFAP-Cre transgene in a Rosa26 background was used that is expressed in all

telencephalic progenitors during embryonic development. Its expression also persists in the adult stem cell compartment.

When neurofibromin or NF1 is knocked out, the thalamic projections arrive at the cortex as in controls, but the cortical neurons fail to arrange around them to form the barrel cortex[1]. Giving an ERK inhibitor can rescue this phenotype *in vivo*.

Likewise, knocking out NF1 in the cerebellum disrupts the formation of the cerebellum. In the normal cerebellum, the stem cells are in the rhombic lip embryonically and then migrate to form the outer nuclear layer. These cells then migrate inward to form the beautiful, dense structures of the cerebellum.

NF1 ablation during cerebellar development disrupts the migration of the granule neuron progenitors from the outer layer to the inner nuclear layer. Again, giving these mutant mice an ERK inhibitor rescues the phenotype.

However, when PTEN is knocked out in the hippocampus — this is a mouse model of autism — it disrupts the mossy fiber tract of the hippocampus and results in abnormally large dendrites. Giving these mice rapamycin reverses the phenotype, and these mice once again begin to behave socially.

However, when patients with neurofibromatosis are given ERK inhibitors, PI3K inhibitors, or both, the tumors still persist. At least in cancer, the simple notion of reverting the original mutations appears to be largely insufficient.

Necessary and sufficient:

In the 1990s, 3 molecular features of GBM were known: NF1 is a predisposing factor for the disease, p53 mutations can be discerned in some samples, and the PTEN tumor suppressor may play a role.

Mice with ablation of NF1 and p53 in neural precursors develop gliomas that are indistinguishable from human gliomas with 100% penetrance. By contrast, Synapsin1 Cre, which is only expressed in neurons, does not lead to gliomas. It turns out that any Cre that generates gliomas includes expression of the adult stem cell compartment.

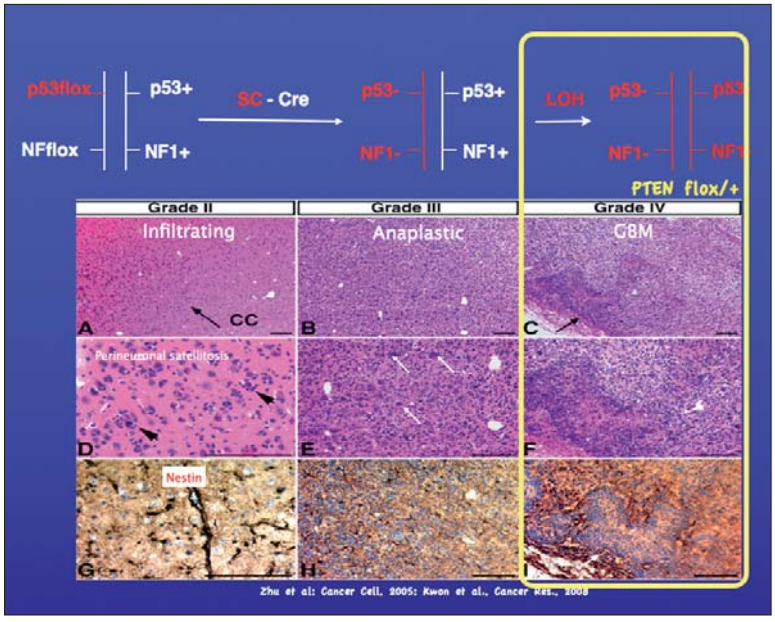

Figure 2
Neural precursor ablation of NF1 and p53 elicits malignant gliomas, and additional ablation of PTEN causes *de novo* high-grade tumors.

Additional loss of PTEN from the NF1/p53 mice leads to de novo high-grade tumors without progressing through low grade, suggesting that PTEN loss is a transitional mutation in GBM[2,3].

The cancer genome atlas project has found that 3 of the 5 most frequently found mutations in sporadic GBM are p53, PTEN and NF1. Somatic mutations in p53 are among the most frequent events in GBM,

and an estimated 14% of GBM patients have mutations in NF1. In fact, the only oncogene in this configuration that's significant is EGFR. NF1 and EGFR may be two alternative ways of mildly activating the RAS pathway, as opposed to the way in which a RAS oncogene might activate it.

Based on these observations, the hypothesis is that loss of p53 and NF1 in neural precursors is sufficient to initiate secondary glioma. This is therefore a stem/progenitor cell disease. The mutations arise in the subventricular zone (SVZ), then the cells glide out into different regions of the brain, where they form the GBMs. Additional PTEN loss causes *de novo* primary glioma.

Nestin-CreErT2 mice provide a means to temporally control induction of Cre recombinase specifically in the SVZ, the stem cell niche. Tamoxifen given at 4 weeks results in beautiful staining of the SVZ, the rostral migratory stream (RMS) and neurons of the olfactory bulb.

When Nestin-creERT2 $NF1^{f/+}$ $p53^{f/f}$ $PTEN^{f/+}$ $Rosa26^{f/+}$ mice are pulsed with tamoxifen, at 8-10 months of age 100% of the mice develop GBM, and the cells are all those originating in the SVZ.

If AdenoCre is added in different regions of the brain to NF/p53/PTEN floxed mice, only some of the injections result in tumors. But 100% of the injections that hit the SVZ develop GBM.

These experiments indicate that tumor suppressor ablation in the stem/progenitor compartment is both necessary and sufficient to elicit glioma, and there is a precursor-to-product relationship between the stem cells and GBM[4]. By heat map analysis, these three tumor suppressors generate tumors that are indistinguishable from the two most divergent kinds of GBMs analyzed by others.

Tumor hierarchy:

Before the notion of cancer stem cells became popular, people thought only about cancer cells, and did not consider the entire microenvironment of the cancer. Solid tumors were thought to be composed of essentially equivalent cells.

But if cancer stem cells exist, solid tumors may have a hierarchy in which at the apex is a cell type that is the only one with the capacity to self-renew. For a therapy to be effective, these cells have to be targeted because they're the ones that will reconstitute the cancer.

To explore the notion of cancer stem cells in GBM, a well-characterized Nestin promoter enhancer was used, but this time in a transgene with a GFP-tagged HSV-thymidine kinase gene.

When the cells in a normal SVZ zone and RMS are stained, the blue cells are the quiescent stem cells and the red are the transit amplifying cells that enter the RMS and depart. When this transgene is introduced, all of the blue cells become green because they express the GFP transgene, but also the proximal transit amplifying cells are green as they enter the RMS.

If this mouse is now given gancyclovir for 2 weeks with an osmotic mini-pump, there is a depletion of

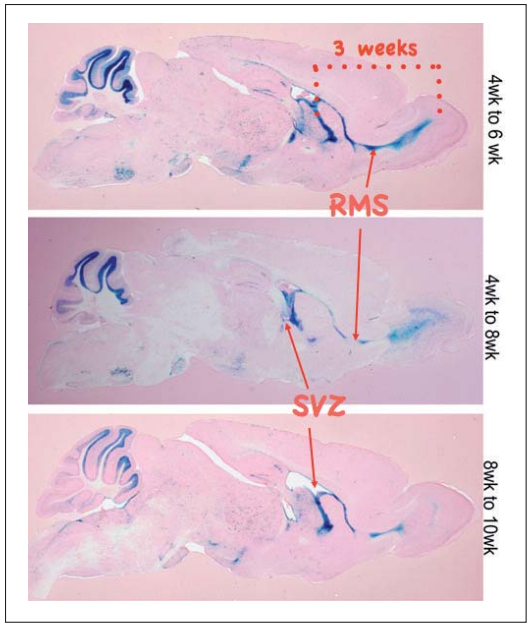

Figure 3
Nestin-CreErT2 mice provide a means to temporally control induction of Cre recombinase in the subventricular zone.

the RMS because all of the emergent cell cycle transit amplifying cells are killed by gancyclovir, and all that is left are the quiescent cells that have yet to enter the cell cycle. This is a tidy way to label quiescent stem cells and pragmatically eradicate neurogenesis.

If the GFP transgene is introduced in the NF/p53 and PTEN tumor background, there is a variable proportion of GFP-positive cells in every single tumor. Tumor development in the presence of this transgene is identical to tumor development in its absence, indicating that the transgene is not toxic and doesn't in any way affect tumor development.

In mice with the transgene, gancyclovir significantly extends survival compared with controls. The tumors in these mice also have well-defined borders and are less invasive, compared with typical GBM tumors.

In these tumors, cells that express ki67 almost never stain with GFP and *vice versa*. However, every section has 1 or 2 cells that co-express GFP and ki67. This is exactly the case in the normal stem cell niche: There are quiescent cells that every once in a while enter the cell cycle and give rise to transit amplifying cells.

GBM is usually treated with radiation therapy and temozolamide, a DNA alkylating agent. In virtually 100% of cases, the tumors recur, and they recur exactly at the tumor resection site.

When the GBM model mice are treated with temazolimide, the tumors stop growing for a while, but resume growth and proliferation when the drug is removed. Pulse-chase experiments with temazolimide and BrdU suggest that the drug kills the rapidly dividing transit amplifying cells, but not the stem cells. Pulse-chase lineage tracing indicates that new endogenous tumor growth arises specifically from quiescent Nestin-HSV-TK-GFP+ cells.

This may be the first demonstration *in vivo* in a spontaneous tumor of a cancer stem cell satisfying the prediction that it's the cell that gives rise to new tumors after therapy.

In fact, if the mice are given temazolimide to kill the transit amplifying population followed by gancyclovir, there is almost no evidence of tumors in the mice. By killing both populations of cells together, as in a putative combination therapy, the tumors can be eradicated. GBM therefore seems to adhere in the true sense of the word to the cancer stem cell hypothesis.

Sequential screens:

With a physiologically relevant mouse model in a defined genetic background, a large number of tumors that are essentially in the same phase of tumor development can be pooled. Even in low passage, this generates enough cells to do a high-throughput screen. The experiments must be conducted in 5% oxygen because the transcriptomes are different in regular tissue culture incubators.

About 200,000 compounds were screened in an ATP Cell-Titer-Glo assay that was very reproducible. This initially yielded about 4,500 compounds that markedly reduce ATP metabolism in these cells.

In the first secondary screen, wild type mouse embryo fibroblasts (MEFs) were taken from the same mouse strain at low passage, and anything that killed the MEFs was discarded. The same process was repeated for wild type astrocytes and SVZ stem cells.

In the end, these secondary screens narrowed the 4,500 compounds down to 61, of which 30 are exclusively toxic to the cancer stem cells and another 30 to the cancer stem cells and SVZ cells. None of them are toxic to wild type dividing astrocytes or MEFs.

EC50s on all 61 compounds are in the nanomolar range, and at the concentrations at which these compounds are toxic to the stem cells, they're toxic to control cells as well. They don't distinguish between normal cells and cancer cells.

Using a differentiation assay, the 4,500 compounds can be filtered down to 27 compounds, about half of which were in the original screen. The EC50s of these also are in the nanomolar range, but these compounds don't induce cell death, they induce cell differentiation and cytostasis.

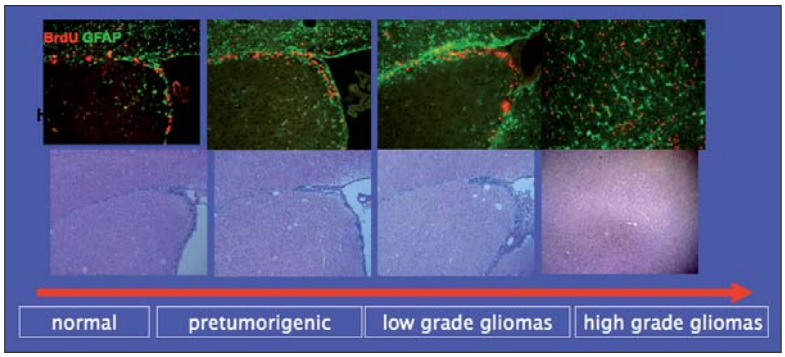

Figure 4
Neural stem cells progress to invasive malignant glioma.

The 61 compounds from the original screen fall into 13 chemical types. Three of these compounds have been selected for further study. Primary human GBM cells are sensitive to these compounds at the low nanomolar range.

Four independent primary cell cultures from four GBM patients have also been tested with the 61 compounds. Interestingly, three of the cell lines behave identically to GBM cancer stem cells, and the fourth cell line seems unresponsive at three times the EC50. At five-fold the EC50, however, it also responds to most compounds, suggesting that it has a slight resistance, but still a huge therapeutic window compared to the wild type cells.

Some of these compounds exert their effects within 6 hours, whereas others take longer. These sorts of results may help lead to target pathway discovery. By transcriptional profile, one of the compounds that acts at 6 hours up-regulates many genes only in the glioma stem cells, but not in astrocytes or MEFs.

There are a variety of independent secondary screens, and they're finding the same compounds in different ways. An siRNA screen is under way, as is human validation.

In all likelihood, these compounds do not block these targets through classic mitosis or other such pathways. They may be hitting synthetic lethality targets, which are fragile in cancer stem cells but not in other cells.

Adult stem cells from every organ are also being tested because any stem cell subpopulations that are sensitive to a given compound might predict that the cancer originating from those organs is also a viable target for those compounds.

More than 50% of compounds already cross the blood-brain barrier without modification and many have also been demonstrated to have *in vitro* and *in vivo* stability.

In conclusion, GBM is generated by mutation of the three tumor suppressors NF, p53 and PTEN, which are highly prevalent in sporadic GBM. These three are sufficient to recapitulate the disease in its complete entirety when this is done in the stem cell population.

This mouse model may be able to irrefutably demonstrate the cancer stem cell hypothesis for GBM. By understanding these cells, it may also be possible one day to treat this cancer.

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Developing a molecular biography of lung cancer

A report on a lecture by
Tyler Jacks
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Tumors in mouse models of adenocarcinoma have been initiated through the somatic activation of oncogenic KRAS, either through a spontaneous recombination event or via Cre-mediated recombination. The p53 tumor suppressor limits tumor progression in this model. Expression of oncogenic KRAS in the absence of p53 leads to the development of distant metastases after long latency. Gene expression and DNA copy number analyses of metastasis-derived cell lines and primary lung tumors have uncovered a metastasis-associated gene expression signature and recurrent genomic alterations. In particular, the transcription factor NKX2.1, which is required for normal lung development and differentiation, becomes down-regulated as tumors progress to metastasis in this model. Cell-based gain- and loss-of-function experiments show that NKX2.1 influences tumor seeding and metastatic potential of tumor-derived cell lines. Using conditional targeting of NKX2.1, either at the time of tumor initiation or in established tumors, the effects of loss of its function in vivo have been examined. Absence of this key regulator of differentiation promotes tumor progression and causes a fate change in developing tumor cells. Finally, several tumors from this model, as well as a series of tumor-derived cell lines, have been subjected to whole-exome sequencing. ***Tyler Jacks*** *presented results from ongoing studies that are providing an increasingly complete view of tumor evolution in this model system.*

One of the key messages from work on cancer models is to pay attention to mutations that occur in the human disease. For example, in human non-small cell lung cancer (NSCLC), mutations in KRAS occur 30% of the time and mutations in p53 about 50% of the time.

In a mouse model of NSCLC, a floxed allele of KRAS is combined with a floxed allele of p53, with Cre introduced on the back of a virus. Introduced into the lung of the animal, this results in individual cells becoming mutated[1]. Tumor development can then be followed over time by, for example, histological analysis[2].

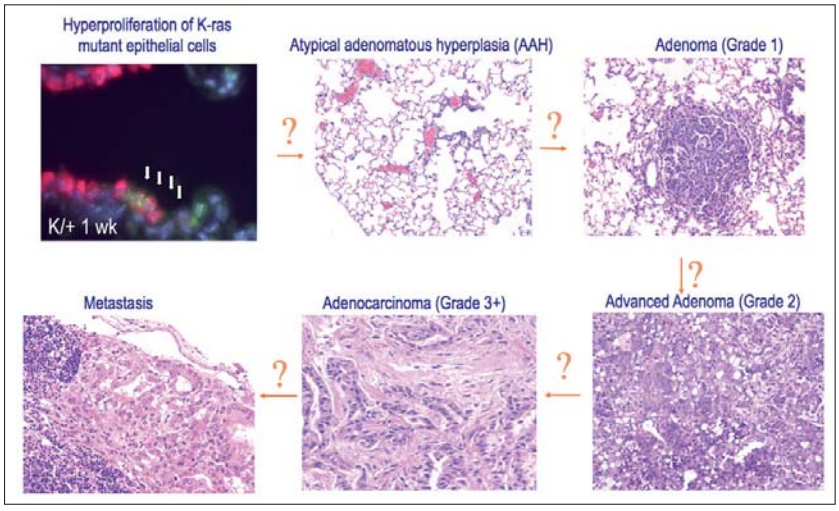

Figure 1
Lung tumor progression.

Tumors in these models go through the various stages of the disease, beginning with the hyperproliferation of

the cell-of-origin, hyperplasias, adenomas and advanced adenomas. These will at some frequency in this genetic background transition to adenocarcinomas, and a fraction of the tumors will progress to metastasis.

In the KRAS-p53 compound mutant mouse, after 4-6 months, about 50% of animals will have developed metastasis. One longstanding question has been to understand what the transitions between the stages mean in molecular terms, and what they translate to in terms of cell biological, genetic and biochemical events.

For example, in the transition from advanced adenoma to an adenocarcinoma, the signal downstream from oncogenic KRAS gets amplified, there is increased signaling down the canonical MAPK pathway and the tumors go from being phospho-ERK negative to phospho-ERK positive.

If the tumor is p53 deficient, the cells will be allowed to transition to this advanced stage. But if they have an intact p53 gene, they respond to a failsafe pathway, which is the up-regulation of the ARF tumor suppressor, an inducer of the p53 pathway.

As a result of the reactivation of p53, those cells are eliminated by an unknown mechanism[3]. The mechanism seems not to be apoptotic, and is likely to be a result of clearance of the cells by the immune system.

The transition to metastasis is also important to understand, as it's the lethal phase of the disease. A gene expression-based study comparing cell lines derived from non-metastatic primary tumors with those derived from metastatic tumors found changes in two proteins that correlate well with the transition to metastasis: down-regulation of NKX2.1 and up-regulation of HMGA2.

Functional studies confirm that these expression changes are important. Artificially manipulating NKX2.1 in cell lines can make non-metastatic cells metastatic, and *vice versa*. The same is true of HMGA2.

Master regulator:

NKX2.1 is a transcription factor in the homeodomain family. There are several of these proteins, all of them important in embryogenesis and development. This one is restricted in its expression to the lung, thyroid and parts of the brain. Based on its role in the thyroid, it is also called TTF1.

In these tissues, NKX2.1 is a master regulator that is required for their development. For example, mice lacking NKX2.1 don't develop lungs. The protein remains expressed in the adult lung, and is used as a marker of lung epithelial cells, including in lung tumors.

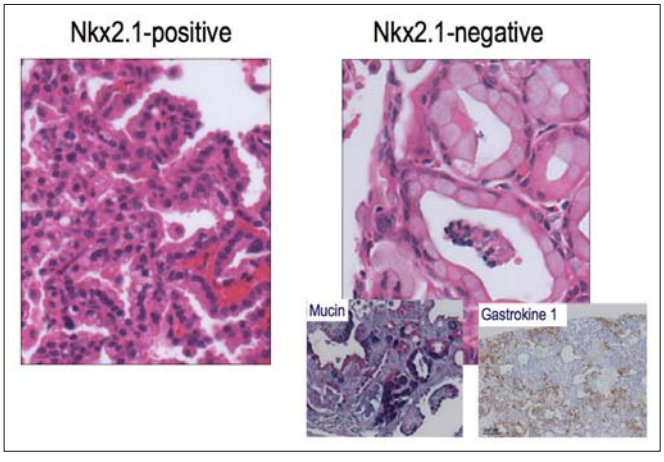

Figure 2
Loss of NKX2.1 alters adenocarcinoma differentiation.

Loss of expression of NKX2.1 correlates with poor prognosis in patients with lung cancer, suggesting a role as a tumor suppressor. Paradoxically, genomic methods have found it to be amplified in a subset of NSCLC, which suggests that it functions as an oncogene. The data discussed below support the view that it inhibits tumor progression.

In human cancers, the levels of NKX2.1 correlate with the degree of differentiation of the cancer tissue as well as clinical prognosis. Well-differentiated tumors have high levels

of this transcription factor — which is not surprising, as it is needed for the development of lung cells. Conversely, poorly differentiated tumors have low levels of the protein. Also, patients who have tumors with detectable levels of NKX2.1 have a better prognosis than those whose tumors show no staining for NKX2.1.

In both mice and people, NKX2.1 is expressed at high levels in early-stage disease in non-metastatic cell lines, whereas metastatic primary tumors as well as their metastases dramatically down-regulate its expression.

In a tumor with a region of well-differentiated NKX2.1-positive cells, a single clone may grow out, having figured out a way to down-regulate the transcription factor, change its biology, and presumably become more aggressive. However, mutations in this gene have not been found in lung cancers, suggesting that epigenetic silencing may be involved.

Change of fate:

During embryogenesis, embryos form a solid ball of cells and, in the process of gastrulation, form the gut tube and the gut endoderm. This is in response to the expression of specific transcription factors, such as the FOXA1/2 transcription factors. The gut tube is important for forming the gut, but it's also the embryological origin of other epithelial tissues, including the pancreas.

Under the control of PDX1, the cells change their developmental state and potential to turn into the pancreas. Likewise, about a day later, NKX2.1 and other lung-specific transcription factors are expressed, causing these cells to change their developmental potential and form the lung.

