4th Annual Symposium
Giovanni Armenise-Harvard Foundation

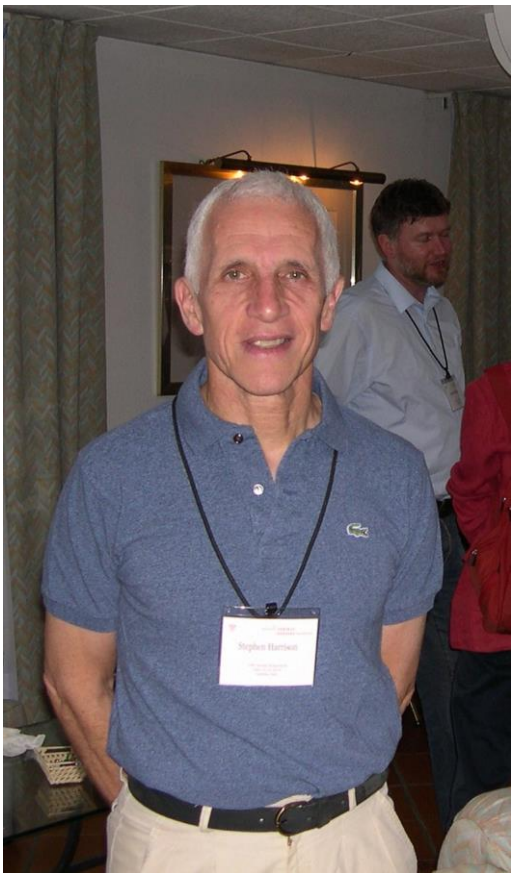
June 19-20, 2000
Bretton Woods, New Hampshire

Contents

About the Symposium

The 4th Annual Symposium of the Giovanni Armenise-Harvard Foundation was held in late June in an emerald valley at the foot of Mt. Washington, the highest peak in the Northeastern United States. In this spectacular setting the Foundation held its first scientific meeting of the new millennium, which was the largest so far with more than 130 Italian and American scientists in attendance.

In his opening remarks, Dr. Stephen C. Harrison of Harvard Medical School reminded participants of the historic past of the Mt. Washington Hotel. It was here that representatives from 44 nations convened, in July 1944, for the landmark Bretton Woods Conference. With World War II drawing to a close, international financial and policy experts gathered to plan for post-war economic stability. They created the International Monetary Fund as a means for stabilizing currency and exchange rates, and the World Bank as a lender to help member countries rebuild and develop after peace was restored.



"It is therefore a totally appropriate site for a conference sponsored by a foundation set up by the generosity of a banker," said Dr. Harrison, paying tribute to Count Giovanni Auletta Armenise, who along with his late wife laid the groundwork for the Foundation in 1994. A few days before this symposium began, a building at Harvard Medical School was named for Count Armenise as a permanent reminder of his contributions to international scientific advancement.

For the past four years, the Foundation has been the catalyst for new collaborations among Harvard Medical School's basic science departments and between HMS and scientific centers in Italy. Much as financial programs born at the Bretton Woods Conference benefited millions of people during the last half of the 20th Century, international scientific cooperation now promises to make life better for future generations. Since its inception, the Foundation's philosophy has been that basic science discoveries will have far-reaching impact on fields as seemingly disparate as medicine and agriculture. During this year's symposium, a series of presentations on the common defense strategies of plants and animals demonstrated that this promise is being fulfilled.

Findings from Armenise-supported research are shared not only during the annual symposium, but at other conferences focusing on basic biology topics. The Foundation also underwrites a broad range of joint ventures, including exchanges of technology and personnel. Five leading Italian institutions conduct Armenise-sponsored research: the European Institute of Oncology in Milano, the University of Padova, the Institute for Cancer Research and Treatment at the University of Torino School of Medicine, the Dipartimento di Ricerca Biologica e Tecnologica (DIBIT) at Scientific Institute San Raffaele in Milano, and Universita' Di Roma La Sapienza. At HMS, the four Armenise centers are: structural biology, neurobiology, cell signal transduction, and human cancer viruses. All were represented on this year's scientific program.

