12th Annual Symposium
Giovanni Armenise-Harvard Foundation
For Advanced Scientific Research

Cancer: From Genes and Proteins to Pathways and Therapeutics

Grand Hotel Bristol
Stresa, Italy
June 20-23, 2008

Count Armenise, 2008 Symposium

About the Symposium

Stresa stretches along the western shore of Lago Maggiore like a kilometer-long perennial border. Visitors stroll waterfront parks where palm trees and hydrangeas mingle with Japanese maples and magnificent conifers. Grand hotels with ornate facades line the inland side; on the other are postcard-perfect views of the three Isole Borromee. The air is scented with jasmine and flowers are blooming everywhere.

More than 80 participants converged on Stresa for the 12th Annual Symposium of the Giovanni-Armenise-Harvard Foundation, held June 20-23 at the Grand Hotel Bristol. The theme for this year was "Cancer: From Genes and Proteins to Pathways and Therapeutics," and the setting was more fitting than one might think.

To the uninitiated, gardens appear to be all about growth. But true gardeners know that the real secret is *controlling* growth. Without judicious trimming and pruning, invasive plants run wild, stealing nourishment and light from others, and the garden suffers and may ultimately die. And this is essentially what happens when cancer cells escape normal control and proliferate in humans and other creatures.

The program grouped 21 oral presentations into three sessions: basic findings about pathways and mechanisms involved in tumor development, insights from model systems, and news about drug development and cancer treatment. "This meeting is about how basic science moves us closer to the clinic," said Ed Harlow, head of the department of biological chemistry and molecular pharmacology at HMS, who presented an overview of the conference during the closing session. Twenty-three poster presentations covered similar ground during a well-attended session on Saturday afternoon.

Harlow said the conference talks were much like the excellent meals enjoyed by the participants: a series of courses, showcasing many flavors and combinations, which sparked lively conversations. Recurring themes included gene discovery, cell metabolism, systems-level analysis of complex phenomena, epigenetic control of gene activity, using genetic information to improve treatment, and evolving perspectives on the stem cell theory of cancer.

Harlow praised keynote speaker Hans Clevers for offering up "a whole meal" instead of a single course. Clevers, who was initially interested in immunology, made what Harlow called, "an extraordinary series of discoveries about how cells grow and divide in intestinal crypts." As a result, Harlow said, he has secured a place as "one of the world's leaders in stem cell research."

On hand for the proceedings were Count Giovanni Auletta Armenise and HMS Dean Jeffrey Flier, participating in his first Armenise-Harvard symposium since being named dean in July 2007. Members of the Foundation's Board of Trustees, Scientific Advisory board and Italian Scholarship Advisory Committee also participated.

U.S. scientific delegates came from Harvard Medical School and three affiliated hospitals in Boston. Italian delegates traveled from 13 Italian universities and research institutes, some as close by as Milano and others as far south as Napoli and Palermo. Senior scientists from multinational pharmaceutical companies were also present.

Speakers and delegates included young scientists who have benefited from Junior Faculty Grants and Career Development Awards supported by the Foundation. Since last year's symposium, four HMS junior faculty have received new support: Chenghua Gu, Tom Bernhardt, Monica Colaiacovo, and Johan Paulsson. Past grant recipients Marcia Haigis and Adrian Salic presented posters at this year's conference.

Two new Career Development Awards, which help outstanding young Italian researchers establish independent laboratories following post-doctoral training abroad, have been made to Rosa Bernardi and Nico Mitro. Bernardi joined six other Career Development Awardees for dinner with Count Auletta during the Stresa gathering.

In addition to promoting scientific research in Italy, the Foundation has also supported extraordinary reporting opportunities for Italian journalists who cover the science beat. Alice Andreoli and Silvia Bencivelli, who report for broadcast and print outlets, are the latest recipients of the annual Science Writer Fellowships. They participated in the symposium, joined previous winners for a science writing workshop in Milano on June 23rd, and later travel to Boston to research stories of their choosing at HMS.

Keynote Address

Identification of Stem Cells in Small Intestine and Colon by a Single Marker Gene *LGR5*

Hans Clevers
Hubrecht Institute, Uppsalalaan 8, 3584CT Utrecht, the Netherlands

In fiction, the word "crypt" conjures up images of stillness and death. But in the intestine, crypts are wellsprings of life – tiny factories generating cells at a rate that makes the intestinal epithelium the fastest self-renewing tissue in adult mammals. In the small intestine, crypts are nestled between finger-like villi that cover the intestinal walls like shag carpet. In the colon, they are pockets in a smooth surface.

Scientists long suspected that the deep recesses of these crypts harbored pluripotent stem cells, even though these cells had not been precisely described. Nothing else could explain how such tight quarters could give rise to four distinct cell types that proliferate at different rates, migrate to different locations, and perform such varied tasks as secreting protective mucus, extracting nutrients from food, and ferrying messages between bowel and brain.

For nearly two decades, the thin line between normal development and cancer has been the focus of Hans Clevers' lab at the Hubrecht Institute for Developmental Biology and Stem Cell Research in Utrech, The Netherlands.

In his keynote address to the 12th Annual Symposium of the Giovanni Armenise-Harvard Foundation, Clevers told how he and his colleagues initially "stumbled" into the study of crypt stem cells – an enterprise that paid off last October with a ground-breaking characterization of these elusive cells in *Nature*.

At the start, “we were looking at the canonical Wnt pathway, which in colon cancer is locked in the ‘on’ state,” he said. This signaling pathway is also essential for normal development, and it was not clear how the normal pathway went awry in cancer.

In 1996, Clevers and other investigators discovered that Tcf-Lef transcription factors are the ultimate effectors in the Wnt cascade in colon cancer cells. In these cells, defects in the APC (adenomatous polyposis coli) gene cause β-catenin to accumulate. When the gut-specific transcription factor Tcf-4 comes on the scene, it locks onto β-catenin and forms a stable complex that turbo charges the proliferation of colon cancer cells.

The implications of this finding extend beyond colon cancer: up to 90 percent of all cancer cells carry APC mutations, and Tcf-4 activity is found in several other types of tumors.

The Clevers lab next turned its attention to normal tissue renewal in the gut, where the Wnt pathway –and especially Tcf-4 – is essential for normal crypt formation. When the investigators compared Wnt signaling in normal and knockout mice, they found that Wnt activity peaks at the bottom of the crypt and weakens in cells higher on the crypt walls.

In mouse models, Clevers said his team found nearly 250 Tcf-4 target genes in normal crypt cells and tumors. In the normal crypt, most of these genes are expressed in “transit amplifying cells,” which proliferate rapidly for a few days before traveling to their posts elsewhere in the intestinal lining.

Clevers hypothesized that one or more of these genes might be definitive markers for hard-to-define stem cells supposedly lurking in the crypts. One likely marker was a Wnt target gene, Lgr5, which the researchers already knew was expressed only in cycling crypt base columnar (CBC) cells. Previously suspected as stem cells, these scarce cells are interspersed with terminally differentiated Paneth cells in the crypt bottom.

Lgr5 is leucine-rich-repeat-containing G-protein-coupled receptor 5, which is also known as Gpr49. It is abundant in cycling columnar cells at the crypt base and was also detected in rare cells in several other tissues. Using an inducible Cre knock-in allele and the Rosa26-*LacZ* reporter strain, the researchers traced cell lineages in adult mice. The *Lgr5*$^{+ve}$ crypt base columnar cell (CBC) did exactly what a stem shell should: it generated all epithelial lineages over a 60-day period.

CBC cells cycle every 24 hours for the life of the mouse, Clevers said, and he showed dramatic pictures of what he called a “clonal conveyor belt” that ferries cells from the base of the crypt to the tips of the villi. Each stem cell produces 30-40 clonal cells a day that climb the crypt walls and differentiate into every cell type found in the epithelium.

In a series of in vitro experiments, the investigators watched CBC cells struggle to recapitulate the crypt-villus structure in an artificial matrix. Without normal stroma, however, the best they could manage was a bizarre assortment of shapes, Clevers said.

Clevers and his colleagues also screened a variety of adult stem cells and tumor types for Lgr5 expression, and found it in some mammary, liver, retina and brain cells. The presence of Lgr5-expressing cells in many tumors raised the possibility that these might be “cancer stem cells” that help tumors grow and expand. In that case, they might be natural targets for chemo- or radiation therapy. When he and his colleagues examined

APC negative intestinal adenomas, however, they found that the percentage of Lgr5-expressing cells decreases steadily as tumors grow.