To investigate the consequences of NKX2.1 down-regulation during tumorigenesis, a conditional allele of NKX2.1 was deleted in early- and late-stage disease. The conditional allele of NKX2.1 was crossed with both the KRAS lox-stop-lox mouse ($KRAS^{LSL}$) to look at the early stage of disease, as well as a KRAS/p53 compound mutant mouse ($KRAS^{LSL}$/$p53^{f/f}$) to look at the effects at later stages.

Surprisingly, loss of NKX2.1 has a dramatic effect on tumor initiation. Two weeks after infection with two floxed alleles of NKX2.1, mice show no discernible histological lesions. But loss of the protein dramatically changes the number and size of discernible lesions, and the proliferation of initiated cells. This is inconsistent with it being an oncogene, and suggests that it is more a classical tumor suppressor gene[4].

In later stages of tumor development, NKX2.1 loss triggers a dramatic increase in tumor burden. There are also interesting effects on the phenotype of the tumors.

In controls with a functional NKX2.1, the lesions look typical and the cells look like alveolar type-2 cells, at least structurally and by gene expression. By contrast, NKX2.1-deficient tumors produce mucin in their cytoplasms, and look like they belong in the gut, suggesting that they have changed their developmental fate from lung to gut. More strikingly, the tumors up-regulate genes such as gastrokine 1 that are normally expressed in the stomach.

Rapid changes:

This is an interesting observation, but it's artificial, in the sense that NKX2.1 is deleted at tumor initiation, when it was discovered it in the context of tumor progression. A better experiment is to allow a tumor to develop and then delete NKX2.1.

These experiments relied on a frt-stop-frt KRAS mouse, and used FlpO, delivered either by an adenovirus or a lentivirus. The beauty of this system is that tumor formation can be initiated with flp/frt, leaving Cre for use at a later time to delete NKX2.1. This is a powerful approach for looking at the biology of the tumor,

but also, for example, to examine potential therapeutic targets.

Using this system, when viral FlpO is used to initiate a tumor, the tumor cells still express NKX2.1 and its targets, such as surfactant protein C. The tumor also has papillary, well-differentiated morphology. When the animals are then treated with high enough doses of tamoxifen, NKX2.1 is deleted in virtually all the cells of the tumor.

Strikingly, the cells change almost overnight. Within a week, the cells show changes in their morphology, their nuclei, their gene expression and their interaction with neighboring cells. These cells change their entire fate.

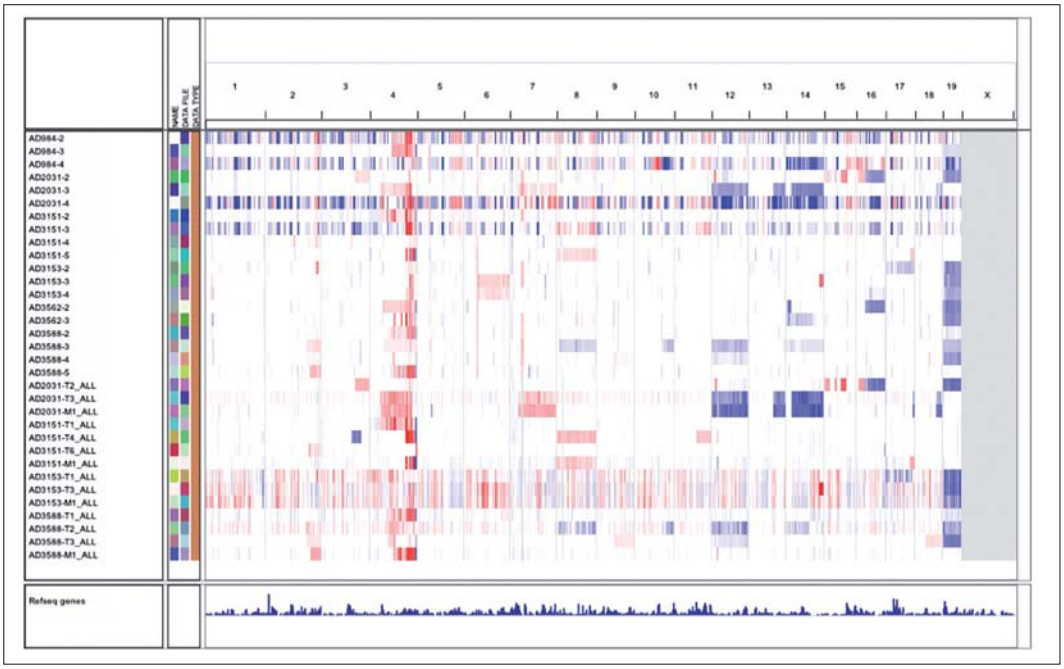

Figure 3
Copy number variation is common in small cell lung cancer.

The loss of NKX2.1 also promotes their proliferation, but an additional consequence is that it unlocks the cells' developmental potential, allowing them to sample other developmental fates. Specifically, these cells turn into the foregut by gene express analysis. In a sense, the loss of NKX2.1 reverses development.

What is responsible at the molecular level for the shift in gene expression profile? The transcription factors FOXA1 and FOXA2 bind along with NKX2.1 on genes involved in lung development and differentiation. As such, these same transcription factors that in other contexts drive the expression of gut genes are sequestered away from those promoters by binding to NKX2.1.

Chromatin immunoprecipitation reveals that, in controls, FOXA1 is bound to lung promoters and not to gut promoters. But in NKX2.1 +/- cells, FOXA1 appears to move to the promoters of gut genes, hinting at the mechanism by which the fate of the lung cells is changed to gut.

Acquiring mutations:

A separate project aims to understand the transitions between the various stages in tumor progression using genome resequencing and exon capture methods. Driver mutations are likely to contribute to transforming

an initiated cell into a fully developed cancer cell. But the presence of various passenger mutations makes it difficult to interpret the findings. The focus on mutations also too frequently ignores the contributions of copy number variations and epigenetic variation.

One study published several years ago found that MET-induced liver cancers almost always acquire β-catenin mutations. Of 21 samples in the study, 20 had activating mutations in β-catenin.

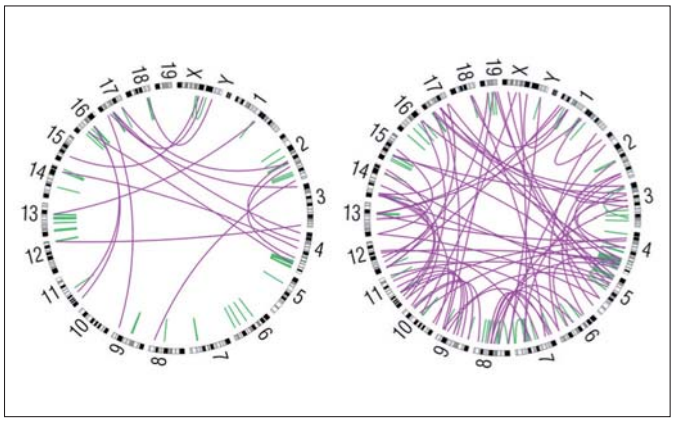

Figure 4
Evidence for chromosomal events in small cell lung cancer.

In a new study, a mouse model for lung cancer relied on potent mutations: KRAS activation and p53 loss at the time of tumor initiation, or loss of p53 and RB at tumor initiation. Still, additional mutations may be acquired as those cancers progress.

The study looked at 72 samples, including whole-exome exon resequencing of 25 NSCLC tumors, as well as whole-exome and whole-genome sequencing of 19 SCLC tumors.

Analysis of the 25 KP NSCLC found both non-synonymous and synonymous mutations. Each tumor had roughly 20 non-synonymous, protein-altering mutations.

However, mutation calls are influenced by how frequently the mutant allele is present within the tumor, the so-called non-reference allele frequency or NRAF. A low NRAF such as 10% does not inspire confidence, but with a higher one — with, say, 15-20% of cells having the mutation — the number of bonafide mutations per tumor is low.

Some tumors have just three protein-altering mutations and one cell line has a single protein-altering mutation. That suggests that a small number of point mutations is enough for tumorigenesis and begs the question, is activation of KRAS and loss of p53 sufficient?

Copy number analysis of these tumors shows that almost every tumor has a change on chromosome 6, where KRAS is located. KRAS is amplified in these tumors as they progress, an example of a secondary event that takes place in the majority of tumors. Whole-genome sequencing might help identify other genomic events that may also contribute to tumor formation.

Epigenetic changes may also plan an important role. For example, NKX2.1 does not get mutated, and yet it clearly changes.

Finally, in the case of SCLC initiated by loss of function of RB and p53 simultaneously, there is a slightly higher number of protein-altering mutations. Depending on the NRAF frequency, each tumor has between 15 and 40 mutations, more frequent than in NSCLC. The recurrence rate of mutations also seems to be higher than in NSCLC.

The most interesting among these is PTEN, which is mutated in 3 of the 19 samples. PI3K signaling appears to be a driver in this disease. The fact that the recurrence rate is higher than in NSCLC also suggests that there are drivers. In this case, tumor initiation occurs with loss of function of two tumor suppressor genes, and there is no potent oncogene, so it may be that the cells require an oncogenic signal to initiate tumor progression.

Copy number changes also occur frequently in SCLC. Intrachromosomal and transchromosomal translocations also both occur, providing another source of mutations in the system.

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Modeling sarcomas in the mouse

A report on a lecture by
Mario Capecchi
University of Utah, Salt Lake City, USA

Although sarcomas are not as common as carcinomas, they predominantly affect an important sector of our population, children and young adults. Further, because genome instability is not a hallmark for most sarcomas, identifying the players of the molecular pathway responsible for progression of sarcomas is greatly simplified. Many sarcomas are also noted for their extremely aggressive nature, which may in part explain their predominant importance for pediatric oncologists. Finally, the majority of sarcomas appear to be initiated by a specific reciprocal chromosomal translocation that defines the sarcoma, providing the modeler with a defined starting point. For instance, synovial sarcomas arise as a result of a translocation between the SYT gene on chromosome 18, and an SSX gene on the X chromosome. ***Mario Capecchi*** *shared his experiences with modeling human synovial sarcoma in the mouse, beginning with the genetic and molecular characterization all the way up to clinical trials of promising therapeutics.*

It is important to model sarcomas for three principal reasons: They affect a critical segment of the population, young children and young adults; they are extremely aggressive: and they are not extensively studied because they're relatively uncommon.

The initiating event for most sarcomas appears to be a reciprocal chromosomal translocation. From a modeler's perspective, sarcomas are not as complex as carcinomas. For example, synovial sarcoma has a normal set of chromosomes and often the only observed change is the reciprocal translocation. That should make it easier to identify the downstream genetic events responsible for cancer progression.

Synovial sarcomas arise in the joints. Histologically, there are three subtypes: monophasic, which are spindle mesenchymal cells; biphasic, which in addition have focal differentiation of epithelial cells; and poorly differentiated, common to several sarcomas, and comprised of small cells with large nuclei.

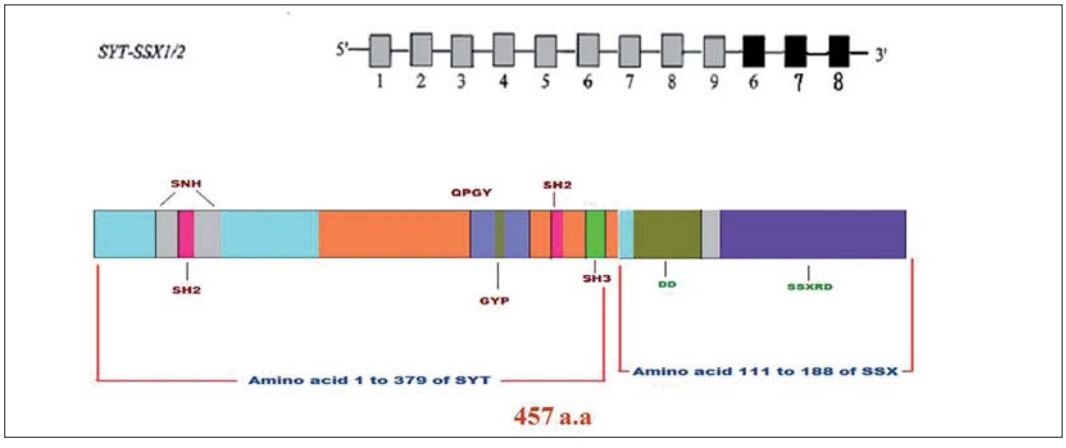

Figure 1
The SYT-SSX fusion protein.

In this case, the translocation is between the SYT gene on chromosome 18, also called SS18, and the X chromosome. On the X chromosome, there are 12 SSX genes in two subfamilies in mice and 9 SSX genes in humans, 3 of which appear in sarcomas[1].

SYT or SS18 is not associated with a DNA-binding domain, but rather with activation domains. The fusion gene contains almost the entire SYT gene, which includes the activation domain, and the carboxy terminal half, including the repressive domain, of an SSX gene.

Ideally, the reciprocal translocation should be used to initiate the tumor model. Unfortunately, the reported efficiency for generating Cre/loxP-based reciprocal translocation doesn't generate a large enough pool of cells containing the translocations to allow the spontaneous genetic events needed for cancer progression such that at least one tumor is generated in every mouse.

In addition, with a chromosomal translocation, four products are generated. There are the two reciprocal gene products, but also the loss of two alleles that are involved in making the translocations. In knock-in models, investigators normally only utilize one of these products thought to be critical for tumor formation. Undoubtedly there will be cancers in which more than one product will be critical for tumorgenesis. Fortunately, synovial sarcoma is not one of those cancers.

In this knock-in model, the Rosa locus was used as the fusion gene expression locus because SYT is ubiquitously expressed. In the presence of Cre, the fusion gene is made, and the cells turn green. When the fusion gene is expressed in every cell of the mouse using HPRT as a driver, it generates a green blob. It has all three embryonic cell types, but the tissues are completely disorganized, are quickly absorbed, and do not lead to tumor formation.

Because the tumors arise near the joints, the experiments focused on the muscle lineage, and used Cre drivers for different states of muscle lineage, such as PAX3-Cre, and PAX7-Cre, which label progenitor cells. Mice that utilized these Cre-drivers also die and do not form tumors.

MYF6-Cre, which is expressed in fully differentiated muscle, gives rise to classical myopathies in the mice, but the mice still do not develop tumors. In myoblasts, MYF5 or MyoD have apparent overlapping functions. If one is knocked out, nothing happens, but in double mutants, no myoblasts are formed and therefore no muscle is made.

If MYF5-Cre is crossed into the mouse containing the SYT-SSX fusion gene targeted to the ROSA26 locus mouse, multiple tumors arise, at a 100% penetrance, and these are indeed synovial sarcomas.

Muscular origins:

The above tumors metastasize, with a pattern of metastasis that is similar to that of human synovial sarcoma, with the most common metastases being in the lymph node. These are most likely metastases because they are much smaller than the primary tumors and are in regions of the mouse that do not expression MYF5.

Histologically, there are both biphasic and monophasic tumors, and they are indistinguishable from human sarcomas. SSX1 leads to primarily biphasic tumors in humans, and SSX2 primarily to monophasic ones. In the mouse model that utilizes SSX2, the tumors are 10:1 monophasic.

There are 25 different markers that typify human synovial sarcoma and all of them are expressed in these mouse tumors. For example, the human tumors have high levels of BCL2 expression, which is also seen in the mouse tumor. By microarray expression analysis of thousands of genes, these tumors are again similar to the human tumor, and specifically group with synovial sarcoma.

Muscle is essentially made by fusing thousands of myoblasts together, so in this case, the muscle would

be expected to be green. However, this muscle is not green. Earlier in embryogenesis, at about 11.5 days of gestation in the mouse when the muscle is just starting to form, the green myoblasts expressing the SYT-SSX2 fusion gene are there, but they do not participate in making the myofiber because they're massively dying.

A TUNEL assay shows the cells rapidly dying except wherever there's cartilage, which is also associated with joints. The cells in close proximity to cartilage survive, continue to divide and then acquire the secondary genetic events that allow tumor progression. It's not known what is made by cartilage that allows survival of those cells.

Although 100% of the mice get tumors, these mice run around and are pretty healthy[2]. This is surprising because muscle fibers are formed by fusion of hundreds, if not thousands, of myoblasts, including MYF5-expressing myoblasts, which are massively dying in the presence of the SYT-SSX2 fusion gene. That suggests two possibilities of redundancy with respect to MYF5 and MyoD.

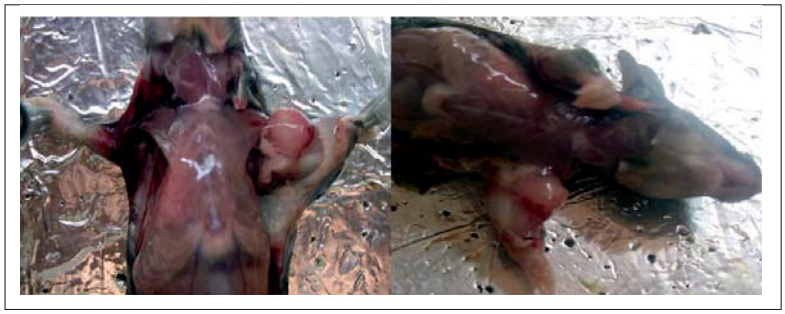

Figure 2
Synovial sarcoma in mouse.

One is that MYF5 and MyoD are in the same cells and are functionally redundant. Alternatively, there can be independent lineages, one expressing one and the other expressing the other, so that the redundancy is at the level of cell lineage.

If diphtheria toxin is introduced into the Rosa locus and crossed with MYF5 Cre, the mice are happy although all MYF5-expressing myoblasts are dying. In the presence of Cre, the toxin is expressed, but the muscle is still there, and the mice run around. The MyoD lineage begins to take off as the MYF5 lineage is dying off. This indicates that there are independent myogenic lineages. If one is knocked out, the other compensates.

From the above described experiments, by definition, the myoblast is a candidate for making this tumor in humans. None of the terminally differentiated muscle markers are expressed in this tumor, and that's why people didn't recognize this previously as a muscle tumor[3].

Complex disruptions:

Molecular analysis of the fusion protein itself reveals that neither the fusion gene nor either partner has a DNA-binding domain. The way to identify what's happening is to take an antibody specific to the fusion gene and bring down all the different proteins associated with the fusion protein and identify them by mass spectrometry. These experiments were done in collaboration with Torsten Neilson and colleagues at the University of British Columia[3].

ATF2, or activation transcription factor 2, is one such protein. ATF2 is a chromatin activator that binds to cyclic AMP responsive elements. It then normally recruits histone acetyl transferases, and activates transcription of genes containing such responsive elements. Another co-precipitated protein, TLE1, is a groucho repressor that attracts histone deactylases and also members of the polycomb group complex that silence transcription.

On a glycerol gradient, ATF2 and TLE1 co-sediment, and that co-sedimentation is dependent on having the fusion gene present.

If in-frame deletions are made across the fusion gene, and the deletions are used to map out where different proteins bind, ATF2 binds to the N-terminus of the fusion gene, and TLE1 binds to the C-terminus. siRNAs to the fusion gene can disrupt formation of the complex.

Based on those kinds of data and many other experiments, the model is that the fusion gene acts as a scaffold to bind ATF2 at one end and TLE at other end. This then recruits the polycomb complex and the histone deactylases to the complex. This complex then inhibits targets of ATF2, including tumor suppressor genes such as EGR1, or early growth factor response element 1.

If the complex is disrupted using siRNAs to TLE, ATF2 or to the fusion gene, that reduces the growth rate in human synovial sarcoma cells, in mouse synovial sarcoma cell lines, and also primary cells derived from mouse tumors. It also reduces plating efficiency and clone formation, and increases apoptosis as judged by a loss of cells, as well as induction of caspase 3.

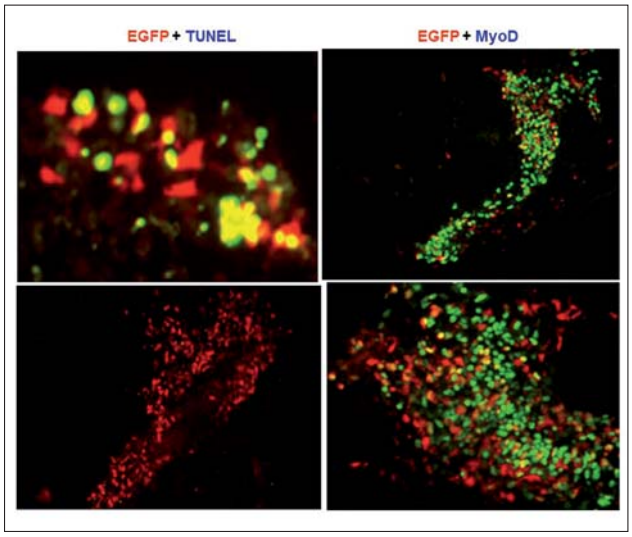

Figure 3
MYF5-Cre/SSM2 – E 11.5.

Over a dozen target genes have been identified for ATF2. On a comparison heat map of synovial sarcoma compared to other sarcomas, there are 10 different target genes, as well as SSX1, SS18 and TLE1. So the pattern for synovial sarcoma is distinct.

By ChIP sequencing, the complex co-localizes with the cAMP element on the EGR1 locus. This has been repeated for the other ATF2 target genes. If the complex is inactivated with an RNAi to either ATF2 or SYT, all of those target genes are up-regulated several-fold.

Trial and error:

The complex can also be disrupted with HDAC inhibitors, which separates the complex into two components — ATF2 and the fusion gene, and the rest of the complex, which is TLE1 plus all of the other inhibitory components. What's more, the HDAC inhibitors also reduce cell growth and plating efficiency, and induce apoptosis.