The 4th Annual Symposium featured 20 formal lectures and 32 posters grouped into five sessions:

- Signal Transduction, Oncogenes, Development
- Cellular Differentiation
- Activities of Nerve and Muscle
- Pathogens and Defense
- Cell Cycle, Senescence, Programmed Cell Death

The organization of this report mirrors the symposium program. Each section begins with introductory remarks on the general topic, followed by summaries of individual presentations.

Signal Transduction, Oncogenes, Development

Overview

These presentations illustrate how signal transduction has snowballed over the past decade from a relatively narrow field into a broad discipline that touches nearly every facet of cell biology and medicine. In earlier times, a typical signal transduction experiment examined the effects of growth factors on cells in culture, Dr. Stephen C. Harrison said when he introduced this session. "Then, groups of people suddenly realized they were working on the same thing." Cancer cell biologists who originally explored the relay of signals within cells were joined by developmental biologists who studied the regulation of form and pattern and the differentiation of cells into structures such as skin, hair, or heart. More recently, clinical investigators jumped on the signal transduction bandwagon as they realized that this field might unlock an array of human diseases.

The four papers in this session represented a wide spectrum: a classic look at oncogenic conversion, followed by two studies of cytoskeleton proteins, and finally an examination of signaling's role in the architecture of the heart. Each offered a detailed analysis of the function and structure of some of the players in some of the pathways that help determine larger phenomena. A common theme of these presentations, in Dr. Harrison's view, was that "signal transduction is taking over studies of the cytoskeleton." Without the microtubules, actin filaments, and other proteins of the cytoskeleton, eukaryotic cells would be shapeless and immobile. Cells require a whole library of elaborate computer codes to go about their business, and Dr. Harrison described signal transduction events as "microscopic subroutines within those codes."

Presentations

Molecular mechanisms underlying oncogenic conversion of scatter factor receptor
Paulo Michieli
Institute for Cancer Research and Treatment
University of Torino Medical School

Hepatocyte growth factor (HGF) is one of several scatter factors that play a pivotal role in normal embryonic development. These scatter factors stimulate branched morphogenesis, a process essential for formation of neural, epithelial, and some mesodermal-derived tissues, including muscles and bones. HGF's receptor is Met, encoded by a member of the MET/RON/SEA oncogene family. These receptors are connected to Ras, a signaling pathway that is an established contributor to malignancy and metastasis.

Dr. Michieli and his colleagues have been studying scatter factors and their receptors for many years, and recently they have focused on mutations in the tyrosine kinase domain of Met. Such mutations have been identified in papillary renal carcinoma, a human kidney tumor, and the researchers wanted to pin down the mutation's contribution to disease. To do this, they analyzed the biochemical and biological properties of numerous Met mutants, and observed whether such mutations were sufficient to turn ordinary mouse fibroblasts into cancerous cells. Most of the mutants stepped up catalytic activity in the cells, and those with the greatest transforming potential had the highest kinase activity and the strongest link to signal transducers. The best transformers hyperactivated the Ras signaling pathway, while the less aggressive ones protected against apoptosis.

But there was a catch. In epithelial cells, which don't make HGF on their own, even the mutations that raised catalytic activity to the highest levels could not turn cells malignant unless recombinant HGF was added to the mix. In mouse fibroblasts, which produce HGF, numerous mutants could turn cells malignant. Transformation was easily blocked, however, by adding HGF antagonists or by using site-directed mutagenesis to keep the Met receptor from binding HGF. These data suggest that although Met mutations may have the capacity to cause cancerous changes in cells, this won't happen in the absence of HGF. It is as though they know how to dance, but will only perform when they hear music. *In vivo*, the kidney and liver have abundant HGF, which makes it highly likely that the Met mutations found in human kidney tumors are indeed pathogenic.