Going forward, Clevers continues using TCF transcription factors and LGR receptors to explore the cancer stem cell hypothesis – a major theme in this year's Armenise-Harvard Symposium. Do stem cells give rise to cancer? Or does cancer arise in a later, more differentiated cell? Although no one knows for sure, Clevers currently believes that Lgr5 stem cells are probably initial targets for oncogenic transformation.

Session 1 – Carcinogenic pathways and mechanisms

Metabolism of Cancer Cells

Lewis C. Cantley
Department of Systems Biology, Harvard Medical School and Division of Signal Transduction, Beth Israel Deaconess Medical Center

"Make sure you get enough to eat," busybodies urge pregnant women and small children.

"Don't eat too much," they caution mature adults.

As annoying as these comments may be at a family gathering, the fact is that the yentas have tapped into a biological truism: mature cells turn nutrients into energy required to maintain basic, day-to-day functions. But a different type of metabolism kicks in when cells, tissues or organisms are growing fast.

During growth spurts, cells shift away from using nourishment to generate energy in the usual way, by synthesizing ATP through glycolysis, and move toward using nutrients as carbon sources for manufacturing proteins, DNA and lipids. Under normal conditions, this changeover happens when growth factors activate tyrosine kinase signaling pathways that enable cell growth and division.

Cantley has a long-standing interest in the biochemistry of metabolism in normal and malignant cells. Ten years ago, his lab discovered phosphoinositide 3-kinase (PI 3-K) and its role in normal glucose metabolism and oncogene-mediated transformation of cells. Activation of the PI 3K-Akt-mTOR signaling axis can turn on HIF-dependent genes that regulate glucose uptake and glycolysis.

"When growth factor says grow, and the cell senses it has enough nutrients and energy to grow, it will: through activation of PI 3-K and consequent changes in glucose metabolism," Cantley said. This same signaling axis, however, "is the most mutated pathway in cancer." Mutations and amplifications in PI 3-K are found in many tumors, including those of breast, colon, brain and liver.

Recent experiments in the Cantley lab indicate that pyruvate kinase, one of the genes regulated by hypoxia-inducible factor, or HIF, plays a key role in the growth of cancer cells. During normal development, the embryonic form of the pyruvate kinase protein – called PKM2 – uses nutrients to drive cell growth. But as the organism matures, PKM2 is replaced by PKM1 – which uses nutrients to maintain cell function.

Cancer cells, however, do not surrender PKM2. In the March 13 issue of *Nature,* members of the Cantley lab report that the M2 isoform is expressed in cancer cell lines and in murine tumors. Structural studies indicate that cancer cells can switch back and forth – from one metabolic demand to the other – depending on whether they need to generate energy or make macromolecules.

The Cantley team is working to uncover more details about how the PI 3-K pathway determines the metabolic options of tumor cells. Mediating this pathway is a matter of intense interest among drug developers and oncologists, and Cantley said that several compounds show promise in his laboratory.

PKM2 may also be useful as a noninvasive marker for various cancers. German researchers have found it in fecal samples from colon cancer patients, and it has been detected in pleural effusions from lung cancer patients.

A unique role for Cdk2 in suppressing oncogene- and stress-induced cellular senescence

Bruno Amati

European Institute of Oncology (IEO), IFOM-IEO Campus, Milan, Italy.

When a cartoon character wrestles with temptation, his determination to resist is represented by an angel on his right shoulder, while the urge to get drunk, steal a car, and seduce innocent virgins is embodied by a devil perched on the other side.

The dilemma is essentially the same when an activated oncogene tempts cells to turn malignant. What is surprising, however, is that the oncogene triggers both the demonic urge to proliferate and the righteous impulse to suppress tumor growth. Tumor suppressing mechanisms include apoptosis, senescence, and/or DNA Damage Responses (DDR) – and cells go wild when these tumor fighters are overpowered by the oncogene's co-conspirators.

Some of these partners in crime are epigenetic factors that modify chromatin structure or otherwise act to promote or suppress tumor growth. In recent years the Amati lab has shed light on how modifications in chromatin structure affect whether tumors grow or not.

The lab's ongoing quest is to understand the many actions of the c-myc oncogene and its protein, Myc. In its role as an anti-proliferation angel, Myc is associated with apoptosis. The Ras oncogene, in contrast, is known to induce senescence. Now it appears that Myc also induces a separate senescence response under certain conditions, Amati reported.

Ras and Myc share the capacity for inducing DDR, and although the details aren't fully known, Amati said that both responses rely upon the ARF-p53 tumor suppressor pathway, with senescence depending also upon $p21^{Cip1}$ (induced by p53) and the $p16^{INK4a}$-pRb pathway.

Recent experiments have examined whether a cell cycle regulator, the cyclin-dependent kinase 2 (Cdk2), might promote tumor growth by disrupting Myc-induced senescence. If it does, that raises the possibility of fighting cancer with drugs that inhibit Cdk2.

Although Amati emphasized that his lab is a long way from developing new drugs, they do have encouraging new results from experiments in mouse embryo fibroblasts (MEFs) and Cdk2 knockout mice. In quiescent cells, ectopic Myc activation promoted cell cycle

entry; in proliferating cultures, Myc activation accelerated cell cycle progression. These effects were equivalent in WT and *Cdk2*-/- mouse MEFs, indicating independence from Cdk2.

In *Cdk2*-/- MEFs, however, a frenzy of cell proliferation was followed by a profound senescence-like growth arrest: this response was genetically dependent upon ARF, p53, $p21^{Cip1}$, $p16^{INK4a}$ or pRb, and was associated with selective induction of $p21^{Cip1}$ and $p16^{INK4a}$. The mutant cells also underwent premature senescence upon oxidative culture shock, suggesting that Cdk2 may generally serve to suppress stress-induced senescence.

In Em-myc transgenic mice, deleting *Cdk2* enhanced cellular senescence at pre-tumoral stages and retarded the onset of B-cell lymphoma. The investigators conclude that although Cdk2 acts like other Cdks during cell cycle progression and development, it has a unique role in bypassing Myc-induced senescence and promoting tumor progression. Amati characterized Cdk2 as a switch in DDR damage response.

Other investigators have shown that chemotherapy can induce senescence in tumor cells, and the next question Amati will tackle is whether inhibiting Cdk2 might induce a clinically valuable senescence response in apoptosis-defective tumors.

Genomic Alterations in Human Cancer

Matthew Meyerson
Dana-Farber Cancer Institute; Department of Pathology, Harvard Medical School; and Broad Institute of Harvard and MIT

Biologists dreamed of studying all a cell's genes at once long before this "genomic" approach to cancer became a reality. Once upon a time it took months – sometimes years – to identify and sequence a single abnormal gene in tumor cells. But that began to change when the race to sequence the human genome drove development of faster, cheaper sequencing equipment.

Today, high-throughput genome analysis tools enable researchers to detect somatic alterations in cancer cells including point mutations, copy number alterations, translocations, or even changes caused by infections. In late 2005, the National Cancer Institute launched The Cancer Genome Atlas (TCGA), a collaboration of 11 centers using sequencing and gene arrays – as well as computational firepower – to catalog all the genomic changes in cancer.

"To find copy number alterations, we have now analyzed over 2,600 cancer samples with arrays representing 250,000 mapped single nucleotide polymorphisms (SNPs), or most recently, over 1.8 million mapped probe sets," said Matthew Meyerson, a TCGA investigator at HMS and the Broad Institute, which Harvard and the Massachusetts Institute of Technology operate together.

Genomic technology generates panoramic views of what's going on in cancer cells. When Meyerson and his colleagues subjected 500 lung cancer samples to this scrutiny, they found 57 recurring genetic changes – 15 of them in genes already know to be bad actors in lung cancer. While it is reassuring to encounter the same oncogenes over and over, it's even more exciting to finger new villains. The investigators were surprised to find that the *NKX2-1* gene, known to regulate normal cell growth in the alveoli, is clearly amplified in lung cancer cells. This finding was published late last year.

More recently, Meyerson and fellow investigators discovered that a gene functionally similar to *NKX2-1* is amplified in squamous cell carcinomas. During normal development, the gene *SOX2* partners with a transcription factor to differentiate foregut cells into esophagus – paralleling NKX2-1's role in differentiating trachea. In both cases, Meyerson speculates that alterations in normal function of either gene may be involved in transformation of normal cells.

The goal of genome analysis is to improve treatment for individual patients, and already there are steps in this direction. Meyerson helped discover why the EGFR inhibitor gefitinib (Iressa) works well in 10% of lung cancer patients but doesn't help the rest. And results from a large-scale analysis of 500 lung tumor samples are helping physicians decide which patients are likely to benefit from Avastin, an anti-angiogenesis agent. (A separate symposium presentation by Bruce Johnson focused on advances in lung cancer therapy.)