The HDAC inhibitors romidepsin and SB939 also increase the expression of the inhibited ATF2 target genes. These inhibitors are going into human clinical trials for management of synovial sarcomas.

About 98% of the tumors express BCL2 at very high levels, indicating that BCL2 might be another interesting target in synovial sarcoma. Moderately effective inhibitors of BCL2 are available, including BH3 mimetics such as Navitoclax. They have been shown to be partially effective with follicular cell lymphoma, and small cell lung cancer.

However, a problem with these drugs is that, even though BCL2 is effectively turned off, there are other members of the BCL2 family that don't have a BH3 domain which can be up-regulated in the tumors. In particular, up-regulation of MCL1 and BCL2A1 provide the most frequent escape route from these antagonists.

In synovial sarcoma, MCL1 and BCL2a1 are specifically down-regulated by the same mechanism described

above. That indicates that these tumors are particularly sensitive to Navitoclax as a single agent.

Navitoclax is also extremely effective in combination with doxorubicin, the most commonly used chemotherapy drug, and in fact, appears to greatly sensitize synovial sarcoma cells to doxorubicin for synovial sarcoma. These tests have been carried out in both human and mouse synovial sarcoma cell lines.

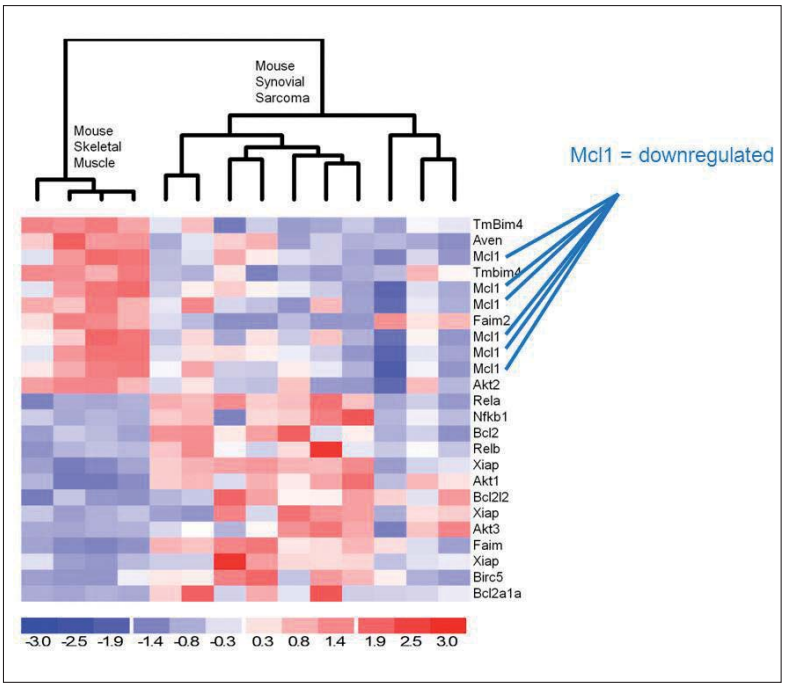

Figure 4
MCL1 is consistently down-regulated in expression arrays of anti-apoptotic genes.

If the drug is given to SSM2/MYF5 mice *in vivo* beginning at week 12, by week 15, it reduces the number of tumors, which are predictably present in these mice, compared with controls. It also rapidly reduces tumor mass.

The above results suggest that the drug may be useful in clinical trials. With respect to developing therapeutics, the most scarce resource is the patients themselves. A mouse model that is carefully authenticated for the disease should help pre-screen potential therapies[4].

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PART IV: Lessons for therapy and resistance from mouse models

Mouse models for pathogenesis and therapy of acute promyelocytic leukemia

A report on a lecture by
Hugues de Thé
INSERM, CNRS and Université Paris Diderot, Paris, France

Acute promyelocytic leukemia (APL) is characterized by a specific t(15;17) translocation, generating a PML/RARA fusion protein. This protein disrupts PML nuclear bodies, which are associated with control of senescence and self-renewal of stem cells. Two therapeutic agents, retinoic acid (RA) and arsenic trioxide (As03), variably induce differentiation of promyelocytes in vivo and clinical remission of APL patients. Both agents directly trigger PML/RARA degradation by the proteasome, but the mechanism underlying each is different. Genetic and pharmacological evidence have demonstrated that RA-induced differentiation and APL clearance are uncoupled events. Instead, APL clearance is associated with a cell-cycle arrest signature, characterized by p53 target genes involved in senescence. Accordingly, p53 inactivation in APL cells allows RA-induced differentiation, but abrogates the anti-leukemic effect. Strikingly, the normal PML allele is required to activate p53 and ensure APL regression, suggesting that PML/RARA degradation by anti-leukemic agents triggers PML nuclear body reformation and activation of a senescence program by p53. Preclinical studies in several mouse models of APL have shown that RA and As03 dramatically synergize for APL clearance although they antagonize for differentiation. This has led to a series of clinical trials with an RA/As03 combination that cures virtually all patients. ***Hugues de Thé*** *suggested that oncogene degradation as a therapeutic strategy may be effective in some other cancers.*

In 1985, retinoic acid (RA) was first introduced in Shanghai, China, as a treatment for acute promyelocytic leukemia (APL). RA was found to induce differentiation *in vivo*, resulting in transient clinical remissions.

Unfortunately, however, the disease almost always relapses when RA is given alone. A combination of RA and chemotherapy yields a jump in the cure rate from 30% to 70%. A few years later, arsenic trioxide (As03) was introduced and shown to cure 70% of APL patients as a single agent. In combination with RA, As03 induces a 90% cure, in some cases without any chemotherapy.

After RA treatment for 2 weeks, APL cells in the marrow mature to granulocytes and are cleared away by the spleen. This led to the molecular characterization of the hallmark alteration in this disease, the 15-17 translocation,

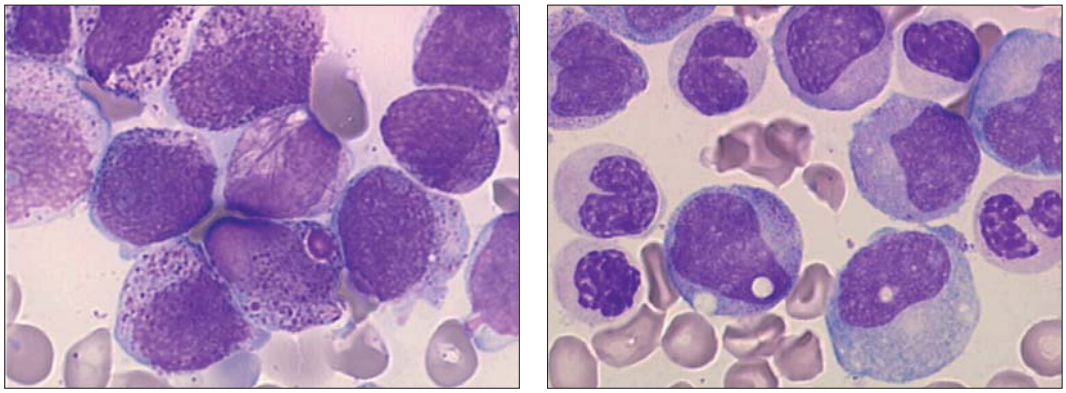

Figure 1
APC cells untreated (left) and treated with retinoic acid (right).

which fuses the RA receptor alpha (RARA) and a gene named PML to create the PML-RARA fusion.

This fusion is found in virtually all APLs, and is the only important genetic alteration in the disease. There are other very rare translocations that involve the PLZF gene and are associated with clinically RA-resistant leukemias.

Work done by several groups suggested that PML/RARA homodimers must be responsible for inducing tight transcriptional repression through enhanced binding of co-repressors and chromatin modification. According to this model, high doses of RA would reactivate the target genes, reopen the chromatin, trigger differentiation and, eventually, induce clinical remission.

This model was very appealing and fit well with cell line data. However, two things did not fit well with the data.

First, it did not explain why enormous doses of RA, at least three logs above physiological levels, are needed. Second, the model did not explain how AsO3, which does not significantly alter RA-signaling, can cure patients as a single agent.

There is another part to PML/RARA-mediated cellular changes: When PML/RARA is transfected, it displaces normal PML and the associated proteins on to distinct micro-speckled sites. This feature is characteristic of APL and can be used to rapidly and accurately diagnose APL.

Like RA, AsO3 also degrades PML/RARA and, as a result, reforms nuclear bodies. Primary APL cells treated overnight with therapeutic concentrations of RA or AsO3 both induce the loss of PML/RARA by proteasome-mediated degradation. However, the targets and the molecular mechanisms implicated are not identical.

RA binds to the RARA part of the fusion and induces exposure of the AF2 transactivating domain that directly binds proteasome components. This process, which is universally conserved among nuclear receptors, also involves RA-induced phosphorylation of RARA.

By contrast, AsO3 not only induces degradation of PML-RARA but also of PML. AsO3 induces SUMOylation of PML or PML/RARA, and this then triggers polyubiquitination by RNF4 and degradation by the proteasome.

How does AsO3 selectively trigger PML SUMOylation? Arsenic induces the aggregation of normal PML on to nuclear bodies. This is due to redox-mediated disulfide bonding of PML and formation of the bodies, together with the direct AsO3 bonding onto PML. Importantly, this precedes SUMOylation and degradation, and is therefore likely to be the initiating event underlying degradation.

In more recent work, mouse models have shown that although RA and AsO3 both induce differentiation, it's not the basis of the cure.

The RA-AsO3 association is antagonistic for differentiation, but highly synergistic for eradication. As expected, the PLZF/RARA mice show full differentiation, but no eradication, which is in full line with the clinical findings. This again implies that resistance to differentiation is not why the disease is not eradicated[1].

Senescence signature:

So, if differentiation is not the driving force, what cures the disease? A large transcriptomic analysis after 6 and 12 hours of treatments indicates that there are two different responses.

The first, as expected, is differentiation, observed in every circumstance. But even at early time points, there is a second effect, and that's a cell cycle arrest signature. This is observed at a standard dose of RA with PML-RARA only, and not with a point mutant that fails to degrade, or in PLZF-RARA.

This signature correlates perfectly with disease eradication. And a large number of the individual genes are primary p53 target genes implicated in senescence.

Other groups have found a connection between PML, PML/RARA and p53. For example, PML is implicated in

p53-mediated senescence, because RAS-induced senescence is not observed in PML-/- cells.

PML nuclear bodies recruit p53, and this is believed to facilitate p53 activation, possibly through acetylation. The PML/RARA transduction in primary cells triggers not only p53 degradation, but also some X-ray resistance, as shown by previous studies.

In leukemic mice, PML-RARA degradation is extremely rapid. The standard dose achieves complete degradation at 12 hours, and with a high dose it is complete after only 6 hours. This is accompanied by activation of a p53 checkpoint, as seen by p53 stabilization *in vivo*.

Experiments with shRNA show that this p53 activation is important for disease eradication. When shRNA is transduced against p53 in the bone marrow, there is the classic 80% prevalence of leukemic cells as assessed by GFP. But following RA treatment for 6 days, the disease is no longer detectable. If p53 is inactivated, however, abundant leukemic cells remain.

The same is true in leukemias generated from p53 null cells or PML null cells. Indeed, the normal allele of PML is implicated in the activation of p53. This is consistent with the fact that PLZF does not show any p53 activation, and suggests that PLZF-driven leukemias are resistant to RA because they fail to activate this senescence checkpoint.

When PML +/+ mice are treated with a standard dose of RA for a week, there is a clear-cut extension of survival, although the mice are not cured. If the same experiment is repeated in a PML null background, there is only a marginal survival advantage despite the fact that the leukemic cells show full differentiation. The absence of PML does not change the features of the untreated leukemia, but it has a drastic effect on the response to RA.

After 3 days or 6 days of RA treatment, wild type cells show a dramatic elimination of leukemia-initiating cells. In p53 null cells, by contrast, there is complete abrogation of the decrease in leukemia-initiating cells, implying that the p53-mediated senescence checkpoint is essential for clearing clonogenic cells from the bone marrow *in vivo*.

In contrast to PML inactivation, p53 inactivation also drastically accelerates the onset of leukemia. The mice die in less than 20 days, whereas wild type p53 cells take about 40 days, so there is a clear enhancement of the intrinsic clonogenic activity of those leukemias.

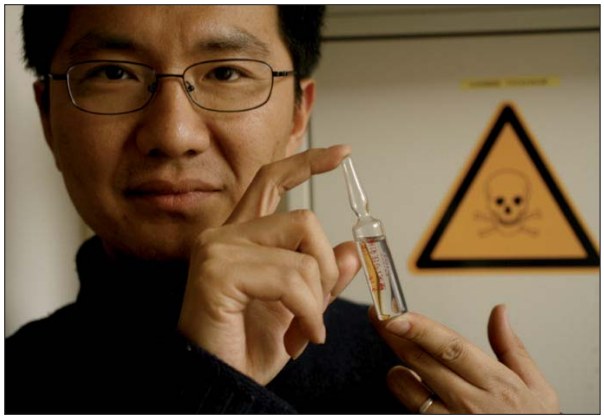

Figure 2
Arsenic, a new therapy for APL.

New model:

This leads to a renewed model of PML-RARA response to therapy. In this model, PML-RARA sequesters PML and also induces transcriptional extinction of target genes. Treatment with RA reactivates transcription from those targets and triggers differentiation.

Also, and key to the clearance, when AsO3 or RA degrade PML-RARA, it allows the reformation of PML bodies and activates p53, leading to senescence. This model also explains why, if the remaining unrearranged allele of PML or p53 is knocked out, the differentiation response is maintained, but the cells no longer go through senescence.

In the rare patients who are therapy-resistant, some p53 mutations have been described. There is also one

example of a patient resistant to RA who has a truncating mutation in the unrearranged allele of PML.

Surprisingly, however, in the more than 10 years that people were doing transcriptomic analyses in cell lines, this senescence program had never been seen. That's because there are only two available APL cell lines, and both are p53 mutants. In fact, one of the cell lines was derived from an RA-resistant patient.

RA and AsO3 antagonize each other for differentiation of primary cells from APL patients. RA alone results in complete differentiation, but adding both RA and AsO3 blunts the differentiation response. These experiments prevented doctors from giving AsO3 to patients.

In fact, when RA and AsO3 are given together, there is dramatic synergy for clearance of disease. In 3 days, the combined treatment almost eradicates the disease, and certainly eradicates clonogenic activity[2].

This was initially thought to be the reflection of better PML/RARA degradation, but new data suggest that the mechanism may actually be enhanced nuclear body reformation.

For reasons that are not entirely clear, giving RA for 6 hours dramatically induces expression of the normal PML allele. At the protein level, there is not just degradation of PML-RARA, but massive induction of the unrearranged PML allele.

AsO3 also induces aggregation of the normal PML, resulting in faster reformation of nuclear bodies, and therefore activation of p53. AsO3 enhances reformation of the bodies through redox stress and the formation of disulfides. On the array data, some primary p53 senescence target genes are hyperactivated, suggesting that the accelerated reformation of bodies enhances senescence.

In a randomized trial in Shanghai of ATRA, AsO3 alone or a combination of both, those patients who received

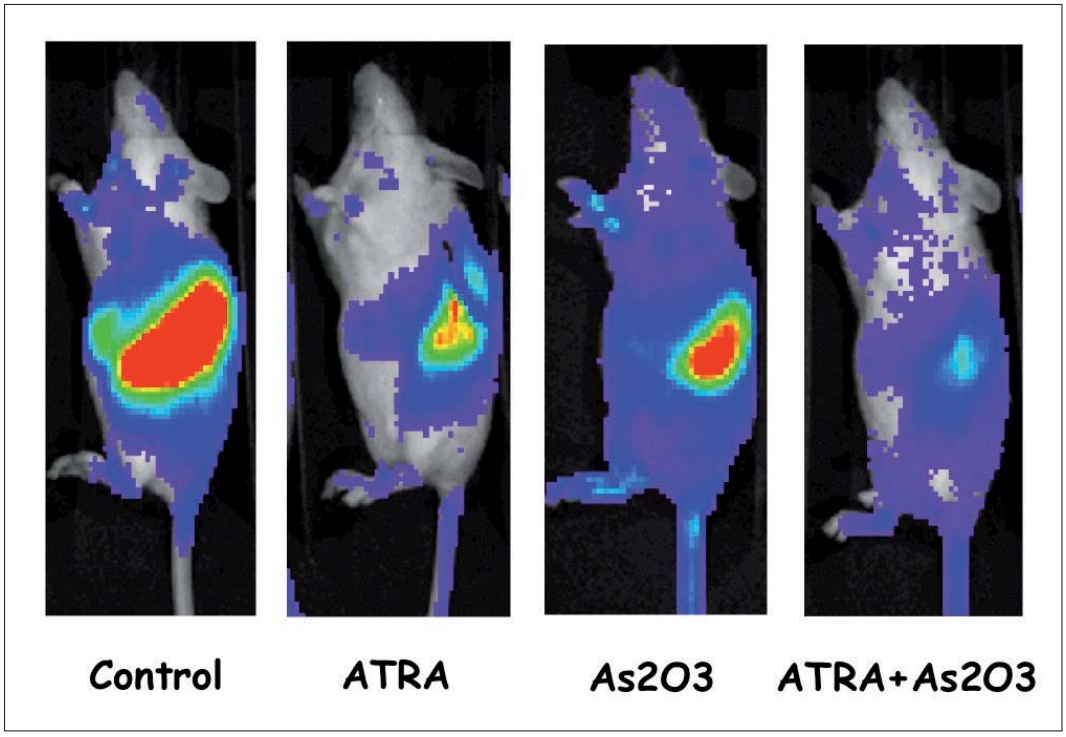

Figure 3
Retinoic acid/arsenic synergy in APL mice.

the combination showed not only a dramatic and clear-cut survival advantage, but also synergy for PML-RARA cDNA clearance[3].

The first paper on this synergy was in 1999, the first clinical trial with 5-year follow-up in 2004, and it took another 5 years before global opinion leaders strongly endorsed the combination. It's used in many places in the U.S., in China and India, and several trials are ongoing in Europe.

Oxidation effects:

There is some unpublished data indicating that transcriptional activation is not needed to trigger differentiation and that, probably, the loss of repression *in vivo* is sufficient to induce differentiation.

A Selex approach showed that the DNA-bound PML-RARA is not only a dimer as initially proposed, but is a heterotetramer, with two PML-RARA bound to two RXRA co-receptor. Having RXRA in the complex is indispensable for triggering disease.

This has two important implications. First, four DNA-binding domains bind DNA more avidly than one. Second, the DNA binding site specificity is dramatically altered. This may explain the oncogenic property of this fusion protein, making it a loosely-specific transcription factor that represses genes in an unspecific manner.

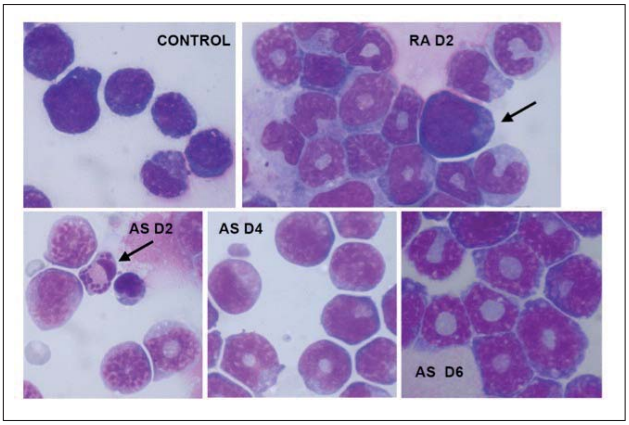

Figure 4
Arsenic induces differentiation *in vivo*.

Surprisingly, knocking out RXRs *ex vivo* or *in vivo*, in the absence of RA or any other treatment except tamoxifen, results in terminally differentiated granulocytes that still express PML-RARA microspeckles. From a physiopathological point of view, this argues that there are no irreversible epigenetic marks implicated in the differentiation arrest.

In retrospect, this probably also explains why AsO3 *in vivo*, but not *ex vivo*, triggers a complete differentiation that is indistinguishable from the one triggered by RA, except that it takes 6 days, rather than the 2 days with RA.

As mentioned above, oxidation-induced disulfide bonds initiate the formation of nuclear bodies, followed by SUMOylation of PML, ubiquitination of poly-SUMOylated PML and proteasome-mediated degradation. This suggests that oxidation-induced disulfide bonds are the initial events triggering PML-RARA degradation[4].

If this is true, it should be possible to trigger a similar effect with oxidants other than AsO3. Indeed, Paraquat, a chemical normally used to trigger oxidative stress in the brain has a strong anti-leukemic effect on PML-RARA, but no effect whatsoever on PLZF/RARA leukemic mice.

One group in Brazil has also shown that a vitamin E derivative, alphaTOS, which is known to trigger oxidative stress from the mitochondria, can induce prolonged remissions in APL mice with exactly the same type of efficiency as RA or AsO3.

This suggests that ROS- and disulfide-mediated bonding of PML and PML-RARA is a key step in arsenic-induced degradation of the disease. It may also explain why the disease is known to be sensitive to anthracyclins, which not only induce DNA breaks but also induce massive oxidative stress.