From Ras to Rac: not just a matter of guanine nucleotide exchanges (GEFs)
Giorgio Scita, Research Assistant
Department of Experimental Oncology,
European Institute of Oncology

Growth factors set off a series of chain reactions in cells, and while one regulates cell cycle a second may be molding the shape of the cell itself. Dr. Scita's laboratory has examined the cross-talk between the Ras pathway and Rac, a small guanine nucleotide (GTP)-binding protein that is a crucial organizer of the actin cytoskeleton. Rac has been identified as a key downstream target in Ras signaling, and the researchers performed a series of experiments aimed at identifying intermediaries that carry signals between the two. One player is a substrate of receptor tyrosine kinases called Eps8, which binds to a protein designated E3b1/Abi-1. Dr. Scita's team recently showed that Eps8 and E3b1/Abi-1 participate in the transduction of signals from Ras to Rac by regulating Rac-specific guanine nucleotide exchange (GEF) activities.

The plot thickened when the researchers realized that *in vivo*, Eps8 and E3b1 form a tricomplex with a GEF protein called Sos-1. When all three work together, they enable Rac to organize actin filaments into "ruffles" of the cell membrane. But if either Sos-1 or E3b1 is blocked, the ruffles don't form. Further experiments indicated that although Sos-1 acts on Rac when it is part of this tricomplex, it functions quite differently if hooked to a different partner. When the Sos-1 protein is coupled with a Grb2, an adaptor protein, it is recruited to the plasma membrane where it activates Ras by catalyzing the exchange of guanosine diphosphate for guanosine triphosphate.

Although Sos-1 is a versatile player that can function either upstream or downstream of Ras, it cannot play both roles at once. *In vitro*, it is clear that Grb2 and E3b1 compete for binding to Sos-1. *In vivo*, E3b1 overexpression kept Grb2 from associating with Sos-1 and favored the formation of the Eps8-E3b1-Sos-1 tricomplex. This complex has Rac-specific GEF. Additional experiments provided further evidence that Sos-1's specificity as a GEF depends entirely on how it is complexed: a receptor-Grb2-Sos-1 complex results in Ras activation, whereas an Eps8-E3b1-Sos-1 complex regulates Rac activation.

Recognition of a proline motif in beta-dystroglycan by an "embedded" WW domain in human dystrophin
Michael Eck, Assistant Professor
Department of Biological Chemistry and Molecular Pharmacology,
Harvard Medical School

Several years ago, Dr. Eck's laboratory was the first to determine the crystal structure of the protein encoded by *Src*, the first human oncogene to be discovered and the flagship of the tyrosine kinase class of cell-surface receptors. Src is like a switch, made of several different components, that can be flipped by a variety of stimuli. One part of the switch is the Src homology-3 (SH3) domain, a structure that Dr. Eck's team recently found not in cancer, where one might expect to see it, but in a hereditary disease of muscle.

Dystrophin, the protein that is defective in Duchenne and Becker muscular dystrophies, serves as a scaffold for signaling molecules and forms a structural link between the actin cytoskeleton and the extracellular matrix. Dystrophin is linked to the cell membrane through a protein called beta-dystroglycan. The C-terminal region of dystrophin binds the cytoplasmic tail of beta-dystroglycan, in part through the interaction of a WW domain on dystrophin with a proline motif (PPxY) in the tail of beta-dystroglycan. This WW domain is homologous with SH3; in this setting, it is stabilized by an adjacent helical region that contains EF hand-like domains. The crystal structure of the dystrophin and beta-dystroglycan complex shows that beta-dystroglycan peptide binds a composite surface formed by the WW domain and one EF-hand. Embedded in a larger binding molecule, the WW-domain recognizes the PPxY motif much as SH3 would do.

In a separate series of experiments, Dr. Eck's team found another Src-like structure, called SH2, in a negative regulator of signal transduction called CBL. This protein down-regulates tyrosine kinase receptors by marking them for proteolysis. The N-terminal of CBL contains an SH2 domain, again combined with an EF-hand structure, that grabs phosphorylated tyrosine kinases.

Like Legos, standard modules crop up in different settings and their functions are at least partly determined by context. These observations show how efficient nature is at reusing the same hardware for different purposes, Dr. Eck said.

How hearts are made: The genetics behind the induction and patterning of the heart field
Mark Mercola, Associate Professor
Department of Cell Biology,
Harvard Medical School

One of medicine's holy grails is to be able to repair damaged heart muscle. Dr. Mercola's research is predicated on the idea that if researchers knew exactly how cardiomyocytes develop in the embryo, it might eventually be possible to recreate myocardial tissue for therapeutic use.