Right now, glioblastoma is on center stage in the Meyerson laboratory. The Cancer Genome Atlas consortium has completed preliminary analyses of 206 glioblastoma tumor DNA and RNA specimens, hoping to identify "drugable targets" in these hard-to-treat tumors. Already they see several patterns of gene abnormalities that might be amendable to treatment.

New technologies will speed the pace of discovery, Meyerson said, invoking "Moore's law of genomics: capacity doubles every year."

mTOR Signaling & Cell Growth Control in Normal and Cancer Cells
John Blenis
The Department of Cell Biology, Harvard Medical School

Rapamycin is a drug that wears many hats: it is an antibiotic originally isolated from a soil bacterium, an anti-rejection drug used in transplant medicine, a component in the polymer coating of some coronary stents, an approved treatment for the congenital disease tuberous sclerosis, and now an experimental agent being tested against breast, lung, prostate, and certain head and neck cancers.

All this is possible because rapamycin inhibits a protein kinase that constantly monitors its surroundings for nutrients and mitogens, integrates these extracellular signals, and tells cells when to grow or divide. This structure has been dubbed mTOR, short for "mammalian target of rapamycin."

Before a mother cell can divide, it must "bulk up" – doubling its DNA and protein – in preparation for splitting into two daughter cells, Blenis said, and mTOR helps accomplish this by acting in concert with raptor and Lst8, which together form the rapamycin-sensing complex mTORC1.

The mTOR-raptor pathway has two effector arms, 4EBP1 or S6KI, that are important for normal cell growth and proliferation. Unfortunately, a variety of factors – including growth factors, amino acids, glucose or stress – can throw normal signaling cascades off course and lead to improper development and human disease.

Blenis suspects that alterations in mTOR-raptor pathways will turn out to play a part in nearly all cancers. There is already evidence that gain-of-function mutations in Ras, PI3-kinase, Raf and various receptor and cytoplasmic tyrosine kinases, or loss-of-function mutations in tumor suppressors like PTEN, TSC1/2, NF1, LKB1 and others result in constitutive activation of mTORC1 signaling. Recent work in his lab has focused on understanding how rapamycin inhibits the mTOR signaling network, and how this might block tumor formation.

Researchers have known for the past decade that rapamycin acts on S6 kinase signaling, and in the April 18 issue of *Cell* members of the Blenis lab paint a more detailed picture of how this happens. Experiments conducted by Xiaoju "Max" Ma indicate that mTOR gives spliced mRNAs a competitive advantage over nonspliced mRNAs during the "pioneer" round of protein synthesis.

Ma discovered that S6K1 is recruited to newly spliced mRNAs by SKAR, a normal protein that helps regulate cell size and growth and binds only to hyper-phosphorylated and activated S6K1. Together, the two form a conduit between mTOR checkpoint signaling and the pioneer round of translation under conditions that favor growth.

Although there's more to learn, these experiments provide molecular insights into how mTORC1/S6K contributes to translation initiation and protein synthesis. In addition to shedding light on how rapamycin works, Blenis expects these investigations to lead to other novel compounds that can fight cancer with minimal side effects.

Regulation TGF-β signaling in development and cancer

Stefano Piccolo
Dept Medical Biotechnologies, University of Padua School of Medicine

In 1924, a young Ph.D. candidate named Hilde Mangold painstakingly transplanted the dorsal lip of a newt blastopore to the ventral side of a differently colored newt embryo. She watched as the transplanted cells accomplished two amazing feats: they induced nearby ectoderm cells to become neurons, and they turned what would have been the newt's tummy into its back. This proved, beyond a doubt, that signals emanating from the dorsal lip determined the fate of cells and tissues during development.

And then, just months before these remarkable results were published in a leading scientific journal, Hilde Mangold burned to death in a house fire. She was 26 years old. Nine years later her lab chief and mentor, Hans Spemann, won the Nobel Prize for the discovery of what textbooks now call "Spemann's Organizer" or simply "the Organizer." Only rarely is Mangold's name attached to her discovery.

Decades of research have shown that growth factors are big players in the sort of germ layer induction and patterning that Mangold studied in newt embryos. Stefano Piccolo is especially interested in members of the transforming growth factor-β superfamily, key players both in embryonic development and in maintenance of healthy adult tissues. Asymmetric binding of a TGF ligand, Nodal, is well established as the signal that induces the Organizer on the dorsal lip.

Piccolo uses frog and mouse models to examine what he calls "two sides of the same coin" -- TGF-β signal transduction cascades during normal development and in cancer. In recent years, his lab has published many new and important findings about TGF-β.

For example, the Piccolo team found that instead of a digital, on-off switch, TGF-β responds to graded, analog input. The Smad2/Smad4 complex of transcriptional regulators monitor input like a person "trying to shut out unnecessary noise and hear the information they need," Piccolo said. When the researchers knocked out Smad2/Smad4 in mice, they heard too much and grew oversized and misshapen heads.

In a separate set of experiments, the researchers found that TGF-β teamed with a different partner, a glycoprotein called Emilin1, to maintain normal arterial blood pressure. When they knocked out Emilin1, TGF-β signaling and blood pressure both spiked in the mutant animals.

Now Piccolo and his colleagues have attracted a new wave of attention by demonstrating, for the first time, that endogenous microRNAs play a key role in the generation of Spemann's Organizer. There has been tremendous excitement in recent years about the many and diverse functions of these tiny, single-stranded RNAs, but this is the first indication that they act so early in vertebrate development.

In the September 13, 2007 issue of *Nature,* the investigators describe a new role for two endogenous microRNAs, miR-15 and miR-16, which other researchers have previously implicated in various types of leukemia and lymphoma. These two microRNAs are active only on the ventral side of the frog embryo, where they restrict the size of the Organizer by targeting the Nodal type II receptor Acvr2a, thus pushing it to develop on the dorsal side. miR-15 and miR-16 are not found on the dorsal side because the dorsal-specific Wnt/b-catenin pathway keep them from processing into mature microRNAs. On the dorsal side, beta catenin is "the mother of all pathways," Piccolo said.

These findings illustrate that microRNAs, once too small to merit serious notice, play a key role in the transformation of symmetric structures into asymmetric creatures – one of biology's central events. Hilde Mangold would be amazed.

Breast tumor heterogeneity: causes and consequences

Kornelia Polyak
Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School

""It is not the strongest of the species, nor the most intelligent, but the most responsive to change that survives," Charles Darwin wrote nearly 150 years ago.

And what is true for Darwin's famous finches is also true for cancer cells, according to Kornelia Polyak, who organizes tours to the Galapagos archipelago when she's not analyzing cells from primary breast tumors and their metastases. "Cancer is an evolutionary and ecological process," she said, and the more genetically heterogeneous a tumor tissue is, the worse the patient's prognosis.

Traditionally, physicians have sought to wipe out every cancer cell because they believed that any one cell could reconstitute the tumor by cloning itself repeatedly. However, an alternative hypothesis has arisen called the cancer stem cell model. Scientists in this camp say that the bulk of tumor cells are irrelevant, and that treatment should focus on a small population of cancer stem cells endowed with vigorous self-renewal properties. These stem cells have not been definitively isolated and characterized, however. And regardless of first causes, it is clear that the cells

constituting tumors can undergo genetic and epigenetic changes. Like Darwin's finches, they evolve.

Decades of study have established that breast tumors are composed of various cancer cells with distinct phenotypes, and that metastases are often not the same as the patient's primary tumor. If researchers could understand the molecular mechanisms responsible for breast tumor heterogeneity, Polyak said, this would facilitate development of more effective ways to treat and prevent breast cancer.

Toward this end, Polyak and her colleagues have isolated cells from normal and malignant breast tissue samples and characterized their molecular profiles. Building on earlier studies conducted at the University of Michigan and elsewhere, two of the markers they looked at were CD44 and CD24. CD44+ cells have been described as putative stem cells in breast tumors.

Polyak's experiments showed that CD44+ cells are associated with a poorer clinical prognosis and that these cells express genes involved in invasion and metastasis. Surprisingly, they found that although CD24+ cells are more differentiated and thought to be less tumorigenic in xenograft assays, distant metastases in breast cancer patients were rich in cells with this profile. Within some tumors, the investigators saw evidence that the CD44+ and CD24+ cells probably originated from the same clone but genetically diverged over time.