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Disease progression and therapeutic resistance in murine ductal pancreatic cancer

A report on a lecture by
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Pancreatic ductal adenocarcinoma (PDAC) is the most lethal common malignancy, with little improvement in patient outcomes over the past 40 years. Transplanted tumor models have superior delivery of gemcitabine compared with primary murine PDAC, and this inversely correlates with stromal content. As in mice, human PDAC is profoundly hypovascular. Stromal depletion with a hedgehog pathway inhibitor increases vascular density and gemcitabine delivery, and prolongs survival. Abraxane or nab-Paclitaxel, an albumin-Paclitaxel formulation, also elevates gemcitabine levels in the tumor by a separate pathway. Finally, γ-secretase inhibitors can inhibit the Notch pathway, which is implicated in vessel morphogenesis. These effects are exacerbated by concomitant exposure to gemcitabine, resulting in hypoxic necrosis. A transposon-based genetic screen for genes that cooperate with a sensitizing mutation identified the deubiquitinase USP9X. Correlative studies show that low or absent expression of USP9X is a poor prognostic factor and is associated with increased metastasis at the time of death. Interestingly, USP9X mutations are not observed by genomic sequencing, highlighting the importance of mouse models in unveiling tumor suppressors. ***David Tuveson*** *argued that the lethality of PDAC may be better understood by investigating the unique microenvironment of this cancer and searching for pathways that promote the resistance to cell death. Such findings should serve as vulnerabilities to exploit for clinical benefit.*

Pancreatic cancer has a high mortality rate, with a median survival of 6 months. The disease is characterized by metastasis, cachexia and severe pain. Compared with other carcinomas, pancreatic cancer has the advantage of having a clear initiating oncogenic event, which is likely to contribute in some manner to maintain the tumor. A variety of pathways are found to be dysregulated and mutated in the final cancer.

The popular pancreatic cancer model expresses the oncogenic KRAS allele, largely in the pancreas, either during development or after. The disease that develops is characterized by mucinous metaplasia in the ducts. The acinar tissue and the islets appear to be largely unaffected, although there is evidence of acinar duct metaplasia or ADM.

Over a long period of time, these animals stochastically develop cancer, which is invasive. Including any number of tumor suppressor genes mutated in human pancreatic

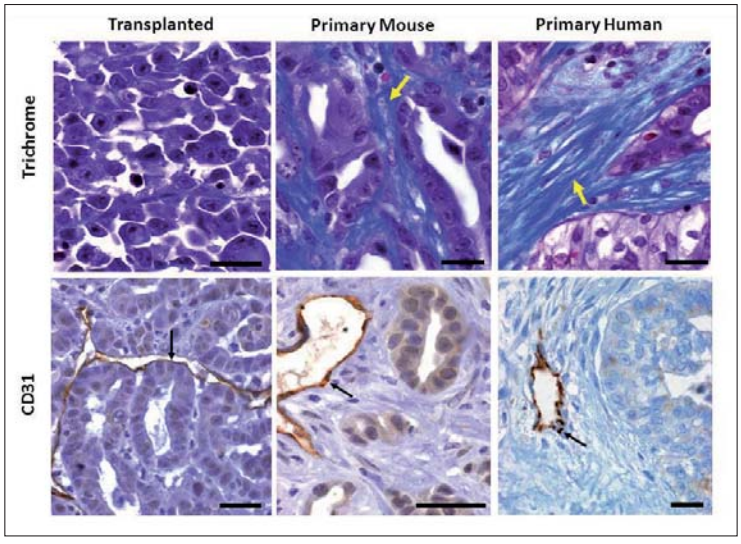

Figure 1
Stromal-rich primary mouse and human PDAC is hypovascular.

cancer — for example, $p53^{R172H/+}$ — speeds up this process and generates a robust model of pancreas cancer with complete penetrance. This is a terrific model to ask diagnostic, therapeutic and biologic questions.

The dual mutant animals die much more quickly than those with just oncogenic KRAS expressed in the pancreas. They present with ascites, tumor at the head of the pancreas, extension of the gall bladder and metastases to the liver, the lung and other relevant sites.

Importantly, these animals also get a cachexia syndrome characterized by skeletal and cardiac muscle loss, and that's a characteristic feature of patients at the end stages of pancreatic cancer.

In the tumor cells, both the wild type and the mutant allele of KRAS are expressed, and mutant KRAS is maintained in all cells. In the case of p53, however, the wild type allele is spontaneously lost, and only the recombined allele with the point mutation is expressed.

By immunohistochemical approaches, p53 protein expression is elevated in invasive cancer cells but not in the pre-invasive precursor lesions. Other canonical tumor suppressors in these cell lines are usually retained and not mutated. If the cells are passaged extensively in culture, however, they select for epigenetic silencing of the INK4a locus.

Under the microscope, the cells have anaphase bridges, which are uncommon and unstable. They also have multi-centromeric chromosomes with nonreciprocal translocations — the type of features present in human carcinomas — but without the manipulation of telomeres as the initiating event.

Enhanced delivery:

To explore why pancreatic cancer trials fail despite promising preclinical evidence, nearly 1,000 cages of mice have been set up. The tumors in these animals are first felt by palpating them, then measured by ultrasound, which is a fairly simple and inexpensive technology.

A variety of studies have shown that this is an invasive cancer and kills animals within 12 days, so there is a tight survival curve. The mice are enrolled when the tumors are around 250-300 cubic millimeters in size.

When transplanted models are compared with these primary genetically engineered mouse models (GEMMs), the transplanted models, but not the primary, respond to gemcitabine.

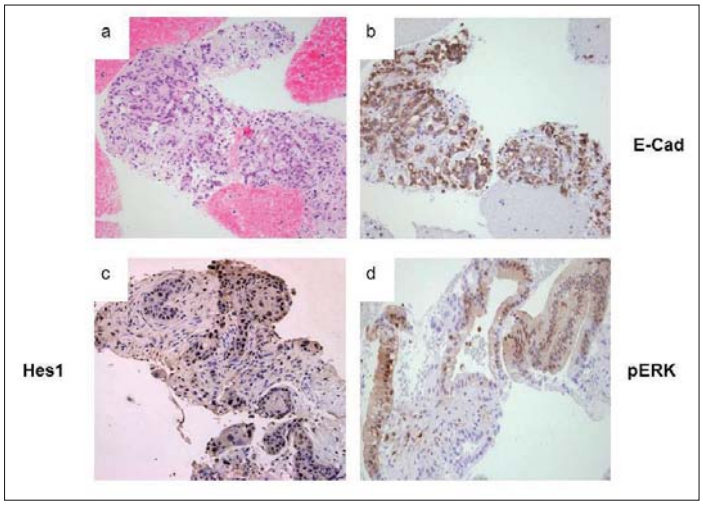

Figure 2
Mini-cores from EUS-FNA are suitable for immunohistochemistry.

There are differences in perfusion between these tumors: Microbubbles on an ultrasound machine, or a paramagnetic substance, gadolinium DTPA, both get into transplanted tumors but not into GEMM tumors.

There are also differences in stroma. The stroma is rich in human pancreas cancer, modest but present in mouse pancreas cancer, and largely absent in transplanted models.

Human ductal pancreatic cancer is hypovascular. There are lots of blood vessels present in the acinar

tissue, but hardly any in the invasive part of tumor. By density, there is roughly about a quarter of the number of blood vessels in a mouse pancreas tumor compared with normal tissue.

Those blood vessels that are present are compressed because the abundant desmoplastic stroma exerts a lot of interstitial fluid pressure. The vessels are also distant from the cancer cells, so it takes longer for an agent to get there through the tenacious extracellular matrix. This observation led to the speculation that this may have something to do with therapeutic resistance.

The hedgehog signaling pathway is relevant in many tissues as a paracrine mesodermal-endodermal interaction. The hedgehog pathway inhibitor IPI-926 is a semi-synthetic derivative of cyclopamine, a natural product. It's a potent inhibitor of Smoothened, and has a long half-life.

In response to IPI-926, the tumor stroma disappears rapidly, and blood vessel content goes up. This results in enhanced delivery of small molecules, including tracers such as doxorubicin and the active form of therapeutics including gemcitabine-triphosphate[1].

By mass spectrometry analysis, Gem-triphosphate in the tissue peaks 2 hours after administration, and that correlates precisely with the peak of cell death in the tumor. These are the types of mouse studies that can then be rolled out into early phase trials in patients.

Gemcitabine delivery improves when it is given after IPI-926. The animals live longer when treated with both drugs. At 10 days after treatment, the vascular density goes back down, so there is a temporary change from hypovascularity to normal vascularity and then back to hypovascularity. The stroma at the end is also different in that it stains differently with chemicals, and is likely to have a different biochemical composition.

Better biopsies:

Translating these studies into the clinic is difficult because typical biopsies for pancreatic cancer are done with a 22-gauge needle and don't preserve stroma or tissue architecture.

But the same needle can be gently and repeatedly advanced and retracted to generate microcores that are quickly fixed in formalin. This preserves the stroma and the ductal epithelial structures. It makes the samples better suited for immunohistochemistry, laser capture and other studies.

This approach has been used to test the effects of Hedgehog pathway inhibitors in patients prior to surgery. More than 30 patients have been assessed and 2 have been enrolled.

In a phase Ib trial of IPI-926 with gemcitabine, 5 of 16 patients with pancreas cancer had a partial radiological response, which is promising. The best responder, who had metastatic pancreas cancer, showed shrinking in both primaries and metastases. But when the randomized phase II was unblinded, the trial hit a futility score. The reason for this clinical failure is currently unclear.

Another therapy that is available in America is abraxane, or nab-paclitaxel, which is a formulation of albumin combined with paclitaxel. When gemcitabine and abraxane are given together in patient-derived xenografts, the combination is extremely active, resulting in significant tumor shrinkage.

In that model, the proposed mechanism is that the stroma is depleted rapidly, inducing the vasculature to proliferate and promoting more perfusion and delivery of gemcitabine[2].

Giving this combination to transgenic mice is complicated because human albumin is extremely immunogenic in a mouse with an immune system. Still, the combination of abraxane and gemcitabine elicits anti-tumor activity in a RAS/p53 double mutant model of pancreas cancer, in which tumor regression is rare.

However, the stroma in these mice looks the same in mice that get the combination treatment, vehicle or abraxane alone. The real difference is in the epithelial cancer cells, which die in large numbers, as seen by co-localization of cleaved caspase 3 (CC3) and E-cadherin. It turns out that there is a massive synergy between abraxane and gemcitabine, but abraxane must be given first.

Mice given abraxane first have a lot more gemcitabine and gemcitabine-triphosphate in the tumor tissue, compared with controls. Gemcitabine typically comes into cells via equilibrated nucleoside transporters, and rapidly gets either degraded or phosphorylated.

The degradation enzyme is cytidine deaminase (CDA), and the phosphorylation enzymes are either TK2 or DCK. A variety of RNA and protein-based assessments show that treating mice with abraxane and gemcitabine together promotes the loss of CDA protein[3]. A proteasome inhibitor rescues this effect, suggesting that abraxane destabilizes the enzyme.

CDA is sensitive to oxidative stress. It's also known that taxanes induce a burst of reactive oxygen species when it hits cells. Giving abraxane decreases CDA levels, and N-acetyl cysteine rescues that effect. This suggests that drug sequencing and antioxidant use by patients should be further investigated clinically.

The last chemical example is of a γ-secretase inhibitor (GSI), which targets presenilin. Presenilin enzymes are relevant in development, and probably relevant in cancer because they modulate the Notch signaling pathway. GSIs were originally developed as potential treatments for Alzheimer's disease.

Notch drives the expression of many genes that are pertinent in proliferation, differentiation and cell death, and there is evidence that it may be involved in ADM transition to pancreas cancer. GSIs have been shown to prevent onset of pancreas cancer in a mouse model that does not have a p53 mutation.

Treatment with a GSI for 72 hours dramatically decreases HES1 in the nuclei of pre-neoplastic cells, as seen by immunohistochemistry. When GSI is given along with gemcitabine, there is less gemcitabine and less GSI in the tumor compared with controls, suggesting that the combination decreases drug delivery.

In this model, the combination of GSI and gemcitabine causes vascular collapse and tumor necrosis. This suggests that the two drugs synergize very differently than the combination of gemcitabine with abraxane. The vascular collapse promotes hypoxia, which activates Notch and Notch-dependent pro-survival signals. Treatment with a GSI potentiates cell death and causes tumor necrosis[4].

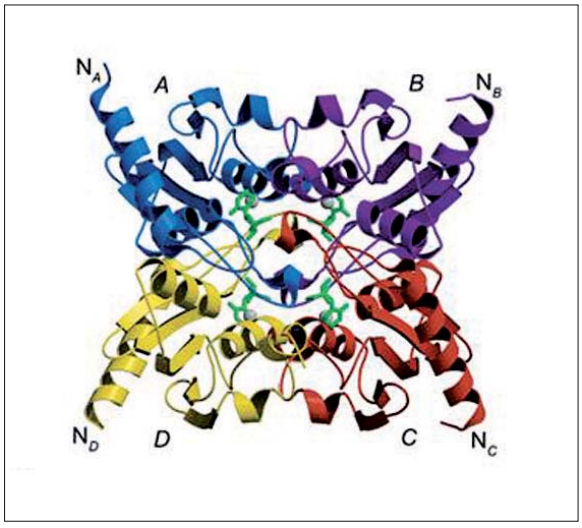

Figure 3
CDA contains 7 cysteine residues, 3 of which are sensitive to oxidation.

Screen shot:

When transposons are used as a way to rapidly screen for genes that cooperate with a sensitizing mutation (see Adams, page 21), the top hit is a gene called USP9X, located on the X chromosome[5].

USP9X is a large gene with 46 exons, therefore challenging to transfect into cells. About 50% of the tumors have this as a common insertion site or CIS. Cells lacking this gene grow fine in 2D cultures. However, loss of USP9X promotes survival in pancreas cancer cells when cultured in suspension.

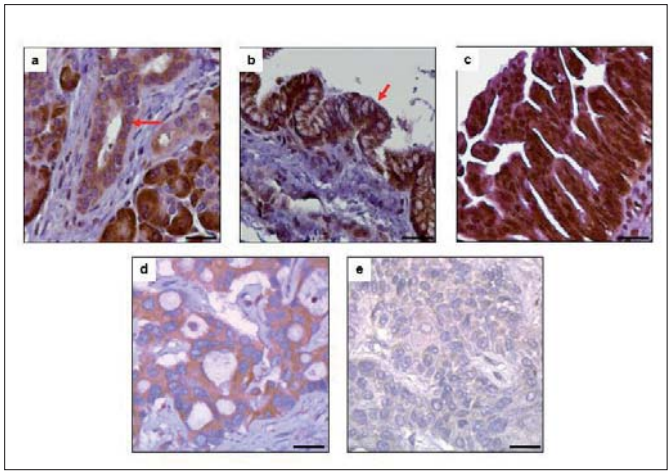

Figure 4
USP9X protein loss is late in human PDAC.

Several papers in 2008 showed that USP9X regulates the cycling of SMAD4 through the nucleus. It deubiquitinates SMAD4, and influences its trafficking and function. However, USP9X does not regulate SMAD4 in pancreatic cancer cells. The loss of USP9X also decreases levels of ITCH, an E3 ligase, and increases levels of a protein that may be mono-ubiquitinated by ITCH.

With wild type USP9X, ITCH levels go up. Restoration of wild type USP9X expression suppresses colony formation dramatically in pancreatic cancer cells that lack USP9X. By contrast, a mutant version of USP9X that lacks deubiquitinase activity does not have this effect. USP9X protein loss is stochastic, and occurs late in pancreas cancer in both humans and mice.

Exome sequencing does not reveal any mutations in USP9X. However, three different clinical cohorts suggest that low expression of USP9X correlates with poor outcome in pancreas cancer. For example, patients in a German cohort who didn't show detectable levels of USP9X had a shorter survival. In a cohort at Johns Hopkins, 50% of patients who had a lot of metastases at the time of death had low levels of USP9X compared with 20% of those who had few metastases.

Finally, data from the ICGC show that patients who have 10 percentile or lower expression of USP9X mRNA, compared with those who are in the top 90th percentile, have a poor outcome.

A conditional knockout of USP9X, made using the PDX-Cre driver, combined with KRAS rapidly develops advanced PAN-INs, micro-invasive PDA and invasive cells.

In summary, this potentially identifies a new pathway, at least in pancreas cancer, in mice. USP9X acts to suppress tumors in mice. It also suppresses anoikis, which may be useful for screening pathways that can be combined with other therapies in efforts to kill pancreas cancer cells.

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Studying therapy response and resistance in mouse models of BRCA1-associated breast cancer

A report on a lecture by

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Mouse models of human cancer provide powerful tools to study drug resistance mechanisms in a realistic in vivo setting. To study the role of BRCA1 loss-of-function in the development of basal-like breast cancer, genetically engineered mouse models based on Cre/loxP tissue-specific deletion of p53 and BRCA1 have been generated. Mammary tumor models carrying specific BRCA1 mutations have also been made. BRCA1 deficiency in patient-derived xenografts is driven either by genetic mutation or by BRCA1 promoter methylation. The tumors that arise in these various models show strong similarity to basal-like breast cancer and are all characterized by a high degree of genomic instability. The tumors are highly sensitive to DNA-damaging agents such as platinum drugs and PARP inhibitors, which indirectly induce double-strand breaks by inhibiting DNA single-strand break repair. However, none of these drugs is capable of eradicating tumors. In vitro functional genetic screens and in vivo genotype-phenotype correlations show that therapy response and resistance is affected by several factors, including the type of BRCA1 founder mutation, drug efflux transporter activity and 53BP1 status. ***Jos Jonkers*** *reported that a cell-based screening approach has found that bifunctional alkylators such as nimustine may cause remission of BRCA-deficient mouse mammary tumors.*

Mouse models, both genetically engineered mouse models (GEMMs) as well as patient-derived xenografts, help explore therapy response and therapy resistance for various cancers.

One of the added values of these models, especially of GEMMs, if designed well is that they develop single tumors driven by mutations as they occur in patients. As such, they can be used as surrogate patients. In cases in which the mice respond well to treatment, they can be used to study minimal residual disease and acquired resistance.

BRCA1-mutated breast cancer represents a small fraction of breast cancers. BRCA1-like breast cancer is a much larger subgroup because it makes up a substantial proportion of triple-negative breast cancer, which accounts for 10 to 15% of all breast cancers.

A mouse model in which a large deletion in BRCA1 is combined with a large deletion in p53 in a tissue-specific fashion shows accelerated tumor development, resulting in BRCA1-null tumors[1].

These tumors show increased genomic instability, as seen by array-based comparative genomic hybridization (aCGH) analysis. There is some instability in the p53 null tumors, but that is greatly amplified in the

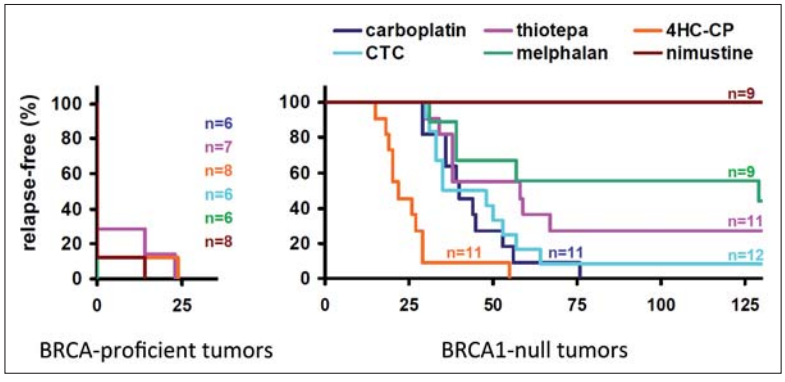

Figure 1
In vivo validation in the BRCA1-null model.

tumors that also have concomitant loss of BRCA1.

Group-wise analysis of these tumors shows a pattern that is highly reproducible, so there is heterogeneity, but there are also recurrent copy number gains and losses. These copy number variations are not unique to BRCA1 deficiency because they occur in the p53 group as well, just to a lesser extent, indicating that BRCA1 functions as a caretaker of genomic stability in tumor suppression.

Another advantage of the BRCA1 model is that it grows mammary tumors that mimic not just the genomic instability phenotype, but also other aspects of the human disease. Typical features include a high-grade undifferentiated nature and the pushing margins, as well as hormone receptor negativity and HER2 negativity.

DNA-damaging agents and certain targeted therapies directly or indirectly induce double-strand breaks that are critical lesions in these tumors, because the absence of BRCA1 makes the tumors defective in double-strand break repair.

Platinum drugs and PARP inhibitors identified in the mid-2000s have all been tested as key synthetic lethal drugs that would cause reduced tissue toxicity and efficacy as chemotherapy agents.

The experiments relied on a spontaneous model of BRCA1-null mammary tumors, as well as BRCA1-null tumor allografts generated by orthotopic transplantation of tumor fragments into wild type animals. The latter allows for interventions at an accelerated pace, and parallel treatments on genetically identical tumors.

The bottom line with chemotherapy interventions is that in most cases, there is acquired resistance after the initial responses, mimicking the clinical pattern. But this is not true for drugs such as cisplatin and carboplatin. Tumors fail to acquire resistance, but at the same time they cannot be eradicated, meaning that this is an ideal model to study minimal residual disease. Apparently, even this perfect tumor drug combination doesn't hit all cells.

Selective screen:

On the basis of this, a compound library screen was performed using isogenic cell line pairs created by reconstitution of BRCA2-deficient mammary tumor cell lines with BAC clones that encode BRCA2. This is a selective screen of 1,280 pharmacologically active compounds. It picks up 97 compounds, including platins, one false-positive compound, and 5 compounds that give selective toxicity in BRCA2-deficient cells[2].