In order to actually form a heart, prospective heart tissue must receive signals from two adjacent tissues: endoderm that will form the floor of the pharynx and dorsal midline mesoderm that will form the notochord and head mesoderm. Once induced, the heart field is then subdivided into distinct myocardial and non-myocardial compartments, in part by interactions with neurogenic tissue. Recent experiments in his laboratory have focused on two systems that mediate these processes; one involves a growth factor and the other a receptor-ligand pair.

Wnt is a diffusable growth factor, and Dr. Mercola's team has found that the dorsal midline mesoderm secretes several Wnt antagonists, such as Dkk1 and Frzb, that induce genes needed to begin turning non-cardiogenic mesoderm into myocardium. Although additional signals from the endoderm are required for progression to a heart tube, he believes that the location and extent of cardiogenic mesoderm in the embryo depends on the distribution of these endogenous Wnt antagonists. Tissue where Wnt is unopposed will not become part of the heart.

Genes encoding the transmembrane receptor Notch1 and its ligand Serrate1 are expressed in a pattern that strongly suggests they subdivide the heart field into myocardium and non-muscular components such as valves. When the Notch pathway was activated through the downstream transcription factor Su(H), myocardial gene expression was inhibited and non-myocardial genetic markers increased. When Notch and Su(H) function was blocked, mesoderm differentiated into cardiomyocytes. Moreover, lineage analysis showed that cells where Notch signaling was activated did not contribute to myocardial tissue. Clearly, cells will only choose to become myocytes in the absence of Notch signaling.

Cellular Differentiation

Overview

One of biology's greatest wonders is that a fertilized egg gives rise to an embryo made up of myriad cell types that are not only chemically different, but also arranged in a specific, three-dimensional pattern. Cells that inherited exactly the same genetic material from the egg diverge into brain and bone, hair and heart. Such chemical and architectural variety is possible because genes are switched on or off, and are expressed differently in diverse tissues.

This session, like the opening one, also concerned development, Dr. Tullio Pozzan said in his introduction. The first presentation focused on a novel method for identifying genes that are activated in normal and malignant growth of epithelial cells. The other three explored various facets of neuronal tissue development.

Presentations

The transcriptional response of epithelial cells to scatter factors
Enzo Medico
Institute for Cancer Research and Treatment,
University of Torino Medical School

In normal development, scatter factors stimulate epithelial cells to execute a complex program that culminates with polarization and the formation of tubules; in invasive tumor growth, this normal process is subverted and cells proliferate and invade in abnormal ways. One way to understand the differences between normal and malignant growth is to compare which genes are switched on in each. In order to do this, Dr. Medico's team created a "gene trap" – a novel fusion protein that can be used to screen a cell's entire genome for activated genes, whether or not their sequence and function are known.

They built a promoterless retroviral vector carrying a reverse-oriented splice acceptor (or ROSA) gene and the sequence for a green fluorescent nitro-reductase (GFNR) fusion protein that serves as a marker. When this gene trap encounters an active promoter, the trap construct will integrate itself downstream and the marker will be expressed. Cells that have taken up the trap can then be identified using FACS analysis. Conversely, the nitro-reductase moiety allows pharmacological selection against constitutive GFNR expression. A mouse liver cell line was stimulated with hepatocyte growth factor (HGF), a scatter factor, and screened with some traps set to select for HGF-induced genes, and others designed to pick out genes suppressed by exposure to HGF. Some 60 different traps were used to categorize genes and pick out the most promising HGF targets. Several responsive clones were isolated, and regulated expression of the trapped gene was confirmed at the RNA level. When Dr. Medico and his colleagues sequenced the regions around trap sites, they found genes that had never before been linked to scatter factor biology. The goal of future studies will be to shed light on transcriptional response in normal and cancerous cells.

The establishment of neuronal identities in the developing nerve cord
Stefan Thor, Assistant Professor
Department of Neurobiology,
Harvard Medical School

The long-range goal of Dr. Thor's work is to understand the molecular genetic mechanisms that control establishment of motor neuron identities. His laboratory uses *Drosophila* as its primary model, and although the fly has a relatively simple nervous system it still features about 100 distinct types of cells that can be classified as neurons, glia, or interneurons. Recent experiments have focused on LIM homeodomain proteins, a family of transcription factors that are expressed in discrete subsets of developing neurons throughout the animal kingdom.