"Analysis of the research backing each concept, including the results of our recent studies investigating putative breast cancer stem cells, suggests how the cancer stem cell hypothesis and the clonal evolution model may be involved in generating breast tumor heterogeneity," Polyak said.

And the bottom line for patients is that the more heterogeneous, the worse the prognosis. Despite these findings, Polyak said she is not pessimistic about treating women with breast cancer. "We know that targeted therapy works but that resistance is always a problem. HER2-targeting drugs work very well in some patients," she said.

The heterogeneity of tumors works in their favor, but as Darwin said, phenotypes change continuously due to evolution. The key to better treatment may depend partly on having new agents, but knowing when to administer them may be just as important.

The Aurora B kinase is a tough target

Andrea Musacchio
Department of Experimental Oncology, European Institute of Oncology, Milan, Italy

Aurora A and B are serine/threonine kinases that play several key roles in mitotic progression, including bipolar spindle formation, microtubule dynamics, chromosome condensation, microtubule-kinetochore attachment, spindle checkpoint function and cytokinesis. These kinases fascinate cancer biologists because they are overexpressed in a variety of solid tumors.

Aurora A is found mainly at the spindle and spindle poles, and just prior to mitosis it helps centrosomes mature. Other researchers have established that it is an oncogene, and drug companies have Aurora A inhibitors in preclinical and clinical development.

Andrea Musacchio has a long-standing interest in Aurora B, a protein that moves about as mitosis progresses. This equatorial kinase localizes at the kinetochore base in prometaphase, controls events in anaphase, marks the central spindle and then localizes at the mid-body. Structural and functional studies carried out by the Musacchio lab have established that Aurora B is a key component of the spindle assembly checkpoint (SAC).

Recent experiments have persuaded Musacchio that Aurora B may be an even better target for anti-cancer drugs than Aurora A; not simply because it is overexpressed in tumors, but because interfering with it will make it impossible for the cell cycle to progress normally.

As an essential part of the SAC, Aurora B acts as a combination usher and stage manager, charged with ensuring that the show – metaphase congression and anaphase – does not begin until all the seats are filled. As a regulator of a different complex, called the KMN network, Aurora B also helped set up the chairs in advance.

During mitosis, chromosomes straggle in like typical theatergoers, some early and others arriving later than they should. Aurora B pushes them into their seats, monitors how full the theatre is, and holds off on raising the curtain until everyone has been seated. This is the equivalent of having all the chromosomes in place, so they can separate – in perfect synchrony – at anaphase.

All this has to happen for the cell cycle to progress. The idea of inhibiting Aurora B is inherently appealing, because without it cells will become polyploid, undergo abnormal mitoses and eventually die as a result.

Unfortunately, Aurora B can be a tough target for drugs. Structural studies in the Musacchio lab led to a working model for activation, and made it possible to examine how it can be inactivated by several small-molecule ATP-competitive inhibitors. The problem is that Aurora B mutates in ways that change its structure and confer resistance to these would-be inhibitors, according to experiments done in collaboration with University of Manchester researchers.

In recent months, Musacchio's team – in collaboration with Riccardo Cortese's lab in Naples – has obtained exciting results using a novel inhibitor called Reversine, which targets the Aurora B active site with an approximate IC_{50} of 10 nM. This compound surfaced in a 2004 report from Scripps Institute researchers, who showed that it reversed the plasticity of a myoblast cell line, C2C12, causing them to differentiate into different cell types.

Biochemical experiments showed that Reversine inhibits nearly 100% of Aurora B activity but leaves Aurora A alone – a specificity not seen with other small-molecule inhibitors. Musacchio knew right away it would be valuable as a tool for unearthing Aurora B's unique functions, and that it might have therapeutic potential as well.

Experiments with Reversine have already shed new light on Aurora B's contributions to the SAC. In addition to being the SAC's tension sensor, letting this cellular machine know when lax, "tensionless" attachments need to be fixed, it also maintains the SAC before chromosomes gather. Essentially it helps maintain the theater between shows.

The therapeutic potential of this molecule has yet to be explored. “Its discovery as an Aurora B inhibitor is only six months old,” Musacchio said. “It should most definitely be tested in the future.”

Breast cancer development through the perturbation of multiple pathways served by BRCA 1 and 2

David M. Livingston
Dana-Farber Cancer Institute/Harvard Medical School

Educated consumers who have never heard of *Ras*, *Myc* or *p53* know that *BRCA1* and *2* are breast cancer genes. If these people are women, especially well-informed men, or of Ashkenazi heritage, they may know that mutations in these genes are also implicated in ovarian cancer.

Ask what these genes do – when they’re not running amok and causing cancer – and few people have a clue. Until recently, biologists were not much better off. Transfixed at first by BRCA1 and 2 mutations associated with inherited breast and ovarian cancers, it took time for researchers to step back and look at the big picture.

David Livingston and colleagues at Dana Farber Cancer Institute are using multiple technologies to develop a systems-level understanding of how BRCA 1 and 2 sustain normal mammary epithelial cell development and how perturbations cause disease. They knew at the outset that these genes encode large nuclear proteins used by many cell types during homologous recombination, proteins that help maintain the integrity of DNA from generation to generation in many cell types. And they recognized that in breast and ovarian tissue these genes normally exhibit what Livingston called “professional tumor suppressing elements.”

Alternative splicing enables BRCA1 to generate a minimum of three different protein products; whether the same is true for BRCA2 is unknown. The Livingston lab focuses on the polypeptide known as IRIS, which is involved in initiating DNA replication and also stimulated S-phase progression during the normal cell cycle. Germ line mutations can turn helpful genes like BRCA1 and 2 into bad actors, of course, and in breast cancer cells IRIS levels soar and proliferation goes into overdrive.

By combining gene expression data with functional genomic and proteomic data, Livingston and his colleagues are seeking a systems level understanding of the networks that breast cancer genes exploit to cause disease.

Cancer-associated mutations occur not only in *BRCA1* and *2,* but also in other genes whose products interact with proteins encoded by BRCA1 and 2. While mutations in BRCA1 are associated with highly penetrant breast cancer development, abnormalities in BRCA2 are associated with less serious disease. Since both genes have tumor suppressing properties, it is unclear what accounts for this difference.

Whatever the case, BRCA1 -/- tumors are more likely to be basal-like and ER/PR/HER2-negative, BRCA2 lesions are more often ER+/PR+. The researchers also found that IRIS is sometimes overproduced in cell lines derived from sporadic, rather than inherited, human breast cancer; in these cells it boosts HER2 expression, probably through a transcriptional route.

Recent experiments in the Livingston lab have manipulated IRIS in a wound-filling model using human epithelial cells, where the investigators have observed that it confers a neoplastic phenotype when alternatively spliced. They have also noticed that these same BRCA1 -/- cells are often missing an intact Xi (inactivated X chromosome, which female cells should have)

In one recent publication from Livingston's group, first author Miquel Angel Pujana reported last fall in *Nature Genetics* that an "omic" (genomic, proteomic) approach enabled him to identify a network of 118 genes with 866 potential functional associations in cells from women with elevated breast cancer risk.

These proteins comprise a network, which normally shape mammary epithelial cell behavior and are collectively disturbed in BRCA1-/-, BRCA2 -/- cells, and certain sporadic breast cancers, Livingston said. System-wide screening will help investigators identify the many coconspirators of IRIS, which Livingston speculates may turn out to be "the most upstream player in erb B2 tumors." In clinical practice, genomic data will ultimately help doctors characterize breast tumors more precisely and treat patients more effectively.

Session 2: Cancer model systems

Biological Properties of Cancer Stem Cells

Pier Guiseppe Pelicci
Department of Experimental Oncology, European Institute of Oncology, and FIRC Institute of Molecular Oncology (IFOM), Milan

Faced with an unruly mob of demonstrators storming the gates of an embassy or a factory, what's the best way for police officers to restore order? Some say a great show of force is best, and that hauling huge numbers of protesters away in paddy wagons is the solution. Others contend that the best way to prevent future trouble is to hunt down and arrest members of the vanguard, individuals whose incendiary messages sparked the riot in the first place.

A similar debate is going on among cancer biologists, now that researchers have found that cells with the properties of stem cells (SC) are integral to the development and perpetuation of several forms of human cancer. If eradicating cancer stem cells (CSC) is the key to curing cancer, then drugs aimed at "debulking" tumors could amount to temporarily breaking of a riot while leaving dangerous organizers on the streets. On the other hand, there is no proof that selectively killing CSCs will keep the tumor from growing back.