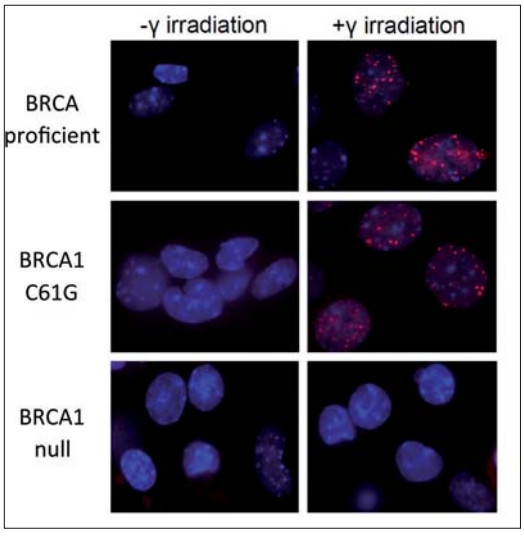

Figure 2
Hypomorphic activity of BRCA1-C61G.

Among these, bifunctional alkylators had not previously been tested in a stratified fashion in BRCA1 and BRCA2 models. Nimustin and, to a lesser extent Melphalan, show complete disease eradication in all or a substantial fraction of the animals.

On the one hand, these bifunctional alkylators somehow outperform cross-linkers such as platin drugs, showing that it's possible to eradicate this disease. Platin drugs create G-G crosslinks, predominantly intra-strand and to a much lesser extent inter-strand crosslinks (ICLs).

By contrast, bifuncitonal alkylators that create G-C crosslinks are much more effective at creating ICLs, so it's possible that the increased load of ICLs in the cells that make up the tumor remnants render the

cells completely defective. In support of this, the G0-G1 cells in the tumor remnants appear to be the cells that give rise to tumor recurrences.

Clinical proof for this concept comes from a retrospective analysis of a study with high-dose chemotherapy, in which patients with advanced breast cancer involving four or more positive lymph nodes were randomized for either 5X standard treatment, or 4X standard plus high dose chemotherapy and autologous bone marrow transfer[3].

Patients with triple-negative breast cancer that have tumors with a sporadic-like genomic profile don't benefit much from one cycle of high dose chemotherapy.

However, those who have BRCA1-like tumors benefit enormously, and their long-term survival goes up from about 30% to close to 80%[4]. This is based on an unplanned long-term retrospective analysis, which is considered a 'dirty' analysis. It also relies on high-dose chemotherapy, which is not very popular these days.

Still, it's interesting and informative for two reasons. It suggests that the right therapies can cure patients with poor prognosis breast cancer. It also suggests that these patients should receive more aggressive chemotherapy.

If nimustine's dose is dropped from 30 to 22.5 mg/kg, tumors regrow and the mice start to relapse. So dose as well as the type of chemotherapy treatment both matter.

With PARP inhibitors, the tumors melt away and in the majority of cases and become non-palpable. But this is a 28-day treatment regimen. After the first treatment cycle, tumors grow back rapidly, and if treatment is resumed, the tumors all become resistant.

Acquiring resistance:

In the BRCA1 null mouse mammary tumor model, acquired resistance to the clinical PARP inhibitor olaparib was in all cases driven by the induction of drug efflux transporters[5]. Combining the PARP inhibitor with a drug that inhibits the pumps re-sensitizes these tumors, but it is unclear whether this is a key issue in the clinic.

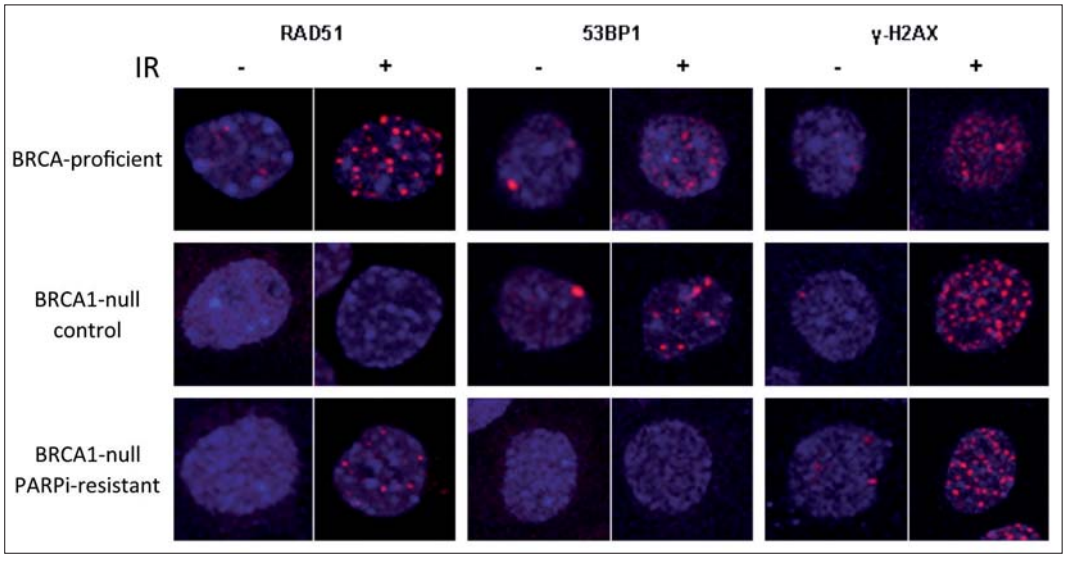

Figure 3
Loss of 53BP1 correlates with partial restoration of RAD51 foci formation.

However, the BRCA1 null mammary tumor model doesn't recapitulate tumors with patient-relevant BRCA1 mutations because it has the artificial deletion of exons 5-13, which prevents re-activation of BRCA1 by genetic reversion. This might also be the reason why the tumors in this model do not acquire resistance to platin drugs.

Research in a BRCA2 mutant cell line has shown that genetic reversion can drive resistance to platin drugs. To investigate this, three additional mouse mammary tumor models with patient-relevant BRCA1 mutations were generated.

Two of them contain mutations in the N-terminal RING domain, and the third has a mutation in the C-terminal domain. Together they make up the most common founder mutations for BRCA1. All three alleles behave like pathogenic mutations, and the genetic profiles of the tumors are indistinguishable.

However, there is an enormous difference in therapy response. The mutations that abrogate the RING domain show rapid induction of therapy resistance without reversing the mutation. This is not true for the allele that contains the C-terminal mutation.

In the case of the BRCA1-C61G mutation, the overall survival falls right in between the BRCA1-null tumors, which show good overall survival, and the BRCA1-proficient tumors. Following tumor volume dynamically over time shows that as soon as the tumors start to shrink, about 5-7 days after inhibition begins, the tumor volume curves start to deviate between the BRCA1-nulls and the BRCA1-C61G, suggesting rapid adaptation rather than clonal selection of rare species.

The same is true for platin drugs. The results are even more striking with cisplatin because the overall survival curve of the C61G-expressing mice is not significantly different from that of mice with BRCA1-proficient tumors.

These alleles are fully pathogenic, but they somehow confer sufficient residual activity to build up rapid resistance to PARP inhibition and to platin drugs. The underlying mechanism is unknown.

Restoring repair:

To identify mechanisms of olaparib resistance other than activation of P-glycoprotein drug efflux transporters, the BRCA1 null mouse mammary tumor model was crossed onto a P-glycoprotein deficient background, yielding a model in which two resistance mechanisms — genetic reversion of BRCA1 and activation of P-glycoprotein — have been genetically eliminated.

The mammary tumors arising in these mice were orthotopically transplanted into syngeneic, wild type recipient female mice, and the resulting tumor outgrowths were treated with olaparib.

It takes several cycles with olaparib before P-glycoprotein-deficient BRCA1 null tumors become resistant. In fact, if olaparib is continuously given, it takes even longer, although resistance eventually develops. Importantly, in all of these tumors, there is still potent inhibition of the drug targets, suggesting that the tumors acquire resistance by somehow rewiring single-strand break repair, or by restoring homologous recombination repair in the absence of BRCA1.

In roughly 25% of tumors, the latter appears to be the case, because exome sequencing shows that in some of the tumors, frameshift mutations occur in 53BP1, leading to loss of the protein. The mechanism for the remaining 75% of tumors is unknown.

In some cases of 53BP1 loss, there are fields that have lost 53BP1, suggesting that there is intra-tumor heterogeneity even at the level of acquired resistance. This indicates that there is a large array of scenarios that could confer resistance to PARP inhibition.

53BP1 seems to work by partial restoration of homologous recombination repair. BRCA1-null tumors completely lose RAD51 foci formation, indicating total loss of homologous recombination repair. However, the resistant tumors that have lost 53BP1 at least partially restore RAD51 foci formation.

Tumor cell line outgrowths that have been depleted for 53BP1 show a survival curve that is as bleak as that of control tumors, whereas the parental cell line shows a survival benefit following treatment with olaparib.

With cisplatin, by contrast, there's a moderate decreased overall survival because the time to relapse decreases. However, tumors remain sensitive to repeated treatments and mice die from cisplatin-associated toxicity rather than from resistant tumors, indicating that 53BP1 loss confers resistance to PARP inhibitors but not to platinum drugs.

What may be going on is that 53BP1 prevents strand resection of DNA double-strand breaks in the absence of BRCA1. In the absence of 53BP1, CTIP — the key enzyme that confers strand resection and therefore sets the switch for homologous recombination repair — can catalyze strand resection, trigger the enzymatic machinery that's independent of BRCA1, and restore homology-directed DNA repair.

Although the clinical relevance of these observations remains to be determined, there is an increased incidence in aberrant expression, that is, a loss or mislocalization of 53BP1 in triple-negative breast cancers[6]. A study exploring whether 53BP1 status stratifies patients in terms of their response to olaparib is under way.

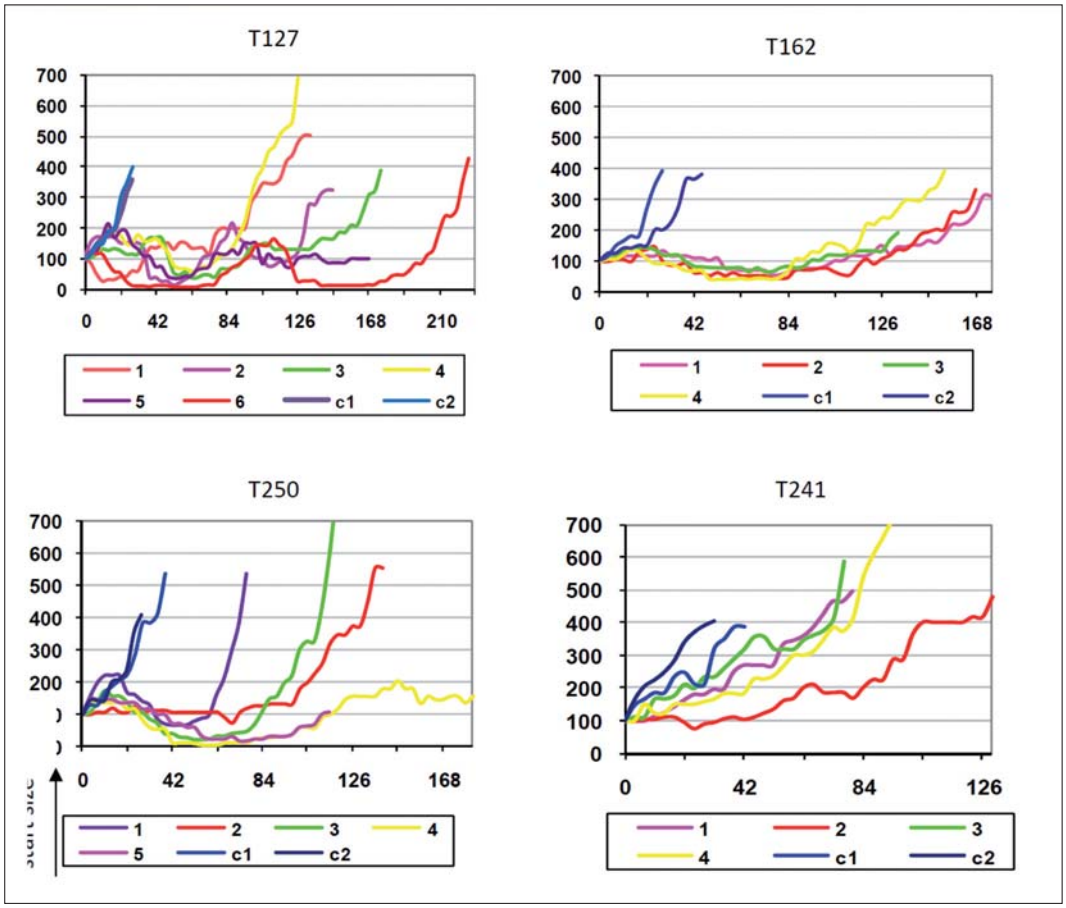

Figure 4
BRCA1-deficient TNBCs acquire resistance to PARPi.

Epigenetic effects:

Since 2008, primary tumor fragments from breast cancer patients have been propagated in immunodeficient mice, comparing both the histopathological phenotype, as well as the aCGH profiles of these outgrowths with the original tumor. For triple-negative breast tumors using unsupervised classification, all of the tumors co-cluster with the originals from the patients.

Of interest within the triple-negative domain are tumors with a central frameshift mutation in BRCA1, but perhaps more importantly, BRCA1-methylated tumors.

When BRCA1-methylated tumors are treated with chemotherapy agents and with PARP inhibitors, they show durable responses going up to 200 days. The time it takes tumors to develop resistance is similar or even longer than that for BRCA1-mutated tumors, so the BRCA1 promoter methylation imprint appears to be very stable.

In the case of the BRCA1 mutated tumor, deletions that restore the reading frame drive the acquired resistance. Among the BRCA1 methylated tumors, not all cases that show resistance have re-expression of BRCA1. More importantly, of those cases that show re-expression of BRCA1, only half show loss of promoter methylation.

This suggests that there is a genetic mechanism that mediates reactivation of BRCA1. Indeed, this turns out to be the case because simple 5' RACE shows that at least in a fraction of the resistant cases, heterologous promoters became juxtaposed with BRCA1 coding sequences.

The BRCA1-methylated tumors are genomically unstable, and rearrangements may therefore occur more frequently in these tumors versus those that are more stable. But it's also possible that changes in methylation, at least when they arise at the level of the enzymatic activity, may have more global consequences, whereas genetic rearrangements are more local and don't affect other regions in the genome.

In summary, the full range of responses is recapitulated in GEMM models. With optimized PARP inhibitors, the disease could be controlled in a chronic setting as long as the compartment of residual tumor cells can be minimized, so that the risk of creating clones that utilize a resistance mechanism is minimized.

In the case of the platin drugs, BRCA1 activity is critically required to build up resistance. The RING domain is not relevant here, but activity at the C terminus is critically required to build up resistance. The observation that certain bifunctional alkylators are capable of eradicating BRCA1-null tumors suggests that it should in principle be possible to target residual tumor cells with optimized combinations of targeted therapeutics.

The second observation is that the synthetic lethal paradigm, in which the only way to escape a perfect tumor drug combination is to revert the lesion, is not entirely true. In addition to inactivation of BRCA1 status, there is a range of other parameters that may determine responses to PARP inhibition in the clinic.

In conclusion, there is a unique value of both genetically engineered mouse models and patient-derived tumor xenograft models for studying minimal residual disease and resistance mechanisms, and genotype-phenotype correlations in relation to therapy response and resistance.

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PART V: Metabolism and beyond in mouse models

Michael Karin
Mouse models of inflammation and obesity's role in cancer

Reuben Shaw
Decoding metabolic reprogramming by the AMPK pathway in cancer and metabolic disease

Neal Rosen
Are mouse models useful for cancer research?

Mouse models of inflammation and obesity's role in cancer

A report on a lecture by
Michael Karin
University of California School of Medicine, San Diego, USA

Inflammation has been suspected for more than 150 years to be a major contributor to human cancer. An estimated 20% of human cancers are thought to be inflammation-related. Another major factor contributing to cancer development is obesity, whose effect is most pronounced in cancers of the liver and pancreas. Despite the important role of inflammation and obesity in human tumorigenesis, the mechanisms through which they operate have remained elusive until the development of appropriate mouse models. These models have elucidated a major role in colon cancer for the pro-oncogenic transcription factors NF-κB and STAT3 and for inflammatory cytokines, such as interleukin-6 and tumor necrosis factor, that control their activity. Inflammation is also important in spontaneous colorectal cancer. Barrier disruption in adenomas evokes the activation of tumor-associated macrophages, which produce IL-23, leading to activation of an IL-17-driven pro-tumorigenic immune response. In liver cancer, IL-6 overproduction in fatty liver leads to sustained STAT3 activation in pre-malignant hepatocytes. These results are in excellent agreement with human studies. ***Michael Karin*** *argued that, in addition to unraveling basic mechanisms underlying tumorigenesis, mouse models are important for developing therapeutic and preventive strategies.*

Inflammation is part of the body's host response. Normally triggered by invasion of microbes, either pathogenic or commensal, inflammatory responses lead to activation of resident tissue macrophages.

Sterile inflammation is elicited not by microbial products, but by normal cellular proteins released by necrosis, but the outcome is the same. How this transient protective response turns into a persistent chronic response is little understood.

Chronic inflammation increases the risk of some cancers, accounting for 15-20% of cancer-related deaths. Most cancers do not develop in the context of underlying inflammatory disease, yet advanced tumors are often full of inflammatory infiltrates, suggesting that inflammation and cancer are somehow related. Inflammation seems to play an important role in colorectal cancer, for example.

Colorectal cancer is the third leading cause of cancer deaths in the U.S. The molecular etiology of colorectal cancer is well known. Activation of β-catenin initiates the cancer, and additional mutations in KRAS and, eventually, loss of TGF-β signaling and p53 convert adenomas to invasive carcinomas.

A rare form of colorectal cancer called colitis-associated cancer develops in patients who have ulcerative colitis. In this case, inflammation precedes tumor development by many years or even decades.

In addition to the standard molecular events that drive colorectal cancer, the activation of NF-κB and STAT3 in intestinal epithelial cells is also of importance in colitis-associated colorectal cancer. In addition, NF-κB activation in lamina propria macrophages is involved in the production of growth factors that stimulate the proliferation of the premalignant cells, endowing them with a survival advantage[1].

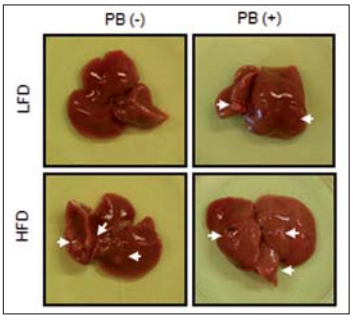

Figure 1
Obesity is a promoter of liver cancer development.

These growth factors include inflammatory cytokines such as tumor necrosis factor (TNF), and interleukins (IL)-1 and -6. This suggests an ongoing interaction between premalignant epithelial cells, and eventually cancer cells, with various components of the immune system, such as macrophages, dendritic cells and T cells[2]. Once oncogenic pathways are established, they lead to increased production of chemokines that further shape the inflammatory microenvironment of the tumor.

The major form of colorectal cancer, however, is the spontaneous type, which is diagnosed by finding blood in stool or by colonoscopy. At stage 1 or 2, patients diagnosed with colorectal cancer have about 80-90% chance of survival, but at stage 3 or 4, survival drops to 10%.

Gene profiling experiments in both mouse models and human colorectal cancer have failed to find any differences between spontaneous and colitis-associated colorectal cancer. Both exhibit the same kind of inflammatory signature, suggesting that by the time spontaneous colorectal cancer is diagnosed, inflammation has been activated.

Useful models:

One important immunoregulatory cytokine is IL-23, which is produced mostly by macrophages and dendritic cells in response to immune challenges. The protein consists of the p19 subunit, and the p40 subunit, which it shares with IL-12. IL-23 is a heterodimeric cytokine, and signals to a heterodimeric receptor, which activates JAK2 kinase and the transcription factor STAT3.

Unlike receptors for other cytokines such as IL-6, the IL-23 receptor is not expressed in epithelial cells but acts within the hematopoietic compartment. Interestingly, samples of human spontaneous colorectal cancer exhibit a pronounced increase in p19 expression, as well as of another cytokine, IL-17a, relative to normal colonic tissue from the same patient.

Many labs use APC^{min} mice to model spontaneous colorectal cancer. However, this is not a good model because most of the tumors are microadenomas that develop in the small intestine, whereas colorectal cancer develops in the distal one-third of the colon. What's more, the tumors must be large enough to dissect, analyze by microarray and other biochemical assays, which is difficult to do with the APC^{min} model.

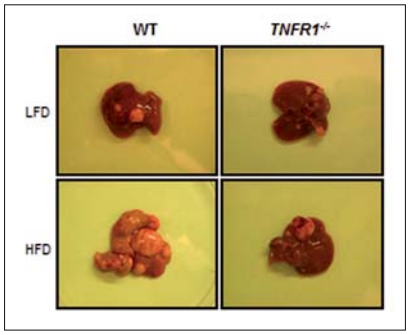

Figure 2
TNFR1 is required for obesity-mediated tumor promotion.

A more useful mouse model is based on inducible loss of one APC allele, relying on loss of heterozygosity to get rid of the second allele, so it mimics the pathogenesis of most cases of human colorectal cancer. Most of the tumors in these mice, called CPC-APC, appear in the right place, are of the right size and have the right histology. They also show an increase in expression of IL-23, IL-17F, IL-17A and GMCSF, and a modest increase in IL-22.

This is interesting because colorectal cancer patients with a strong IL-23/Th17 signature in stage 1 and 2 tumors show rapid progression to metastatic disease within two years. Those who have low levels of IL-17 conversely show slow progression.

Using flow cytometery to isolate the different cell types from colonic tumors in CPC-APC and qPCR analysis reveal that the p19 and p40 subunits of IL-23 are mainly produced in tumor-associated macrophages.

Crossing the CPC:APC model with a knockout of either the p19 subunit or the IL-23 receptor both decreases tumor formation and growth and slows down progression. When radiation chimeras are generated and given bone marrow lacking either the cytokine or its receptor, they show a similar decrease in tumor growth and tumor load.