Direct proof that eliminating CSCs is the way to cure cancer is lacking, Pier Giuseppe Pelicci said, mainly because appropriate model systems are scarce. His laboratory uses mouse, *C.elegans* and *Drosophila* models to study biological differences between normal and transformed CSCs – focusing mainly on leukemia and breast cancer.

Self-renewal is the defining property of SCs, which typically use *asymmetric cell division* to generate one daughter with SC fate and one that differentiates. During development or in response to injury or disease in adulthood, Pelicci said that some SCs proliferate at a rate that outstrips what asymmetric division alone can manage. Recent findings in *C.elegans* and *Drosophila* indicate that SCs use *symmetric cell division* to accomplish

this, generating two SC daughters at once.

In his symposium presentation, Pelicci focused mainly on stem cells in mice where a prolonged, pre-leukemic stage was induced by administering the oncogenic fusion protein PML-RARα. In these pre-leukemic mice, the number of functional SCs is unchanged unless p21 is lost as well. Experiments in the Pelicci lab suggest that PML-RARα induces DNA damage, which in turn up-regulates p21, and that the two abnormalities conspire to maintain a pool of SCs carrying PML-RARα.

If p21 is knocked out, however, pre-leukemic SCs hyperproliferate and die. This suggests that p21 and PML-RARα team up to maintain a population of quiescent SCs even though the DNA is abnormal, he said, and even if their progenitors are lost. It helps that by inhibiting p53, PML-RARα disrupts a checkpoint response (where p21 is also a component) that should ordinarily eliminate SCs with defective DNA. “Our findings suggest that that asymmetric divisions of stem cells function as a mechanism of tumor suppression, that SC quiescence is critical to the maintenance of the transformed clone and that symmetric divisions of SCs permits geometric expansion,” Pelicci said.

Extracellular Matrix Controls Cell Survival through Multiple Mechanisms Including Regulation of Cell Metabolism

Joan Brugge
Department of Cell Biology, Harvard Medical School

Gone are the days when the extracellular matrix, the latticework that gives structure to tissues, was thought of as biologically inert. Decades of research show that the ECM has profound effects on the survival, development, differentiation and death of cells.

In fact, cells separated from their normal attachments behave like a macabre exaggeration of homesick kids at summer camp – they not only go off their feed and stop taking proper care of themselves – many of them actually die.

Cancer cells are different. If they were human, we’d diagnose them as sociopaths because they are so heedless of normal attachments. And this blasé attitude is what cancer researchers seek to understand, because whatever allows cancer cells to proliferate outside the matrix could be an attractive target for therapeutic intervention.

Joan Brugge’s lab focuses on the specialized architecture of the breast, which like other glandular tissues is organized into ducts and hollow spheres. The spheres consist of a monolayer of luminal epithelial cells, and their empty centers are kept clear by mechanisms that confine cells to the walls.

Brugge and her colleagues have found that losing matrix attachment is the beginning of the end for normal cells, and that unmoored cells die in one of three ways: apoptosis, metabolic impairment, or entosis – a novel non-apoptotic mechanism involving invasion of one cell by another and lysosomal degradation. The only cells that survive detachment and keep proliferating have oncogene-induced changes that somehow enable them to cheat death.

In her symposium presentation, Brugge described how her team has used MCF-10A cells (immortalized, non-tumorigenic mammary epithelial cells) as a model for exploring

how loss of matrix attachment impairs normal metabolism. Cells detached from the ECM exhibit a dramatic decrease in ATP level that is independent of the apoptotic program, which is initiated by loss of attachment. In addition, glucose uptake almost disappears within 24 hours of detachment, which Brugge said could explain the fall in ATP level.

ATP levels were easily elevated by administering antioxidants, while glucose uptake was unaffected, suggesting that reactive oxygen species (ROS) contribute to ATP reduction in detached cells. Brugge's team found that antioxidant treatment boosted fatty acid oxidation, giving the cells an alternate source of ATP. They also discovered that overexpressing the oncogene ErbB2 rescued cells from detachment-induced ATP deficiency in an EGFR/Akt dependent fashion. ErbB2 overexpression restored glucose uptake and reduced cellular ROS.

In a more lifelike, three-dimensional model, the investigators found that treating cells with the anti-oxidant N-Acetylcystein (NAC) did indeed promote luminal filling. "Hyperproliferation and anti-apoptosis are both needed to grow tumors," Brugge said, and in these experiments both anti-oxidants and the ErbB2 oncogene promote the survival of cancer cells outside their natural niches.

Brugge noted that high levels of super-oxide dismutase (SOD2) have been found in advanced breast tumors, and her group's findings "Raise questions about whether anti-oxidants are always so good for you – especially if you have early tumor cells."

Myeloid-derived suppressor cells in cancer

Vincenzo Bronte
Istituto Oncologico Veneto and Venetian Institute for Molecular Medicine, Padova

Although vaccines that protect healthy people against Hepatitis B and Human Papiloma Virus also reduce the risk for liver and cervical cancer, campaigns to create therapeutic vaccines for cancer patients have been less successful. "Objective clinical response rate was less than 10 percent in a meta-analysis of all available clinical data for trials using vaccines to fight solid tumors," Enzo Bronte reported, based on an analysis he and his colleagues carried out four years ago.

By the time cancer patients enter trials like these, their tumors are already adept at suppressing and evading the body's protective immune responses. Bronte's symposium presentation focused on how alterations in myelopoiesis interfere with the body's early warning system and its response to immunotherapy.

Several mouse tumors, either spontaneous or arising from transplanted cells, alter normal myelopoiesis and give rise to abnormally large populations of $CD11b^+/Gr\text{-}1^+$ cells in the blood, lymphoid organs and at the tumor site. Several years ago, researchers designated these as myeloid-derived suppressor cells, or MDSCs. Bronte has been a leader in discovering exactly how these cells – recruited by growing tumors – lead to dysfunctions in the immune response.

Working in knock-out mice, Bronte's team used genome-wide expression profiling, biochemical analyses, and functional studies to identify a population of $CD11b^+$, inflammatory-type monocytes expressing the alpha chain of the interleukin-4 receptor (IL-4Rα). These cells are elicited by growing tumors and activated by interferon-γ (IFN-γ) released from T lymphocytes, Bronte said. $CD11b^+/IL\text{-}4R\alpha^+$ cells produce IL-13 and IFN-γ, integrate their signals into a coherent message, and trigger a chain of events that

causes $CD8^+$ T lymphocytes to sit on the sidelines – instead of rushing to the body's defense – when invaders threaten.

Two enzymes – arginase-1 (ARG1) and nitric oxide synthase-2 (NOS2) – metabolize the amino acid L-arginine and take the final steps that keep $CD8^+$ T lymphocytes from doing their job. Assays in the Bronte lab indicate that these enzymes are overexpressed in about 60 percent of human prostate cancer cells. These findings unveiled potential targets for novel therapeutic interventions, Bronte said. Treatments that help restore normal host immunity would improve the odds for success using anti-cancer vaccines or other immunotherapies.

As it happens, three of the world's most heavily advertised drugs – Viagra, Cialis, and Levitra – reverse tumor-induced immune suppressing mechanisms in several mouse models for cancer. These phosphodieserase-5 (PDE5) inhibitors interfere with ARG1 and NOS2 in the MDSCs recruited by tumors and allow T cells to spring into action. In experiments that mimic the microenvironment of prostate tumors in hosts with intact adaptive immune systems, "when we treat with sildenafil (Viagra) we see massive infiltration of CD8 cells into tumor," Bronte said.

He is also working with a pharmaceutical company to investigate whether aspirin that is chemically combined with a nitric oxide (NO) donor might hold promise as an adjuvant for therapeutic vaccines against cancer. In mouse models, adding "nitro aspirin" to the animals' drinking water reduced arginase activity to normal levels, and Bronte's lab has more experiments underway in human MDSCs.

In the meantime, he did not speculate about when television commercials will tout erectile dysfunction drugs as "good…and good for you!"

Systematic Proteomic Analysis of Human Deubiquitinating Domain Containing Proteins Reveals Candidate Targets and Pathways for Ubiquitin Control

J. Wade Harper
Department of Pathology, Harvard Medical School

In households around the globe, an archetypal debate takes place millions of times every day. One person wants to throw something out, but the other insists that the object is needed and must be kept.

The same argument goes on incessantly inside cells, where proteins are at issue instead of old boom boxes. Wade Harper's aim is devising better ways to eavesdrop on all those molecular disputes about what to keep and what to toss out.

For more than a decade, Harper has been studying the cell's highly regulated trash disposal system, the ubiquitin-proteasome pathway. This system is a key regulator of many events in the cell, including the cell division cycle and its checkpoints, which makes it an attractive target for drug discovery.

Ubiquitin-conjugated proteins are bound for destruction by the proteasome, a kind of intracellular shredder. Ubiquitin must be assembled in a three-step process called the E1-E2-E3 cascade before it hooks onto proteins and marks them for destruction. Acting in opposition to the E1-E2-E3 cascade are deubiquitinating enzymes (Dubs), which specifically recognize target proteins and remove or remodel ubiquitin chains.

Over the past decade, Harper's highly productive teams – first at Baylor College of Medicine and now at Harvard –have identified a superfamily of cullin-based E-3s encoding more than 250 E3s that target hundreds if not thousands of proteins for degradation. They have also detected about 95 Dub domain containing proteins (DDCPs) in the human genome.

It is one thing to identify hundreds of molecules that appear to be of a certain class, and quite another to figure out what they actually do. The Harper lab brings many technologies into the quest: biochemistry, mass spectrometry, and now functional screens that use "small hairpin" RNA (shRNA) to silence or knock down genes. Working with Stephen Elledge, also at Harvard Medical School, Harper's team has used shRNA to create libraries of shRNAs targeting the ubiquitin system as well as protein kinases, that are being used in high throughput screens for cell cycle components. Working at this scale, his lab has also developed considerable bio-informatic expertise.

This multi-pronged approach is paying off. Recent RNAi screens revealed that some DDCPs are essential for proper cell cycle progression. In order to begin to understand the biological functions and targets of deubiquitinating enzymes, the investigators performed a systematic proteomic analysis of DDCPs and their associated proteins in human cells. A library of 77 full length DDCPs were tagged with an N-terminal Flag-HA epitope and expressed from a retroviral LTR promoter in 293T cells.

After purification, each DDCP and its associated proteins were identified by LC-MS/MS. In order to pin down selective interactions between proteins and individual DDCPs, a new statistical scoring procedure was developed to distinguish "what is real from what is background information," Harper said. The researchers created software with built-in cross checks aimed at spotlighting important DDCP-protein interactions.

Sixty-percent of DDCPs formed complexes, either with a limited number of proteins or with previously characterized macromolecular complexes. "We found that previously unstudied Dubs are active in transcription, splicing, mRNA and decapping, DNA damage recognition, and cell division," Harper said. At least two Dubs are linked to histone modifications, one in a yeast model, and future efforts will incorporate yeast and C. elegans databases.

Harper and his colleagues have been able to generate interaction maps, showing where some Dubs act and members of the lab are validating these results using technologies including reverse Tag mass spectrometry. "One of our ultimate goals is to merge proteomics data with RNAi data, such as proliferation or spindle checkpoint screens, to gain new insights into biological pathways," Harper said.

Therapeutic potential of cancer stem cell targeting

Ruggero De Maria
Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanità, Rome

As a physician, Ruggero De Maria treats cancer patients at the Mediterranean Institute of Oncology, a cancer hospital in Catania (Sicily); as a researcher, he investigates cancer stem cells (CSCs) – rare, undifferentiated tumorigenic cells that are thought to be responsible for tumor initiation, maintenance and spreading. De Maria knows from

clinical experience that conventional chemotherapy targeting rapidly proliferating tumor cells often fails to cure patients – perhaps because it leaves slower growing cell populations – which might include CSCs – untouched.

If more was known about these CSCs, oncology might be revolutionized by rationally designed therapies that could selectively kill CSCs and thus eradicate tumors. To learn more, De Maria and his colleagues developed a technology that has enabled them to isolate and expand CSCs from biopsy samples of various tumors, including glioblastoma, melanoma, breast, lung, colon, thyroid and ovarian cancer.

When purified CSCs were injected into immune-deficient mice, “patients whose sample gave rise to CSCs had a very poor prognosis compared with patients from whom SCs could not be purified, De Maria said. The outlook was especially poor for patients whose samples tested positive for CD133 and Ki 67. (Many studies, by his group and others, have shown that CD133+ cells are tumorigenic in several cancers, including colon and glioblastoma. Antibody assays for Ki 67 are used to determine what proportion of cells in a biopsy sample are proliferating.)

The ability to recapitulate specific human tumors in mice is a powerful tool for evaluating the therapeutic potential of new cancer treatments. De Maria and his team are using a variety of methods – including genome-wide expression of mRNA, microRNA and proteome profiling – to profile the tumorigenic cell population of a variety of solid tumors. They used Reverse-Phase Protein Microarray technology to profile all the major survival pathways active in CSCs. Homing in on atypical signalling networks in CSCs allowed them to identify several pathway inhibitors that they are testing, alone and in combination, both *in vitro* and in CSC-based mouse xenografts.

The encouraging news from proteomic screening is that CSCs from melanoma and colon cancer samples look “more similar than when we do the same analysis of differentiated cancer cells,” De Maria said. The flip side is that combination therapies will probably be needed to attack both stem and differentiated cells within the same tumor.

Studies using mice with engrafted human tumors have already yielded at least one surprise: erythropoietin helps protect breast CSCs from taxol and doxorubicine. This chemotherapy regimen shrinks breast tumors in humanized mice, but giving Epo at the same time keeps the tumor large and thriving. Having said this, De Maria emphasized that results obtained in mice cannot be used to judge treatment efficacy in humans.

Membrane organization in tissue homeostasis and tumorigenesis

Andrea I. McClatchey
MGH Center for Cancer Research and Harvard Medical School Department of Pathology

As many as one in 25,000 people has neurofibromatosis type 2, a genetic disorder that allows tumors to develop on nerves – especially in the brain and spinal cord. People with this progressive disease inherit one defective copy of the neurofibromatosis type 2 (NF2) tumor suppressor, but do not develop hearing loss or other symptoms until they acquire mutations in the second copy of NF2. Once NF2 function is lost, multiple tumors develop and they may be extremely difficult to treat. Sometimes tumors can be surgically resected, sometimes not. There are no proven pharmacologic treatments.

In contrast to most tumor suppressors, which act inside the cell to control cell division, NF2 localizes and acts at the interface of a cell and its environment. And this is where Andrea McClatchey and her team focus their attentions.

NF2 encodes Merlin, which is closely related to ERM proteins (Ezrin, Radixin and Moesin) that are thought to organize membrane domains by providing regulated linkage between membrane proteins and the cortical cytoskeleton. Although this family connection has been known for about 15 years, therapeutic progress has been slow because Merlin's exact functions have been difficult to decipher. Now it appears this long dry spell may be ending.

The McClatchey lab developed a mouse model for Nf2 and has used this to understand Merlin's tumor suppressing function and how its loss promotes tumor growth. Mutant mice develop a variety of what she called "spectacular phenotypes" – including overproliferation in the skin and intestine, and metastatic tumors of bone and liver – which have helped the researchers see the essence of Nf2's tumor suppressing function.

"We have found that Merlin prevents internalization and signaling of the Epidermal Growth Factor Receptor (EGFR) upon cell:cell contact, thereby mediating contact-dependent inhibition of proliferation and coordinating the processes of junction stabilization and EGFR silencing," McClatchey said. In order for this to happen, Merlin must localize at the cortical actin cytoskeleton. In fact, single particle tracking studies reveal the ability of Merlin to control the movement of individual EGFR molecules on the cell surface.

"If you remove Merlin, you get persistant EGFR signaling," McClatchey said. "This is important because we want to find ways to help patients with Nf2-deficient brain tumors." In cell culture, her team observed that small molecule EGFR inhibitors – such as gefitinib and erlotinib – restored contact-dependent inhibition to Nf2-deficient cells.

The most common tumors associated with NF2 are schwannomas, growths along the auditory nerve that cause patients to lose their hearing and sometimes cause intense pain as well. In Schwann cells cultured from patients, gefitinib treatment returned the cells to a normal phenotype: "we saw a spectacular response of these cells *in vitro,"* McClatchey said.

These results led physicians at Massachusetts General Hospital to try administering Erelotinib (Tarceva) to a small number of schwannoma patients, and "one who had an auditory growth is hearing sounds for the first time in years," McClatchy said.

Back in the lab, she believes that what her team has learned about Merlin's inhibition of EGFR signaling is a paradigm for understanding how Merlin and the ERM proteins provide a coordinated intracellular response to multiple signals from outside the cell. Current studies are pursuing this.