This suggests that both the cytokine and its receptor act in the hematopoietic compartment. Despite this, ablation of IL-23 or its receptor both affect the activation of STAT3 in epithelial cancer cells. However, it is not possible to do conditional knockouts of STAT3 in this model because CPC-Cre deletes STAT3 in the limbs and tail and the mice become paralyzed.

Inactivating IL-23 signaling reduces production of other tumor-associated cytokines, such as IL-6, IL-17 F and A, and IL-22. It also greatly reduces the tumoral content of Th17 cells, the T-helper cells that specialize in the production of IL-17 and IL-22. Knocking out the IL-17 receptor also results in a reduced tumor number and size, effects similar to that of IL-23 inactivation. In summary, IL-23 expression is up-regulated in tumor-associated macrophages compared with adjacent normal tissue. This results in higher tumoral content of Th17 cells, which stimulate tumor growth and progression through IL-17 receptors.

Permeable tumors:

Normally, IL-23 is induced in macrophages in response to bacterial infection, signaling through Toll-like receptors (TLRs). When MyD88, a critical adaptor protein for TLRs, is knocked out, it wipes out IL-23 production. IL-1 and IL-18 receptors also depend on MyD88. An IL-18 receptor knockout has no effect, and the IL-1 receptor is also unlikely to be involved.

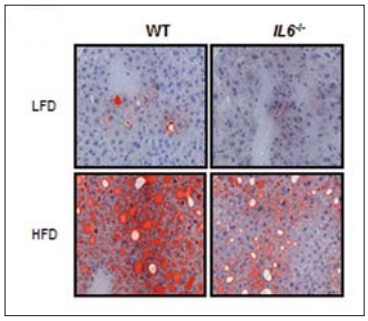

Figure 3
IL-6 sustains hepatosteatosis and steatohepatitis.

Supporting a role for TLRs, which are activated by microbial products, mice treated with a combination of 6 different antibiotics produce much less IL-23 and IL-17 and show reduced STAT3 activation, within 2 weeks of treatment. After 3 months of antibiotic treatment, reduced tumor load is observed.

By PCR sequencing and FISH for bacterial nucleic acids, occasionally there is bacterial 16S RNA within the tumor, but never within the normal tissue. Given that the anatomical location of these tumors is the colon, which is home to trillions of bacteria, the likely explanation for this is that there is increased penetrance of bacterial products. It's estimated that people excrete about a pound of lipopolysaccharide (LPS) endotoxin each day in their stool.

Still, most people don't develop colitis, so it may be that the tumors are more permeable to LPS. One way to assess permeability is to generate so-called colonic loops. This involves clamping the colon of a live mouse and injecting the dye FITC-dextran.

In a normal mouse, there's very little penetrance of the dye into the circulation, but tumor-bearing mice show a lot of fluorescence in the blood within one hour after injecting the dye, indicating a huge increase in permeability. Injecting a more relevant probe, Alexa-488-LPS, shows that the LPS fluorescence penetrates specifically into the tumor, and not into the normal tissue next to the tumor.

There are two major components of the intestinal epithelial barrier. First, mucins provide a coating of mucus, which prevents bacteria from reaching epithelial cells. One of the mucins, MUC2, also has antimicrobial properties and is an important component of the barrier.

Mucins, and specifically MUC2, are expressed by the normal colonic mucosa, but not in tumors. MUC2 knockout mice develop colitis if they're kept in a dirty facility and eventually develop colitis-associated cancer. But they do fine if kept in clean facilities.

Other components of the epithelial barrier are tight-junction proteins such as the junctional adhesion molecules A, B and C. JAMA and JAMB are strongly down-regulated in human colorectal cancer and JAMC is down-

regulated in the mouse adenomas. Claudin 4, another component, is up-regulated at the mRNA level, but the protein is not transported to tight junctions.

The hypothesis based on these data is that defective tight junctions allow bacterial products to penetrate the tumors, eventually increasing expression of IL-23 and IL-17 in the tumor-associated macrophages and Th17 cells. If this holds, patients with a high IL-23/Th17 signature may need to receive small molecules that block Th17 formation, or IL-23 neutralizing antibodies.

Obesity effects:

In the US, an estimated 15% of cancer deaths can be attributed to obesity. The effect of obesity is most significant on liver and pancreatic cancers.

Hepatocellular carcinoma (HCC) is a slow-growing cancer that is associated with chronic liver inflammation. In certain parts of the world, most of this inflammation is a result of viral hepatitis. In the US, much of it is obesity-induced, leading to a condition called non-alcoholic hepatitis (NASH), driven by fat accumulation within the hepatocytes.

A commonly used model used to study HCC in mice has been much criticized, but it has provided a lot of insight into the disease. In the model, mice are given diethyl nitrosamine (DEN), which induces oncogenic mutations and also kills many cells. The hepatocytes that die by necrosis release substances, such as IL1-α, that lead to the activation of Kupffer cells.

Illustrating obesity's effect, combining DEN administration with a high-fat diet in 2-week-old mice substantially increases tumor development. This effect is bigger in males than in females, similar to what happens in human HCC. If the carcinogen is delayed until the mice are 3-4 months of age, it has no effect. However, when it is combined with a tumor promoter, or the mice are given a high-fat diet for 3 months prior to DEN administration, it results in efficient tumor induction.

Insulin is often thought to drive tumor development in obese individuals which are hyperinsulinemic, but is unlikely to be important in the liver, because the liver of obese mice and humans is insulin-resistant.

Obesity down-regulates AKT phosphorylation and p38, but activates JNK, ERK and STAT3, both in tumor and non-tumor tissue. STAT3 activation is mostly due to overproduction of IL-6, which is produced in response to activation of TNF signaling.

The knockout of TNFR1 has no impact on tumor induction and development in lean animals, but it abolishes the tumor-promoting effect of obesity. The knockout mice still get fat, but the TNFR1 deficiency affects the distribution of fat in the body.

In mice that lack either IL-6 or TNFR1, there is very little fat accumulation in the liver and, as a result, less infiltration of macrophages into the liver. This suggests that TNFR1 and IL-6 sustain hepatosteatosis and steatohepatitis[3].

Activation of STAT3 is itself extremely important. Knocking out STAT3 in the liver inhibits tumor development substantially, indicating that STAT3 activity is required for HCC formation and growth.

Inhibiting autophagy:

The other effect of obesity, which is inhibition of AMPK and activation of TORC1, may also be important in HCC. TORC1 activation can lead to inhibition of autophagy. One aspect of this is the accumulation of p62, which is a chaperone for poly-ubiquitinated proteins[4].

When IKKβ is knocked out and NF-κB activation in hepatocytes is inhibited, HCC development is strongly augmented. Mice lacking IKKβ in hepatocytes express high levels of p62 in the liver. Administration of DEN further increases p62 accumulation compared with control mice. In mice lacking both IKKβ and STAT3, this inhibition is relieved by an unknown mechanism.

Knocking out p62 slows down the development of hepatic adenomas in mice that are already compromised by the knockout of ATG7 or ATG5.

However, fatty liver is not NASH. A new model of NASH relies on MUP-uPA mice, whose livers are subject to chronic regeneration. When these mice are given a standard high-fat diet — with lots of fat but no cholesterol — they accumulate fat and develop ballooning hepatocytes. This same feature is seen in human and mouse NASH.

Importantly, this mouse model also develops bridging fibrosis, another feature of NASH. The mice also accumulate p62-mediated protein aggregates called Mallory bodies, a classical feature of human NASH.

When IKKα is knocked out in the pancreas, it results in defective completion of autophagy and a massive accumulation of p62. In addition to tying up poly-ubiquitinated proteins, p62 can also interact with KEAP1. KEAP1 inhibits NRF2 so this can lead to NRF2 activation, which protects cells from KRAS-induced oxidative stress and senescence.

Accumulation of p62 is present in chronic pancreatitis, becomes more apparent in PanIN lesions, and develops into very strong aggregates in ductal adenocarcinoma. This correlates with up-regulation of NQO1, a classical target for the NRF2 pathway.

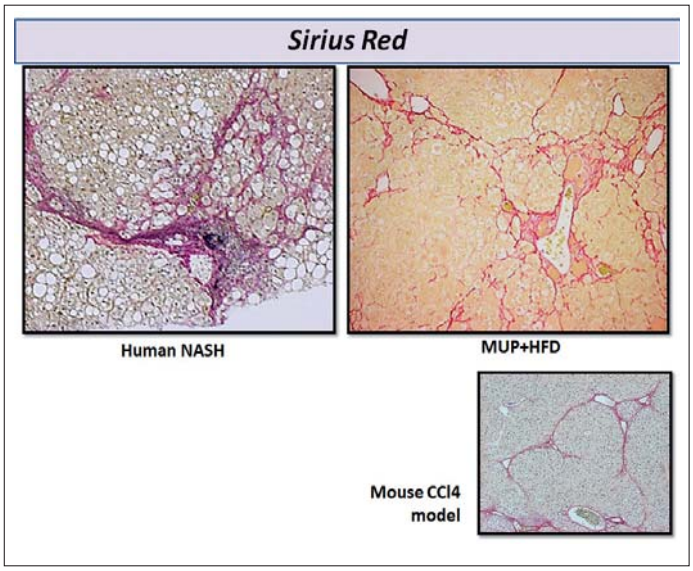

Figure 4
The pattern of fibrosis in MUP+HFD is similar to human NASH.

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Decoding metabolic reprogramming by the AMPK pathway in cancer and metabolic disease

A report on a lecture by
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AMPK is a highly conserved sensor of cellular energy status found in all eukaryotic cells. It maintains cellular metabolic homeostasis by reprogramming growth, metabolism and autophagy in the face of cellular stresses. AMPK is activated by direct binding of AMP and ADP to its regulatory subunits, which enhances its phosphorylation by its upstream kinase, LKB1. Notably, LKB1 is a tumor suppressor gene frequently inactivated in sporadic human lung cancer, cervical cancer and the familial cancer disorder Peutz-Jeghers Syndrome. AMPK is also activated by a number of diabetes drugs, and beneficial effects of AMPK activation have been reported in a variety of metabolic diseases. Genetic inactivation of LKB1 confirms its dominant role in suppression of both cancer and metabolic disease. LKB1 is essential for the anti-diabetic action of metformin, the most widely used type 2 diabetes drug. A three-pronged proteomic screen has identified novel substrates of AMPK and its related family members, also regulated by LKB1, that may mediate the downstream effects on metabolism and growth control. For example, AMPK directly phosphorylates and activates the ULK1 kinase to initiate autophagy, and for mitochondrial homeostasis and cell survival under starvation conditions. ***Reuben Shaw*** *discussed the potential for using metabolic drugs such as metformin and phenformin as anti-cancer agents.*

The AMP kinase (AMPK) pathway is completely conserved throughout eukaryotes. In *Arabidopsis*, for example, mutant AMPK causes growth defects and overgrowth under conditions of altered carbon sources and in *C. elegans*, it ties in with lipid storage, cell growth and aging. Overall, in all the different species in which it has been studied genetically, it has similar phenotypes of deregulation of metabolism as well as cell growth. In humans and in mice, deregulation of this pathway gives rise to cancer.

Oncogenes and tumor suppressor genes acutely drive signaling pathways that not only control cell proliferation, but coordinately reprogram cell metabolism. A tumor suppressor called LKB1, a serine/threonine kinase, is mutated and inactivated in Peutz-Jeghers Syndrome. This disease is an autosomal dominant inherited cancer disorder characterized by gastrointestinal polyps and early-onset tumors. LKB1 is also the third most common mutated gene in non-small cell lung cancer (NSCLC), often coincident with KRAS mutations. It is also commonly altered in human cervical cancer.

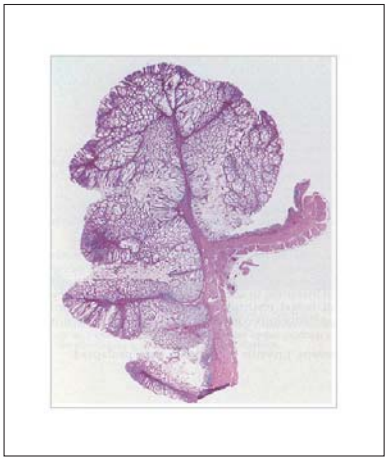

Figure 1
How are cell growth and cell metabolism coordinated?

Interestingly, in both cases, LKB1 seems to harbor a secondary mutation. In NSCLC, for example, the adenomas contain KRAS mutations and LKB1 is only found at the progressive stage. In human cervical cancer, HPV is the causal agent in probably 100% of cases, and LKB1 mutations are secondary.

Surprisingly, AMP activated kinase is among LKB1's conserved substrates. AMPK has been well-studied in the context of metabolism. In mammals, a number of metabolic hormones, as

well as exercise, stimulate and alter AMPK activity. AMPK is also a marker of efficacy of metformin, the most widely used diabetes therapeutic in the world.

There are a number of other pharmacological agents that can activate AMPK in an LKB1-dependent way. Many stresses, hormones and nutrient changes cause changes in the intracellular ATP level. When ATP levels fall, it triggers proportional increases in both AMP and ADP. A regulatory subunit of AMPK conserved across evolution contains three pockets that preferentially bind to AMP and ADP, and that puts the kinase complex into a conformation that favors its phosphorylation by LKB1, its upstream kinase.

This seems similar to ATM activation of CHK2, and the classic DNA damage checkpoint. The stress in the case of LKB1 activation of AMPK is loss of intracellular ATP, and this intracellular circuit of kinases aims to inhibit ATP-consuming processes, particularly biosynthetic processes, and then acutely upregulate processes to restore ATP levels.

'Lazy' mice:

In a wide variety of epithelial lineages, loss of LKB1 is sufficient to trigger hyperplasia, if not frank carcinoma, for example in the endometrial compartment. In other tissues, mirroring the human condition, there is strong synergy of LKB1 with other oncogenes, but LKB1 loss by itself doesn't generate a tumor.

Interestingly, if LKB1 is deleted in other epithelial lineages, including in hepatocytes, it does not lead to hyperplasia, although the blood glucose levels in these mice double within a week and they develop several signs of frank diabetes. If LKB1 is deleted in the skeletal muscle, the muscle architecture is largely normal, but the mice become 'lazy' and have defects in voluntary exercise.

These are interesting and quite distinctive phenotypes, all made with the same floxed allele, so the disparate phenotypes are not due to some off-target effect of one lab's mice. Conversely, if this pathway is activated, for example with metformin, blood glucose drops, using the activator AICAR, the skeletal muscle is programmed and the mice become capable of endurance running[1].

LKB1 regulates a family of 14 kinases related to AMPK, and the molecular function of more than half of these is poorly understood. An iteration of a peptide library methodology identified the key conserved substrate motif of AMPK and related kinases downstream of LKB1 in a search to identify substrates involved in prevention of cancer and diabetes. In this approach, more than 200 peptide libraries are spatially arrayed and screened for selectivity at each individual amino acid position relative to a central fixed residue.

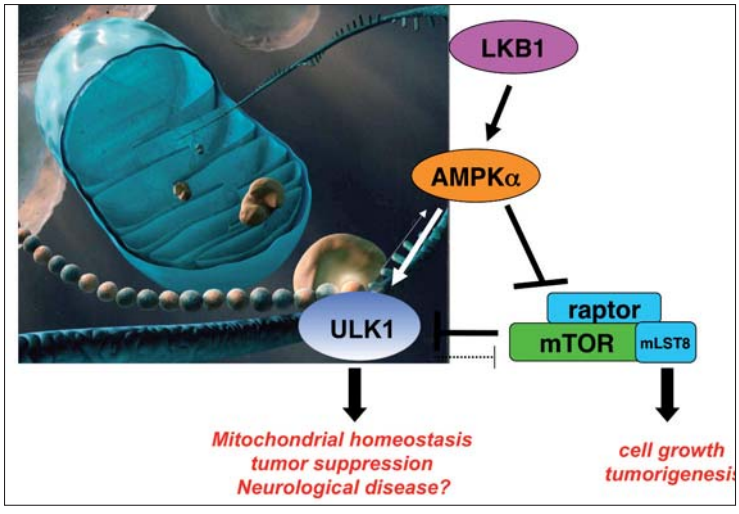

Figure 2
The interconnected activity of kinases may dictate a large amount of cell growth, cell survival, cell fate and metabolic decisions.

This approach shows that AMPK and the related family members are very selective, and require 3 or 4 different residues relative to a central serine in order for something to be phosphorylated *in vitro*.

A three-part screen then identifies conserved AMPK family substrates. The first

part of the screen uses bioinformatics to scan websites such as Prosite or Scansite for *in vivo* sites that match the consensus. However, even with something this specific, there are between 1,000 and 1,500 different proteins in the human genome or the mouse genome that contain an element like this.

The second part of the screen relies on mass spectrometry databases and functional assays, including an AMPK-substrate motif antibody, and immunoprecipitation from genetically defined cells and tissues in an LKB1- or AMPK-dependent manner.

The third arm of the screen is based on the fact that half of the known substrates, when they are phosphorylated by AMPK or a related kinase, create a 14-3-3 docking site on the protein. That means that 14-3-3 can be used as an affinity trap to find proteins that only bind it in a wild type tissue as compared with an LKB or AMPK knockout.

Hunting targets:

One big question in the field is whether it is possible to find targets of AMPK that are involved in known cancer pathways. The only pathway that is consistently deregulated in different tumor types lacking LKB1 is the so-called mTORC1 signaling pathway. Both in LKB1-deficient tumors as well as in normal cells undergoing proliferation, when LKB1 is inducibly deleted, or blocked with siRNA, mTORC1 signaling comes blazingly on.

TSC2 is a target of AMPK, but is not well-conserved through lower eurkaryotes. The critical mTOR binding partner raptor is also a key conserved target of AMPK, and its phosphorylation sites are conserved through yeast and *Arabidopsis*. This represents one of the most fundamental and conserved signals that all cells use for responding to nutrient status in their environment and then coupling that with growth control[2]. The mTORC1 pathway appears to be a common convergence point for LKB1-AMPK and RAS-PI3K pathways.

When cells adapt to changes in their environment, prolonged changes are often adapted at the transcriptional level. In yeast, AMPK is SNF1, for sucrose non-fermenting 1. Although SNF1 has a similar phosphorylation motif, none of its targets are conserved in mammals.

In the liver, there are profound defects in both glucose and lipid metabolism, and they are mediated at the transcriptional level, recently discovered to be in part by a family of conserved HDACs, or histone deacetylases.

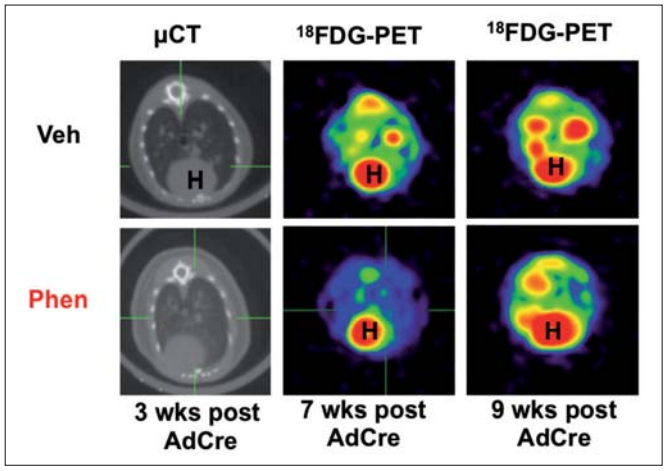

Figure 3
Phenformin induces a therapeutic response in KRAS LKB-/- lung tumors.

There are a few subclasses of HDACs, and elegant genetic studies have shown that the Class IIa HDACs play critical roles in muscle differentiation. These enzymes are true bonafide targets of AMPK and its related kinases. Phosphorylation of two particular N-terminal residues in these proteins by AMPK drives the protein to the cytoplasm and inactivates it. That suggests that the LKB1 pathway acts to inhibit the function of these HDACs by simply driving them out of the nucleus.

In the absence of the class II HDACs, expression of FOXO target genes in liver are completely lost. Class II

HDACs bind to and control the endogenous acetylation of FOXO, with deacetylation activating FOXO. These HDACs are rate-limiting for blood glucose levels, and suppressing them by shRNA in the liver dramatically drops blood glucose levels in a number of diabetes models[3].

In A549 cells, a commonly used lung cancer cell line that is KRAS mutant and LKB1-null, introducing either a wild type copy of LKB1 or a kinase dead copy shows that phosphorylation of HDACs on the two N-terminal sites is LKB1-sensitive. The next step is to find the targets of these HDACs in these lung cancers because the targets there will be different than the glucose synthesis genes they control in the liver.

A transcriptional regulator that is of high relevance but is poorly understood in terms of its role in cancer is the SREBP1 transcription factor. This protein controls fatty acid and cholesterol synthesis by regulating all of the genes involved as a cassette, almost in same way that *E. coli* and yeast regulate metabolism.

AMPK directly phosphorylates Ser372, a conserved site in SREBP1 right near its cleavage site. This inhibits SREBP1 activity, and provides a molecular explanation for how LKB1-null livers have dramatic elevations in lipid.

This result has implications for both diabetes, because drugs that activate AMPK suppress both lipogenesis and risk of atherosclerosis, and for cancer. In cancer cells, lipid and cholesterol synthesis are needed to fuel cell proliferation, so this suggests potential treatment avenues[4].

Autophagy cascade:

It is possible to reprogram metabolism when there are small changes in nutrient levels, but with acute drops in blood glucose availability or in response to stress, the problem must be rapidly fixed. One way to do that is through autophagy.

One of the major hits from all three arms of the screen is the initiating kinase in the autophagy cascade, ULK1 or, in budding yeast, ATG1. ULK1 is a direct substrate of AMPK, with four sites that contain the AMPK consensus.

In the liver, autophagy occurs constitutively on a 24-hour cycle. When ULK1 is deleted, or if the AMPK1 sites in ULK1 are mutated, there is a dramatic accumulation of defective mitochondria. This triggers changes in the mitochondrial membrane potential, which results in increased levels of reactive oxygen species.

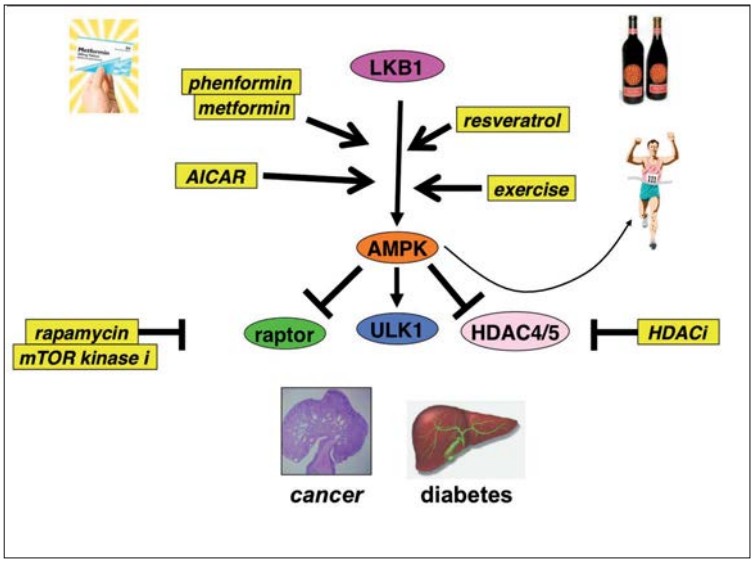

Figure 4
The LKB1/ AMPK pathway sits at the nexus of cancer and metabolism.

In most contexts, autophagy is a cell-survival mechanism. It's a way for the cell to cannibalize itself to stay alive until it can get additional nutrients. When an RNAi to ULK1 is added to cells in starvation media, the cells,

which are defective in autophagy, rapidly start undergoing apoptosis. The kinetics and the magnitude of this effect are dependent on ULK1 kinase activity, as well as on the AMPK sites in ULK1.

AMPK is known to be the central regulator of mitochondrial biogenesis. It's also responsible for targeting defective mitochondria and degrading them, a process called mitophagy[5]. That means that a single kinase sensor encodes the ability to direct the cell to both trash the existing defective mitochondria and then rebuild new mitochondria from scratch.

Deletion of LKB1 in the hematopoietic stem cell compartment results in one of the most dramatic phenotypes in terms of loss of pluripotency. This correlates with the loss of ATP and the strange accumulation of mitochondria even though the ATP levels are lower. The therapeutic implication is that LKB1-null cells can no longer engage autophagy properly, and should be sensitive to any agent that can lower ATP levels.

Many drugs lower ATP levels and increase AMP and ADP. LKB1-deficient cells, including tumor cells, have lost their sensor and have no idea that their ATP levels have fallen until it is impossible to restore them.

In a screening of existing metabolic drugs to find ones that would selectively kill only LKB1-null cells, the metformin analog phenformin is the most potent. Metformin is a widely prescribed diabetes drug, derived from a plant. It's a simple biguanide compound that had no mechanism of action when approved for clinical use in the 1950s.

More than 100 million people have taken this drug, and more than 50 million have been taking it for well over a decade. Early studies support the reduced cancer risk in those who take metformin.

In A459 cells, phenformin appears to drive cell death by stimulating mitochondrial ROS. LKB1-deficient NSCLC cells, A459 cells and others have elevated levels of mitochondrial ROS to begin with, as read out by MitoSOX. Phenformin disrupts the little bit of mitochondrial membrane potential that's left, generating a burst of mitochondrial ROS.

In various NSCLC mouse models, phenformin treatment at least modestly blunts the overall tumor burden, but in the KRAS LKB1-deficient lung model, it significantly decreases tumor burden and increases survival.

Phenformin as a single agent can reduce tumor burden and extend survival, but to effectively treat LKB1-deficient NSCLC tumors, mTOR inhibition, inhibition of subgroups of HDACs or even statins could be combined with phenformin. What's needed is also a clear idea of the genotypes that confer sensitivity and resistance to metabolism-based drugs.

Effective targeted therapeutics should result in the collapse of oncogene-driven metabolism, including glycolysis. This should activate AMPK (and ULK1) as a means to restore homeostasis and stabilize the cancer cell's metabolism. Combining an AMPK or ULK1 inhibitor with a targeted therapeutic may be another effective therapeutic option in advanced cancers that rely on AMPK and ULK1 to help restore energy homeostasis following treatment with targeted therapeutics.

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Are mouse models useful for cancer research?

A report on a lecture by
Neal Rosen
Human Oncology and Pathogenesis – MSKCC, New York, USA

There are primarily four types of models to investigate hypotheses in cancer: cell culture, xenografts, genetically engineered mouse models, and tumor tissue taken from patients. The last one is the only one that is highly likely to generate results that are relevant to human cancer. Results from the other types of models may be irrelevant or even misleading. In particular, studies often rely far too much on genetically engineered mice, without validating the results in patient biopsies. Over the past decade or so, few therapies have resulted from work on mouse models. From an oncologist's perspective, any good model must identify and validate targets and inhibitors. It must also help determine the ideal drug dose, delivery schedule and the degree of inhibition required, and the therapeutic index. Genetically engineered mouse models can't provide any of that information with accuracy, nor can they help test drug combinations. They are also inadequate at modeling pathway inhibition. ***Neal Rosen*** *argued that to be useful, genetically engineered mouse models must be used concordantly with patient biopsies, in order to prove that the models mimic what happens in patients.*

Genetically engineered mice are undoubtedly relevant therapeutic models of human cancer. However, they are sometimes erroneously considered to be the only relevant therapeutic models, to the exclusion of xenografts, cell lines or other models that have led to effective therapies.

In fact, very few successful therapeutic strategies have been formulated from insights derived from genetically engineered mouse models (GEMMs), primary xenografts and three-dimensional (3D) cultures. Although 3D cultures or primary xenografts generate different, provocative data, there is an unfortunate trend where models like these are presumed to be better than they are, without actual proof.

The first step to making these models relevant is to reproduce the findings in human models. For example, one drug company had the first five primary xenografts of RAF mutant melanoma which, when treated with a MEK inhibitor, not a RAF inhibitor, were all cured. But is this any more relevant than xenografts, in which they're never cured?

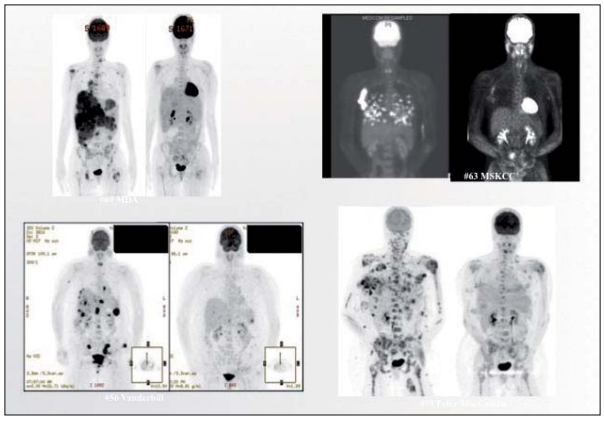


Figure 1
PET Scans at Baseline and Day 15 after PLX4032.

There are arguably four types of models: cell culture, xenografts of some sort, GEMMs, and humans. But results from the first three of these models may be irrelevant or even misleading. Instead, studies should be based on more patient biopsies, at various times before and after therapy, and on comprehensive ways of analyzing the molecular biology of those tumor biopsies. Better mouse models may require fewer biopsies, but at the moment, biopsies are unavoidable.

What do oncologists need from therapeutic models in order to treat patients? First, the model has to identify and validate targets and inhibitors. For example, cell lines are great for tumor autonomous targets. Various drugs make the cells stop growing, or make them die, suggesting which of the drugs might be effective.

Then there are targets that depend on normal tissue interactions. Obviously, cell lines and xenografts are hopeless for these because they don't take into consideration the microenvironment, the endocrine environment and the organismic environment of the tumor.

Oncologists also need to know what dose of the drug to deliver, the schedule at which the target should be inhibited and the degree of inhibition required. There is no evidence that GEMMs can determine any of these details.

Also crucial is the therapeutic index. For example, there are many drugs that can inhibit the PI3K, RAS or ERK pathways, but the toxicity of those drugs is, by and large, skipped. Mice don't usually exhibit toxicity with the drugs, making mice perhaps a bad model for investigating these drugs. There are also some drugs that may be more toxic to mice than to people.

Fourth, oncologists will need combinations of drugs, as single agents are usually ineffective. But developing combinations is difficult to do in mice. The therapeutic index of combined target inhibition is also key, and has to be modeled. Some drugs and combinations may be effective in xenografts, but they don't work in people.

Finally, when an oncogene that has constitutive activity is inhibited, that affects the signaling network in the cell. It reactivates feedback-inhibited pathways and induces stress and anti-apoptotic pathways, and potentially limits the effectiveness of the drug. These events cannot be modeled in xenografts, and have not been adequately tested in GEMMs, but are probably crucial for the development of more effective therapeutic models.

Inadequate models:

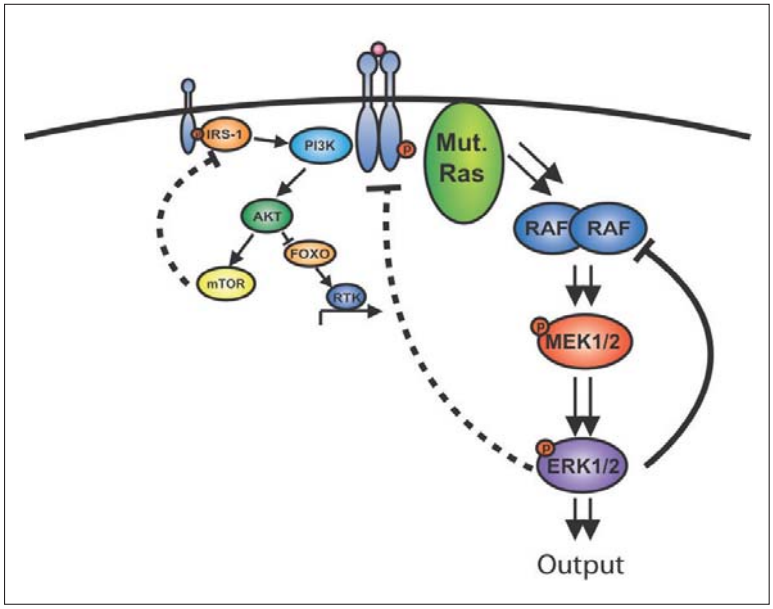


Figure 2
Mutant RAS signaling.

GEMMs are less complex genetically than cell lines or xenografts. Tumor evolution is different when initiated with a single dominant hit. Also, artificial expression of a proto-oncogene at the wrong levels or at the wrong time may not accurately reproduce a signaling network, or target dependency.

It's also difficult to examine the kinetics of drug effects on the target in GEMMs. The experiments would need several identical models, treated and analyzed at short intervals. From a physician's perspective, the

pharmacodynamics and dosing schedule are difficult to define in GEMMs.

Relevance to humans mandates validation in human tumors, and there are several tools available to do this, including tumor samples, sequencing technologies, and '-omes' of various kinds.

The fancy molecular biology answer for why many of the drugs in development fail in the clinic is because they don't eliminate stem cells, or because the tumor is heterogeneous and other things compensate. But from a translational point of view, it's because the drugs are suboptimal, with poor selectivity, poor pharmacokinetics and poor pharmacodynamics, and the drugs are tested in suboptimal trials based on suboptimal hypotheses and schedules.

There are some great drugs available now that are selective for the target to answer biologic mechanisms questions in mice. These drugs should be used in people. Too many researchers don't sequence tumors or do tumor biopsies before using a targeted drug. Tumor biopsies provide invaluable information about what's happening in the tumor, they test the hypotheses, and validate murine models.

Another reason the drugs fail is that mice are inadequate at modeling pathway inhibition, and the pathway inhibition may be limited by on and off target effects of the drugs. So called 'dual specificity' drugs tend to have many other targets.

There is often genetic resistance, with secondary mutations that reduce the biologic effects of inhibiting the driver oncoprotein. There is also adaptive resistance, with physiologic effects that reduce the drug's effectiveness.

So what makes for a great drug? A great drug is targeted against a great target, mutated or otherwise, and is very selective, with limited off-target effects. It has a good PK, great PD, and selectively inhibits a particular, limited subset of tumors. In this case, a subset is defined by lineage or class or is driven by a particular mutation.

Therapeutic index:

For example, one company has an inhibitor to the fibroblast growth factor receptor (FGF-R), screened against 300 cell lines. The drug didn't inhibit the growth of about 280 of those cell lines at greater than 10 micromolar. But it did inhibit about 20 of the cell lines with various FGF-R mutants at 10 nanomolar.

Except for bad PK, nothing should prevent that drug from being given to patients. It's obviously hyper-selective, as it has no effect on 280 cell lines even at three orders of magnitude higher dose. It kills the FGF-R and causes complete cessation of growth of the FGF-R mutant cells. Most of the great results over the past 7 to 8 years, barring the emergence of resistance, have been obtained from data like that.

In another example, a RAF inhibitor inhibits RAF mutant tumors and nothing else because of a biochemical parlor trick of that drug. By contrast, the RAF inhibitor sorafenib inhibits all cells at approximately the same dose, meaning that the drug is non-selective, and doesn't have much of a therapeutic index.

Vemurafenib is a drug that targets a driver mutation. A PET scan two weeks after initiation of the drug reveals that the tumor has imploded. Tumors in about 90% of patients with BRAF mutations regress in response to the drug. Two major advances allowed this to happen: This pattern of inhibition, and a therapeutic concentration formulated by Roche.

In the past 7-8 years, all but one treatment developed in the Rosen lab have been validated in patients, including HSP90 in HER2 breast cancer[1], pulsatile EGF-R with chemotherapy in non-small cell lung cancer and MEK and RAF inhibitors for RAF mutant tumors.

But one approach that did not work is inhibiting ERK and PI3K together in tumors that have co-existing

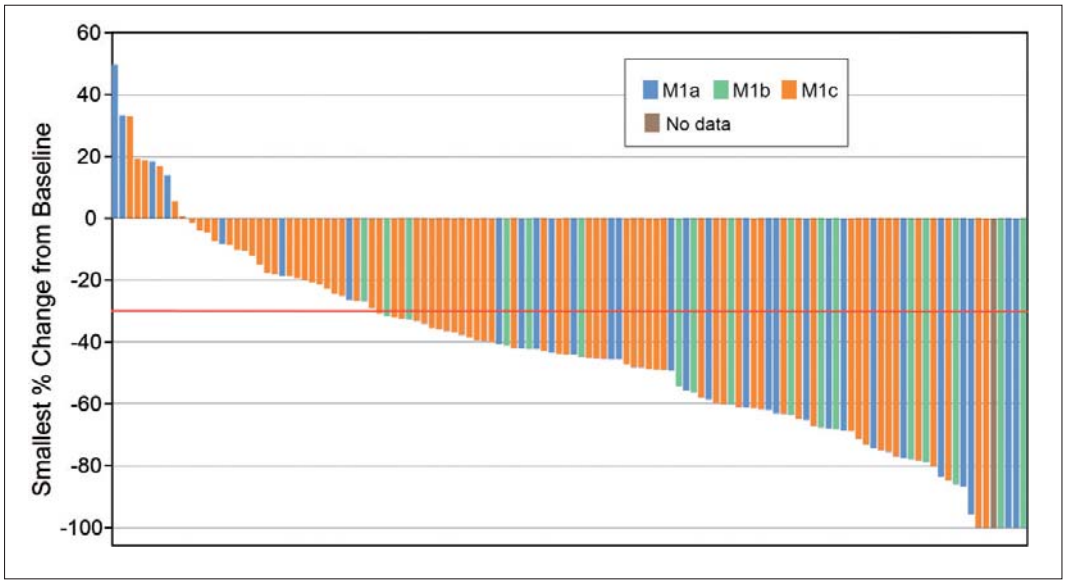
mutations in both pathways. PI3K inhibitors in general have worked poorly, but it also may be that it's difficult to inhibit these pathways together without a lot of toxicity. There may be ways to get around that with dose and schedule, but that must be studied in GEMMs.

Something interesting that should also be modeled in GEMMs is the hypothesis that physiologic adaptation to inhibition of oncoprotein–activated pathways limits anti-tumor activity. This may be because of the induction of stress responses or drug metabolism and transporters, because of changes in the regulators of apoptosis and metabolism or the regulation of the targeted signaling network.

For example, when EGF-R signals, it activates RAS, RAF, ERK, MEK, PI3K and several things. But the hypothesis now is that oncogene activation also leads to sustained negative feedback throughout the network. For instance, the feedback response to MEK inhibition is altered in cells with mutant V600E B-RAF[2]. But can this sort of feedback be modeled in mice?

Regulation of the network and the relief of feedback by inhibitors are completely dependent on the ligand environment, the stromal microenvironment, and the availability of the ligands to activate those receptors. If the signaling network has a constitutively activated protein, it will not just hit specific downstream targets, it will instead exaggerate the normal feedback that regulates the network.

Figure 3
Best overall response (independently reviewed).



Feedback inhibition:

This leads to three hypotheses. First, constitutive activation of the oncoprotein ought to lead to constitutive negative feedback. Activation of the pathway output must require insensitivity of the oncoprotein to feedback. Or, there must be a second hit that attenuates the feedback.

Second, the sustained negative feedback throughout the network reduces the robustness of the cell and causes key processes to become dependent on the oncoprotein alone, and that's responsible for oncogene addiction.

Finally, inhibition of the oncoprotein causes cell death, but also relieves profound feedback throughout the network, restores network complexity and allows survival of the tumor.

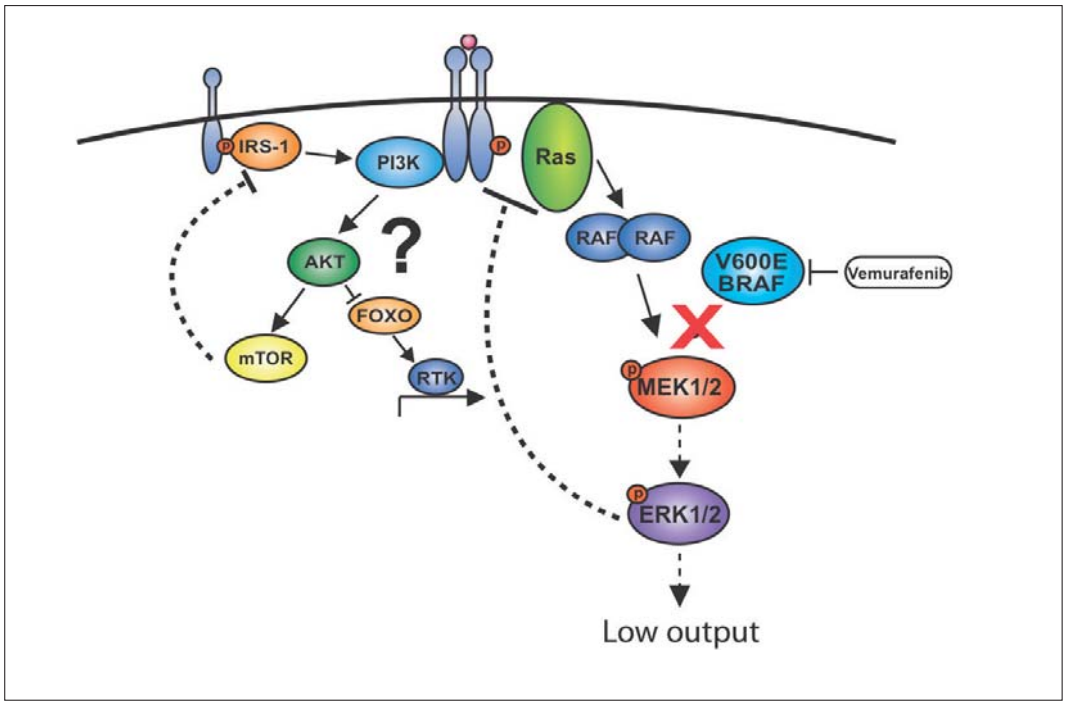


Figure 4
Adaptation to RAF/ERK inhibition.

For example, activation of AKT in turn activates mTOR, which reduces receptor kinase signaling by a complex set of events. AKT activation also inhibits FOXO, and that reduces the expression of various receptors, including HER2, HER3 and IGF1R[3].

In a xenograft, an mTOR kinase inhibitor early on inhibits both AKT and mTORC for physiologic reasons. As a function of those, levels of pEGFR, pHER2, pHER3 and pIGF1R all go up *in vivo*[4]. However, mTOR inhibitors never work in prostate cancer. This can perhaps be explained in terms of feedback.

To deal with tumor adaptation, researchers should develop discontinuous schedules and note which pathways are reactivated when the oncoprotein is inhibited. Tumor adaptation will depend on the ligand environment, which will have to be recapitulated faithfully in models to allow relevant testing of combinations. Otherwise this strategy will depend on comprehensive analysis of post-treatment tumor biopsies.

When AKT signaling is inhibited, many receptors are activated, but HER2 is by far the dominant kinase. In a phase I/II clinical trial, patients with metastatic HER2+ breast cancer who had failed Herceptin and chemotherapy were given the rapalog Temsirolimus and Netarinib, an irreversible HER1/HER2 inhibitor. In this trial, the rapalog had a 0% response rate, whereas the HER2 inhibitor had a 20-25% response rate.