Session 3 – Therapeutic strategies in cancer

Turning Biological Insights into Drug Discovery Projects

Giulio Draetta
Vice President, Worldwide Basic Research Oncology Franchise Head
Merck Research Laboratories, Merck & Company, Inc., Boston

Most of the speakers at this year's Armenise-Harvard Symposium spend their careers wrestling with fundamental questions about the inner workings of normal and cancer cells. And while these basic scientists hope what they learn will eventually improve cancer care, perhaps even for someone they love, few are engaged in the nuts-and-bolts of translating discoveries into drugs or vaccines.

In this year's closing session, however, the spotlight shifted to investigations closer to the clinical end of the spectrum. Merck Vice President Giulio Draetta introduced this session with a panoramic view of a major pharmaceutical company's oncology research portfolio. Such a landscape harbors many potentially drugable targets, and armies of researchers have hundreds of small molecules, monoclonal antibodies and vaccines in preclinical and clinical development.

Major challenges for drug developers include understanding what the drugs do and why they may not have the same benefits for all tumors or all patients. A molecule designed to inhibit a specific signaling pathway, Draetta said, may affect other paths in unexpected ways. Treatment "is never about just one thing – proliferation or resistance to apoptosis – it's about integrating different approaches."

Complicating the picture is the fact that even if a cell population arises from the same progenitor, their response to drugs aimed at blocking specific pathways depends on where they are positioned within the tumor, how much oxygen they're exposed to and the nature of their extracellular attachments. In reality, of course, most tumors contain a mix of cells. "From a treatment approach, it's all about heterogeneity," Draetta said.

Nor are all patients the same, which drives Merck's interest in comprehensive genomic tumor evaluation that will help doctors detect and treat individual tumors as effectively as possible. Ideally, comprehensive biomarker screens will enable physicians to select the most appropriate initial treatment, quickly evaluate response at the molecular level, and change therapies if needed. The company is working with Moffitt Cancer Center in Florida to match the molecular signatures of tumors with clinical trial results, Draetta said, and they're getting better at predicting who will or won't benefit from standard treatments.

The goal of the Moffitt partnership and similar efforts underway in other countries is "to change the treatment paradigm from a standard algorithm to a treatment matched to an individual patient's cancer," he said.

Although this may sound futuristic, Draetta emphasized that "the future is now." Merck researchers have already used genome-scale siRNA screening to identify pathway and network characteristics correlated with an enhanced response to cisplatin or gemcitabine. The two sets of enhancers are different, yielding complementary information that helps physicians match patients with the best therapies.

Working in mouse models, company scientists have also made what Draetta called "baby steps" toward predicting response to vorinostat, a novel histone deacetylase inhibitor. A "second baby step" uses a gastric carcinoma cell line, where it appears that amplification of c-MET and FGFR2 predict in vitro tumor response to inhibition of c-MET.

In conclusion, Draetta expressed optimism about the future. "We are moving in the direction of ideal treatment by starting very close to the target, but ultimately responsiveness is going to depend very much on an integrated view. It's not going to be just MET or Notch in isolation, the state of Myc or p53 might also be involved. All of this must be taken into account as we approach this ideal state."

Notch1 in T-ALL: Initiator, Collaborator, or Progressor?

Jon C. Aster
Department of Pathology, Brigham and Women's Hospital and Harvard Medical School

Harvard's Jon Aster, along with collaborators at the University of Pennsylvania, is determined to develop better treatments for T-cell acute lymphoblastic leukemia/lymphoma (T-ALL), one of the most common childhood cancers.

The key, according to Aster and his colleagues, is to understand the Notch-1 mutations that are found in 50-70% of human T-ALL patients. Called gain-of-function mutations, these abnormalities turn up the volume on one of the most widely used – and best studied – pathways in all of biology. Notch-1 receptors are large transmembrane proteins that bind various ligands, undergo cleavage by gamma secretase, and trigger a chain of messages inside the cell.

In normal development, Notch signaling is tremendously important in helping cells fulfill their proper destinies. But bad things can happen when things go awry.

"Everything depends on context and dose when you talk about Notch," Aster said. Strong gain-of-function Notch1 alleles induce leukemia in mice, but these are much more powerful than the mutations typically found in human leukemia patients. To better understand the effects of the most common mutations found in human leukemia, the researchers introduced Notch1 alleles bearing mutations of varying strength into murine hematopoietic precursors. They used only mutations found in T-ALL patients, predicting that any one would be capable of causing cancer.

But not so. Although rare and powerful gain-of-function Notch1 alleles drove T-cell development and caused leukemia, Aster reported, "most Notch 1 mutations found in human leukemia do not generate signals of sufficient strength to cause leukemia on their own." These weaker, more common mutations also led to rapid depletion of hematopoietic stem cells, possibly by inducing T cell differentiation.

These findings suggest that most Notch1 mutations in T-ALL act as collaborators with other mutations. To these this idea, the Notch1 alleles were introduced into mice engineered to express a K-ras oncogene in T cell progenitors. In this model, the same Notch1 mutations that failed to induce leukemia on their own accelerated onset of disease, confirming that less Notch is needed in a collaborative situation than when Notch acts on its own.

Knowing that Notch1 mutations are common and important in T-ALL, the researchers asked whether gamma secretase inhibition might benefit patients. Pharmaceutical companies initially developed such drugs for treating Alzheimer's disease, and so they were available for a small trial enrolling T-ALL patients who did not respond to other agents. "But the trial failed despite the fact that all the genetics said this was the right pathway," Aster reported. The drug had significant GI toxicity, it did not help, and patients also appeared to develop resistance.

Aster still believes that gamma secretase inhibitors will turn out to be one ingredient in a cocktail therapy for T-ALL. He and his collaborators are screening small molecule libraries and taking a hard look at some drugs already being used for T-ALL, such as dexamethasone and glucocorticoids. "Even though Notch is a secondary player, it is still a good target because it seemingly can collaborate with many oncogenes," Aster said. "The future is in drug combinations."

Signalling through endothelial cell to cell junctions

Elisabetta Dejana
IFOM-IEO Campus and University of Milan, School of Sciences, Milan

Every time the heart beats, the walls of blood vessels take a beating. And if these vessels are not fully and completely formed in the early stages of development, with tight junctions between cells, they will leak or even fall apart when the embryonic heart begins to pump.

The walls of blood vessels recruited during tumor angiogenesis start out "disorganized and fragile," Elisabetta Dejana said, but remodelling stabilizes them and they become tough enough to deliver blood the tumor requires to grow. Anti-angiogenesis factors are already being used to treat cancer patients, and her lab does basic research that could pave the way to more and better therapeutic agents.

The Dejana lab has used mouse models to gain considerable insight into cell-cell junctions in the vascular endothelium. In these endothelial cells, adherens (AJ) and tight junctions (TJ), are formed by transmembrane adhesive proteins linked to intracellular signalling partners and cytoskeletal binding proteins. These are the components of the system that her lab explores in mice, using gene inactivation and blocking antibodies to identify what groups of genes and proteins are essential at specific phases of angiogenesis and vascular homeostasis.

During early phases of vascular development, for example, AJ organization requires a cast of characters including VE-cadherin, which associates with cytoplasmic proteins including β-catenin, plakoglobin, α-catenin, and VE-PTP. When VE-cadherin was knocked out, angioblasts differentiated and the vascular primitive plexus formed, but later vascular remodelling was severely affected. Cells without VE-cadherin gradually disconnected from each other and detached from the basement membrane, leading to vessel collapse, regression and large haemorrhages. Dejana reported. Several vessels had enlarged and /or irregular lumens, suggesting that VE-cadherin is needed to control endothelial cell polarization and proliferation.

Additional experiments revealed a series of interactions between VE-cadherin and cytoplasmic partners that have a dynamic and indirect interaction with the actin cytoskeleton; when α-catenin accumulates at AJs, for example, it reduces branching and

induces bundling of actin filaments. Another important VE-cadherin cytoplasmic partner is $p120^{ctn}$ – an inhibitor of Rho family GTPases that also modulates VE-cadherin membrane stability and endocytosis.

In addition to being components of the AJ complex, β-catenin, plakoglobin and $p120^{ctn}$ can translocate to the nucleus and regulate gene expression. Conditional inactivation of β-catenin in the endothelium using the Cre/loxP system caused lethal defects in early embryos. Blood vessels in these mice branched abnormally, had inconsistent lumen sizes and leaked easily. β-catenin deficient endothelial cells displayed thin and elongated morphology with less junctional area and more fenestrations. Interestingly, the absence of β-catenin rearranged organization of the junctional complex: α-catenin level fell but desmoplakin expression and junctional localization increased dramatically.

Recent experiments in the Dejana lab have used Affymetrix chips to dig deeper into how AJ organization affects the organization of TJs in the vascular endothelium. These experiments have uncovered a novel pathway of Cadherin-mediated gene transcription with implications for tumor angiogenesis. These results are now in press.

Epigenetic targets for therapy in acute myeloid leukemia

Clara Nervi
Department of Histology & Medical Embryology, University of Rome “La Sapienza” & San Raffaele Bio-medical Park Foundation, Rome

Clearly cancer is both an epigenetic and genetic disease, and for the past 15 years Clara Nervi has been studying epigenetic modifications in the acute promyelocytic leukemia (APL) subtype of acute myeloid leukemia. Because epigenetic modifications of DNA and chromatin are reversible, they hold tremendous appeal as targets for treatment.

The benefits of all-*trans* retinoic acid (ATRA) monotherapy for AML patients were first reported in 1990, and this agent has long been approved for treating the APL subtype of the disease. In PML/RARα-positive APL patients, ATRA epigenetically reprograms myeloid differentiation and increases terminal differentiation of APL blasts. The combination of ATRA and chemotherapy is widely used today, and Nervi characterizes this as “a paradigmatic success for epigenetic therapeutic purposes since it increased the APL cure rate up to 75%.”

In acute myeloid leukemias (AMLs), epigenetic modifications silence control regions that regulate cell differentiation and proliferation. Aberrant recruitment of histone deacetylases (HDACs) and DNA-methyltransferases (DNMTs) interfere with normal myeloid differentiation in many ways, but primarily by boosting the activity of three oncogenic fusion proteins (PML/RARα, AML1/ETO, TEL-AML1). “Moreover, the *in vitro* and *in vivo* inhibition of HDAC activities restored the response of non-APL AML patients to RA, in terms of transcriptional activation and terminal differentiation, regardless of their underlying genetic lesion,” she said.

Nervi suspected that drugs working by epigenetic means could be useful for treating AML. In particular, she has focused on how the HDAC inhibitor valproic acid (VPA) in combination with ATRA act at the epigenetic level. In a 2006 article in *Cancer Research,*

her team reported significant reductions in blast levels in eight patients with advanced, refractory AML.

Their patient series has now grown to 14, and of these 51 percent had a “good” response to combination treatment with ATRA and/or VPA followed by the addition of low doses of cytosine arabinoside: five patients experienced periods of complete remission and median survival was 7.5 months. In all cases, toxicity was negligible and treatment was given on an out-patient basis. “Of course we want to expand the number of patients we are treating,” Nervi said.

Members of the lab have confirmed that ATRA-VPA reprograms differentiation in refractory and high-risk AML blasts in vitro and in vivo. “The next step is to use this as epigenetic priming to increase sensitivity to novel interventions such as micro-RNAs,” Nervi said. She is specifically interested in miRNA-223, one of many micro-RNAs that negatively regulate expression of genes involved in development, differentiation, proliferation and apoptosis. Early results indicate that miRNA-223 is involved in the differentiation block of hematopoietic progenitors and AML.

In summary, Nervi said her team’s findings underscore the importance of transcription factors, chromatin remodeling and miRNAs as determinants for hematopoietic differentiation, “thus providing new epigenetic targets for the diagnosis, prognosis and treatment of leukemias.”

The Impact of Genomic Changes on the Treatment of Lung Cancer

Bruce E. Johnson
Lowe Center for Thoracic Oncology, Dana-Farber Cancer Institute and Harvard Medical School

Rarely has a drug received so much attention for what it is not.

But gefitinib, better known as Iressa, is not an ordinary drug. It attracted extraordinary attention in 2003 when the Food and Drug Administration approved it for treating relapsed non-small cell lung cancer (NSCLC) – despite evidence that it had little benefit for about 90% of patients in clinical trials. At the same time, the drug worked twice as well as conventional chemotherapy for the remaining 10% of patients. Their tumors shrank rapidly and they lived about twice as long as those given regular chemotherapy.

One year after gefitinib was approved Bruce Johnson was part of a team that uncovered the biology behind this highly unusual pattern of treatment response. Gefitinib acts by inhibiting a tyrosine kinase that controls epidermal growth factor receptors on the cell surface, and the researchers found that mutations in exons 18-21 of the EGFR tyrosine kinase correlated with dramatic clinical improvement.

Since then, Johnson and his colleagues have learned a great deal more about who does – and who does not – respond well to gefitinib and erlotinib, a related EGFR tyrosine kinase inhibitor (EGFR-TKI). It appears that many common genomic changes that arise in lung cancer patients affect EGFR-TKI sensitivity, including *KRAS* and *PTEN* mutations, secondary T790M mutations in *EGFR*, and *MET* amplification.

US clinical trials indicate that patients with *EGFR* mutations treated with gefitinib or erlotinib have a response rate of approximately 80%, tumor growth is arrested for about

one year, and on average they live for more than two years. Additional information is expected from human trials now underway in Europe and Japan, where NSCLC patients with sensitizing mutations of *EGFR* are randomly assigned to chemotherapy or one of the two EGFR-TKIs.

Meanwhile, Johnson and his colleagues have been scrutinizing the biology of drug resistance. They have uncovered a few cases of *de novo* resistance where tumors grow, instead of shrinking, when they are treated with a tyrosine kinase inhibitor. An insertion mutation into exon 20 is associated with this paradoxical response.

In clinical practice, a more common problem is resistance that crops up after a year or more in patients who initially responded to gefitinib or erlotinib treatment. In about 50% of these cases, a single amino acid change spells the difference between treatable or untreatable lung cancer. When threonine changes to methionine at the 790th amino acid of *EGFR*, this puts the pedal to the metal on cell growth that is no longer inhibited by gefitinib or erlotinib. In 20% of patients who develop resistance, the problem is an amplification of the *MET* oncogene. Some patients carry both the T790 mutation and *MET* amplification.

In the laboratory and in clinical trials, researchers are looking for solo and combination therapies that can overpower drug resistance. Two so-called “irreversible inhibitors,” HKI-272 and PF-299804, have performed better than EGRF-TKIs in preliminary studies enrolling people with resistance and sensitizing mutations. Both experimental agents are in phase II clinical trials and results are expected in 2009.
Researchers are using gefitinib-resistant cell lines with *MET* amplification to screen drug combinations that might help treatment-resistant patients. Concurrent use of gefitinib and PHA 665752, a MET inhibitor, looks promising enough to justify testing in patients.

In addition to more powerful medicines, Johnson said doctors need assays to help match each patient with the best possible treatments. Just two weeks after the symposium, an article in the *New England Journal of Medicine* described an experimental blood test that could distinguish between NSCLC patients likely and unlikely to respond well to EGFR-NTIs. Wishes, it seems, are being granted faster than ever.

Targeting the *MET* oncogene and the invasive growth program

Paolo Comoglio
Division of Molecular Oncology, Institute for Cancer Research, University of Turin

Back in the day, Paolo Comoglio remembers there were sometimes empty seats when he lectured about the *MET* oncogene. Today, he noted with some satisfaction, *MET* and its family members have become so popular that his laboratory has become a popular destination for pharmaceutical companies.

After decades of pioneering research on the scatter factors HGF and MSP, which bind to transmembrane receptors MET or its close relatives, RON or Plexin, Comoglio categorizes MET activity in tumors as either “oncogene expedience” or “oncogene addiction.”

Confronted with a negative microenvironmental condition such as hypoxia, tumor cells mount a practical response. Adverse conditions induce *MET* overexpression, and the tyrosine kinase encoded by the *MET* oncogene switches on a genetic program for

invasive growth that involves cell scattering, invasion, protection from apoptosis and angiogenesis. In a large variety of cancers, deregulated activation of *MET*, therefore, it is a powerful expedient for cancer dissemination.

New technologies make it possible to take a series of “snapshots” of MET signature genes in action, and Comoglio works with collaborators to determine whether a particular expression profile at time of diagnosis correlates with better or worse prognosis down the road.

While some tumors use “oncogene expedience” to grow or spread, others are maintained by what Comoglio called “oncogene addiction.” In these cases, which occur less often, *MET* itself is also the transforming agent, genetically selected for the long-term maintenance of the primary transformed phenotype. In this case, some tumors appear to be addicted to MET -- they need it to sustain their relentless growth.

Because *MET* helps some tumor types metastasize and maintains transformation in others, it is an attractive target for drug development. Which explains why Comoglio’s lab is currently working with 11 different pharmaceutical companies on experimental intereventions, including small molecules, gene therapy, RNAi, decoys and antibodies. He discussed preliminary findings from recent experiments, and sounded optimistic about having more to report in the near future.

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