Ligands are key to making these therapies work. In melanoma, and thyroid and colon cancers, ERK feedback is released and RAS-GTP elevated. The latter is much lower in melanomas than in thyroid or colon cancer, accounting for resistance. Adding a MEK inhibitor plus a RAF inhibitor to models is more effective.

It's crucial to understand this complexity and model it in order to develop relevant combination trials in patients. And this needs to be done concordantly with patient biopsies, to prove that the model mimics what happens in patients.

Most researchers spend a lot of attention on genetics, which is totally justified. But they should also start focusing on biochemistry, physiology and, perhaps, the discredited field of pharmacology.

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Abbreviations and glossary

1. ABBREVIATIONS

ADM	acinar duct metaplasia	**MEF**	mouse embryo fibroblasts
ALL	acute lymphoblastic leukemia	**NSCLC**	non-small cell lung cancer
APL	acute promyelocytic leukemia	**PDAC**	pancreatic ductal adenocarcinoma
CIS	common insertion site	**PDX**	patient-derived xenograft
DEN	diethylnitrosamine	**RMS**	rostral migratory stream
EGFR	epidermal growth factor receptor	**ROS**	reactive oxygen species
EMT	epithelial-mesenchymal transition	**SCLC**	small cell lung cancer
GBM	glioblastoma multiforme	**SVZ**	subventricular zone
GEMM	genetically engineered mouse model	**TAD**	transcriptional activation domains
GSI	γ-secretase inhibitor	**TCGA**	the cancer genome atlas project
HCC	hepatocellular carcinoma	**TNF**	tumor necrosis factor
HDAC	histone deacetylase	**VEGF**	vascular endothelial growth factor

2. GLOSSARY

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

adenocarcinoma	a cancer of the epithelium, which may line the surface of a variety of glands, tissues and organs in the body
angiogenesis	physiological process involving the growth of new blood vessels
apoptosis	the process of programmed cell death that may occur in multicellular organisms
cancer stem cells	cells that possess characteristics associated with normal *stem cells*, specifically the ability to give rise to all cell types found in a particular cancer sample
copy number variation	alterations in the genome that results in the cell having an abnormal number of copies of one or more sections of DNA
epigenetics	heritable changes in gene expression or cellular phenotype caused by mechanisms other than changes in the underlying DNA sequence
exome	the part of the genome formed by exons, the coding portions of genes that are expressed
glycolysis	the metabolic pathway that metabolizes glucose and results in the release of free energy
Insertional mutagenesis	the introduction of mutations into DNA by the insertion of one or more bases
metastasis	the spread of a disease from one organ or part to another non-adjacent organ or part

oncogene	a gene that has the potential to cause cancer
RNA interference	a process within living cells that moderates the activity of their genes
senescence	the change in the biology of a cell as it ages after its maturity
shRNA	a sequence of RNA that makes a tight hairpin turn that can be used to silence target gene expression via *RNA interference*
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	connective, supportive framework of a biological cell or tissue
transactivation	an increased rate of gene expression triggered either by biological processes or by artificial means
transdifferentiation	a process where one mature somatic cell transforms into another mature somatic cell without undergoing an intermediate pluripotent state or progenitor cell type
transcription	the process of creating a complementary RNA copy of a sequence of DNA
transcriptiome	the set of all RNA molecules produced in one or a population of cells
tumor suppressor	a gene that protects a cell from one step on the path to cancer
tumorigenesis	the process by which normal cells are transformed into cancer cells
ubiquitination	the modification of a protein by the covalent attachment of one or more ubiquitin molecules
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
xenograft	cells, tissues or organs transplanted from one species to another

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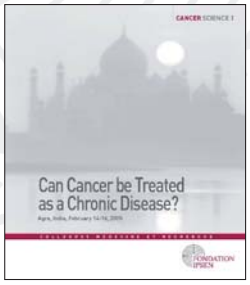

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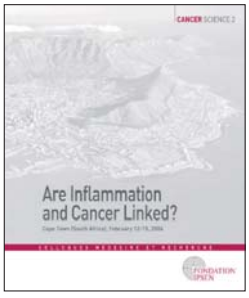

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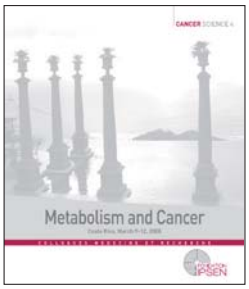
Kari Alitalo *(University of Helsinki, Helsinki, Finland)*, **David Baltimore** *(California Institute of Technology, Pasadena, USA)*, **Mariano Barbacid** *(Centro Nacional de Investigaciones Oncologicas, Madrid, Spain)*, **J. Michael Bishop** *(University of California, San Francisco, USA)*, **Lewis Cantley** *(Harvard Institute of Medicine, Boston, USA)*, **Ann Chambers** *(University of Western Ontario, London, Canada)*, **Hans Clevers** *(Hubrecht Laboratory, Utrecht, The Netherlands)*, **Philippe Clezardin** *(Inserm, Lyon, France)*, **Mario Colombo** *(Istituto Nazionale Tumori, Milano, Italy)*, **Paolo Comoglio** *(University of Torino, Torino, Italy)*, **Hugues de Thé** *(CNRS, Université de Paris, Paris, France)*, **Wolf Hervé Fridman** *(Inserm, Paris, France)*, **Christian Gespach** *(Inserm, Paris, France)*, **Douglas Hanahan** *(University of California, San Francisco, USA)*, **Jean-Luc Harousseau** *(Hôtel-Dieu, Nantes, France)*, **Richard Hynes** *(Massachusetts Institute of Technology, Cambridge, USA)*, **Alain Israël** *(Institut Pasteur, Paris, France)*, **Gilbert Lenoir** *(Institut Gustave Roussy, Villejuif, France)*, **Arnold J. Levine** *(Institute of Advanced Study, Princeton, USA)*, **David M. Livingston** *(Dana Farber Cancer Institute, Boston, USA)*, **Daniel Louvard** *(Institut Curie, Paris, France)*, **Tak Wah Mak** *(University of Toronto, Toronto, Canada)*, **Apoorva Mandavilli** *(The Simons Foundation, New York, USA)*, **Joan Massagué** *(Memorial Sloan-Kettering Cancer Center, New York, USA)*, **Luigi Naldini** *(University Medical School, Milano, Italy)*, **Daniel Peeper** *(Netherlands Cancer Institute, Amsterdam, The Netherlands)*, **Jeffrey Pollard** *(Albert Einstein College of Medicine, New York, USA)*, **Shahin Rafii** *(Cornell University Medical College, New York, USA)*, **Inder M. Verma** *(Salk Institute for Biological Studies, La Jolla, USA)*, **Robert A. Weinberg** *(Massachusetts Institute of Technology, Cambridge, USA)*, **Zena Werb** *(University of California, San Francisco, USA)*, **Owen N. Witte** *(University of California, Los Angeles, USA)*.

METABOLISM AND CANCER • Villa Caletas, March 9-11, 2008



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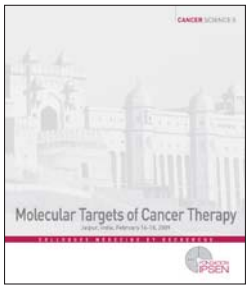

MOLECULAR TARGETS OF CANCER THERAPY • Jaipur, February 14-18, 2009

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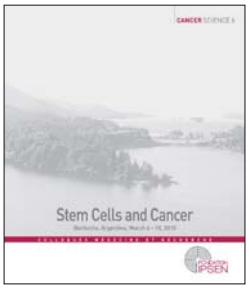
STEM CELLS AND CANCER • Bariloche, March 6-10, 2010



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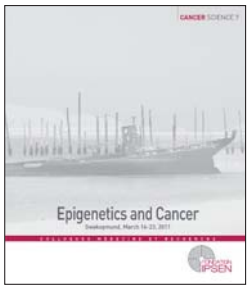

EPIGENETICS AND CANCER • Swakopmund, March 19-23, 2011

ORGANIZING COMMITEE

Inder M. Verma *(Salk Institute for Biological Studies, La Jolla, USA),* **Yves Christen** *(Fondation IPSEN, Paris, France),* **Jacqueline Mervaillie** *(Fondation IPSEN, France)*

SPEAKERS AND DISCUSSANTS

Geneviève Almouzni *(Institut Curie, Paris, France),* **David Baltimore** *(Caltech, Pasadena, USA),* **Stephen B. Baylin** *(The Sidney Kimmel Comprehensive Cancer Center, Baltimore, USA),* **Adrian Bird** *(University of Edinburgh, Edinburgh, UK),* **J. Michael Bishop** *(University of California, San Francisco, USA),* **Xiaodong Cheng** *(Emory University School of Medicine, Atlanta, USA),* **Susan Clack** *(The Garvan Institute of Medical Research, Darlinghurst, USA),* **Hugues de Thé** *(Université de Paris, Paris, France),* **Joseph Ecker** *(Salk Institute for Biological Studies, La Jolla, USA),* **Robert Eisenman** *(Fred Hutchinson Cancer Research Center, University of Washington, Seattle, USA),* **Ron Evans** *(Salk Institute for Biological Studies, La Jolla, USA),* **Edward E. Harlow** *(Constellation Pharmaceuticals, Cambridge, USA),* **Kristian Helin** *(University of Copenhagen, Copenhagen, Denmark),* **Tony Hunter** *(Salk Institute for Biological Studies, La Jolla, USA),* **Jean-Pierre Issa** *(M. D. Anderson Cancer Center, University of Texas, Houston, USA),* **Rudolf Jaenisch** *(Whitehead Institute for Biomedical Research, MIT, Cambridge, USA),* **Peter A. Jones** *(Epigenome Center, University of Southern California, Los Angeles, USA),* **Peter W. Laird** *(Epigenome Center, University of Southern California, Los Angeles, USA),* **Gilbert Lenoir** *(Institut Gustave Roussy, Paris, France),* **Daniel Louvard** *(Institut Curie, Paris, France),* **Charles Sawyers** *(Memorial Sloan-Kettering Cancer Center, New York, USA),* **Maarten von Lohuizen** *(The Netherlands Cancer Institute, Amsterdam, The Netherlands),* **Ali Shilatifard** *(Stowers Institute for Medical Research, Kansas City, USA),* **Craig Thompson** *(Memorial Sloan-Kettering Cancer Center, New York, USA),* **Inder M. Verma** *(Salk Institute for Biological Studies, La Jolla, USA),* **Robert A. Weinberg** *(Whitehead Institute for Biomedical Research, Cambridge, USA),* **Irving L. Weissman** *(Stanford University School of Medicine, Palo Alto, USA),* **Richard Young** *(Whitehead Institute for Biomedical Research, Cambridge, USA).*

MOUSE MODELS OF HUMAN CANCER: ARE THEY RELEVANT? • Ouro Preto, March 10-14, 2012

ORGANIZING COMMITTEE

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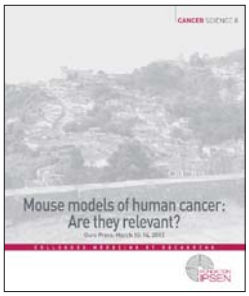
SPEAKERS AND DISCUSSANTS

David J. Adams *(Wellcome Trust Sanger Institute, Cambridge, UK),* **Laura Attardi** *(Stanford University School of Medicine, Stanford, USA),* **Mariano Barbacid** *(Centro Nacional de Investigaciones Oncologicas, Madrid, Spain),* **Daniela Basseres** *(Universidade de Sao Paulo, Sao Paulo, Brazil),* **Anton Berns** *(The Netherlands Cancer Institute, Amsterdam, The Netherlands),* **J. Michael Bishop** *(University of California SF, San Francisco, USA),* **Lewis Cantley** *(Harvard University, Boston, USA),* **Mario Capecchi** *(University of Utah, Salt Lake City, USA),* **Roger Chammas** *(Universidade de Sao Paulo, Sao Paulo, Brazil),* **Hugues de Thé** *(INSERM, CNRS, Université Paris Diderot, Paris, France),* **Vishva Dixit** *(Genentech Inc., South San Francisco, USA),* **Gerard Evan** *(University of Cambridge, Cambridge, UK),* **Ronald Evans** *(Salk Institute for Biological Studies, La Jolla, USA),* **Douglas Hanahan** *(Institute for Experimental Cancer Research – EPFL, Lausanne, Switzerland),* **Tony Hunter** *(Salk Institute for Biological Studies, La Jolla, USA),* **Tyler Jacks** *(MIT, Cambridge, USA),* **Claudio Joazeiro** *(Scripps Research Institute, La Jolla, USA),* **Leisa Johnson** *(Genentech Inc., South San Francisco, USA),* **Jos Jonkers** *(The Netherlands Cancer Institute, Amsterdam, The Netherlands),* **Michael Karin** *(University of California SD, La Jolla, USA),* **Daniel Louvard** *(Institut Curie, Paris, France),* **Scott Lowe W.** *(Memorial Sloan-Kettering Institute, New York, USA),* **Tak Wah Mak,** *(University of Alberta, Toronto, Canada),* **Joan Massagué** *(Memorial Sloan- Kettering Institute, New York, USA),* **Luis Parada** *(UT Southwestern Medical Center, Dallas, USA),* **Neal Rosen** *(Memorial Sloan-Kettering Institute, New York, USA),* **Charles L. Sawyers** *(Memorial Sloan- Kettering Institute, New York, USA),* **Reuben Shaw** *(Salk Institute for Biological Studies, La Jolla, USA),* **David Tuveson** *(Cancer Research UK, Cambridge, UK),* **Inder M. Verma** *(Salk Institute for Biological Studies, La Jolla, USA),* **Robert A. Weinberg** *(MIT, Cambridge, USA).*



In preparation

CANCER IMMUNOTHERAPY • Taormina, March 9-13, 2013

ORGANIZING COMMITEE

Inder M. Verma *(Salk Institute for Biological Studies, La Jolla, USA),* **Yves Christen** *(Fondation IPSEN, Paris, France),* **Jacqueline Mervaillie** *(Fondation IPSEN, Paris, France)*

SPEAKERS AND DISCUSSANTS

Rafi Ahmed *(Emory University, Atlanta, USA),* **James P. Allison** *(Memorial Sloan-Kettering Cancer Center, New York, USA),* **Diego Sebastian Amigorena** *(Institut Curie-Inserm, Paris, France),* **David Baltimore** *(California Institute of Technology, Pasadena, USA),* **Mariano Barbacid** *(Centro Nacional de Investigaciones Oncologicas, Madrid, Spain),* **J. Michael Bishop** *(University of California San Francisco, San Francisco, USA),* **Chiara Bonini** *(Fondazione San Raffaele Del Monte Tabor, Milan, Italy),* **Malcolm Brenner** *(Baylor College of Medicine, Houston, USA),* **George Coukos** *(University of Pennsylvania, Philadelphia, USA),* **Hugues de Thé** *(Inserm, CNRS, Université Paris Diderot, Paris, France),* **Glenn Dranoff** *(Dana-Farber Cancer Institute, Boston, USA),* **Ronald Evans** *(Salk Institute for Biological Studies, La Jolla, USA),* **Jérôme Galon** *(Inserm, Paris, France),* **Philip Greenberg** *(University of Washington, Seattle, USA),* **Douglas Hanahan** *(Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland),* **Tony Hunter** *(Salk Institute for Biological Studies, La Jolla, USA),* **Elizabeth M. Jaffee** *(Johns Hopkins University, Baltimore, USA),* **Karin Jooss** *(Pfizer Inc., La Jolla, USA),* **Carl H. June** *(University of Pennsylvania, Philadelphia, USA),* **Richard Klausner** *(The Column Group, San Francisco, USA),* **Ronald Levy** *(Stanford University School of Medicine, Stanford, USA),* **Daniel Louvard** *(Institut Curie, Paris, France),* **Tak Wah Mak** *(University of Health Network, Toronto, Canada),* **Cornelis J.M. Melief** *(Leiden University Medical Center, Leiden, The Netherlands),* **Miriam Merad** *(The Mount Sinai School of Medecine, New York, USA)* **Giorgio Parmiani** *(Fondazione San Raffaele Del Monte Tabor, Milan, Italy),* **Klaus Rajewsky** *(Max-Delbrück-Center for Molecular Medicine, Berlin, Germany),* **Antoni Ribas** *(University of California Los Angeles, Los Angeles, USA),* **Steven A. Rosenberg** *(National Cancer Institute – NIH, Bethesda, USA),* **Robert D.Schreiber** *(Washington University School of Medicine, St Louis, USA),* **Ton Schumacher** *(The Netherlands Cancer Institute, Amsterdam, The Netherlands),* **Craig B. Thompson** *(Memorial Sloan-Kettering Cancer Center, New York, USA),* **Inder M. Verma** *(Salk Institute for Biological Studies, La Jolla, USA),* **Robert A. Weinberg** *(Massachusetts Institute of Technology, Cambridge, USA)* **Irving L. Weissman** *(Stanford University School of Medicine, Palo Alto, USA),* **Owen N. Witte** *(University of California Los Angeles, Los Angeles, USA).*

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 an area that is exciting for both its medical and fundamental challenges and that is currently in a phase of rapid development: cancer science.

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 several series of annual meetings in collaboration with scientific journals and institutions. Also, the Fondation IPSEN is 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 29 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:

• **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.

• **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.

• **Cancer Science** – Annual experts meetings are organized in collaboration with Inder M. Verma *(Salk Institute for Biological Studies, La Jolla, USA)* and the participation of remarkable leading opinion makers in the field. Challenging topics (see p.135-136-137) have generated outstanding discussions among the participants.

• **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.

• **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.

• **Vascular Tree** – This 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.

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.

Additional series of meetings and partnerships have been implemented since 2007:

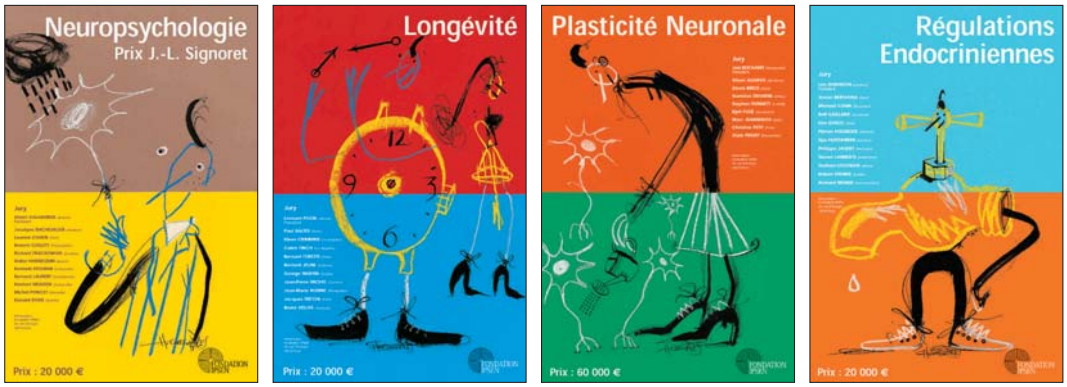
• **Biological Complexity** series *(Salk Institute for Biological Studies, Nature Publishing Group, and Fondation IPSEN)*: Transcription Diseases *(La Jolla, 2007)*, Genes, Circuits and Behavior *(La Jolla, 2008)*, Processes of Aging *(La Jolla, 2009)*, Sensory Systems *(La Jolla, 2010)*, Future Concepts and Trends *(La Jolla, 2010)*,

Immunity and Inflammation *(La Jolla, 2012)*, Molecular biology of psychiatric disorders *(La Jolla, 2013, in preparation)*

- **Emergence and Convergence** series *(Nature Publishing Group and Fondation IPSEN)*: Small RNAs in Development, Immunology and Cancer *(New York, 2007)*, Genome Variation *(2007)*, Epigenetics and Behavior *(Houston, 2008)*, Multiple Sclerosis: From Pathogenesis to Therapy *(Paris,2009)*, Mitochondrial Dysfunction in Neurological Diseases *(Durham, 2008)*, Epigenetic Dynamics in the Immune System *(San Antonio, 2010)*.
- **Exciting Biologies** series
  - *Cell Press, Massachusetts General Hospital*, and *Fondation IPSEN*: Biology in Motion *(Evian, 2007)*, Biology of Cognition *(Chantilly, 2008)*, Biology in Balance *(Buenos Aires, 2009)*, Biology of Recognition *(Singapore, 2010)*.
  - *Cell Press, DMMG, and Fondation IPSEN,* in collaboration with *The Riken Instititute:* Cellular Development: Biology at the Interface *(Kobe, 2011)*.
  - *Cell Press, DMMGF, and Fondation IPSEN:* Forces in Biology *(Dublin, 2012, in preparation)*.
- **Days of Molecular Medicine** series *(AAA Science, Karolinska Institute, Hong Kong University, DMMGF, and Fondation IPSEN)*: Tissue Engineering and Stem Cells: Driving Regenerative Medicine Forward *(Hong Kong, 2011)*

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; Helen Neville, Isabelle Peretz, Robert Zatorre; Catherine Dulac, Michael Meaney and J. David Sweatt.

• **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, Shlomo Melmed, Paolo Sassone-Corsi, Jeffrey M. Friedman and Bert O'Malley.

• **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, Giacomo Rizzolatti, Patricia Kuhl and Cathy Price.

• **The Longevity Award**, 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, Judith Campisi, Tom Kirkwood, Linda Fried and Gary Ruvkun.

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, 27 titles)
- Research and Perspectives in Neurosciences (Springer, 20 titles)
- Research and Perspectives in Longevity (Springer, 5 titles)
- Research and Perspectives in Endocrinology (Springer, 10 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.

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