Dr. Thor's experiments indicate that three LIM-HD genes, *islet (isl), lim3*, and *apterous* act in a combinatorial code to specify motor neuron subtype identity. By attaching markers to mutant versions the genes, he has found that they control two basic hallmarks of neuronal identity – they guide axons toward target cells and specify which neurotransmitters are turned on. Additional genes are probably required to establish the ultimate, unique identity of neurons, and current research focuses on identifying them.

Because LIM-HD programs appear to be highly conserved, Dr. Thor hopes that his findings will ultimately help medical scientists understand vertebrate motor neuron generation and differentiation. With this knowledge in hand, it may someday be possible to replace cells that are lost in spinal cord injuries or neurodegenerative disorders.

***Ebf* genes in vertebrate neural development**
Giacomo Consalez
Department of Neuroscience,
San Raffaele Scientific Institute

A classic family of helix-loop-helix (HLH) transcription factors are the myogenic proteins, which are well known for their role in the differentiation of muscle cells. Dr. Consalez' lab has a long-time interest in a different subclass of transcription factors with the HLH DNA-binding motif, called the Ebfs. This gene family was originally implicated in B-cell maturation and olfactory function. Several years ago, his group identified two family members in the mouse (*Ebf2*, *Ebf3*); more recently they found two more in *Xenopus laevis* (*Xebf2, Xebf3*). Other investigators have cloned Ebf family members from the nematode *C. elegans*.

Just as myogenic proteins can trigger events that turn epidermal cells into myoblasts, Dr. Gonzalez' team has demonstrated that overexpression of *Ebf* genes in *Xenopus laevis* embryos can transform presumptive epidermis into neurons. Ebf expression in frogs begins very early in embryonic development and continues through the tadpole stage, with different genes acting at different times. *Xebf2* operates at early stages of neuronal differentiation, upstream of *NeuroD*, whereas *Xebf3* is a target of *NeuroD* and plays a role in terminal neuronal differentiation. In the mouse, three *Ebf* genes have been shown to advance neuronal differentiation after primary neurogenesis is underway. A tantalizing feature of Ebf proteins is that they have intrinsically different functions, and act at different stages of development, despite having very similar molecular structures.

Signal transduction pathways that regulate neuronal survival in the developing mammalian central nervous system
Azad Bonni, Assistant Professor
Department of Pathology,
Harvard Medical School

For nearly 100 years, scientists have viewed the development of the central nervous system as a life and death matter. Cells are initially produced in huge excess, then whittled away by cell death as development proceeds. Which cells live or die is regulated by extracellular growth factors, such as neurotropins, and Dr. Bonni's laboratory focuses on exactly how these life-or-death decisions are carried out. Using cerebellar granule neurons obtained from rat pups and grown in culture, he has been able to pinpoint the pro-life activities of a polypeptide growth factor called brain-derived neurotropic factor (BDNF).

BDNF appears to promote cell survival in several ways. It activates the Ras-MAPK and PI-3 NKT cascades, which team up to modify BAD, one of a class of proteins that are known to act as gatekeepers of the cell-death machinery. In its native form, BAD promotes cell death by binding to and suppressing pro-survival members of the Bcl-2 family. BAD can no longer kill developing cells, however, if signaling proteins in the Ras-MAPK and PI-3 NKT pathways phosphorylate it at two specific sites.

In addition to this transcription-independent mechanism, Dr. Bonni found that BDNF also promotes cell survival through transcription-dependent means. The MAPK-Rsk path acts on CREB, a transcription factor known to promote cell survival. He hypothesized that while transcription-independent mechanisms might allow cerebellar granule neurons to survive shortly after they are generated on the outer surface of the developing brain, transcription-dependent mechanisms might act later on, as these neurons differentiate and mature in the brain's interior.

Activities of Nerve and Muscle

Overview

Neuroscience is one of the broadest contemporary scientific disciplines, both in terms of its methods and what they are used to study. It encompasses everything from basic biophysics to clinical surveys aimed at linking human diseases with genetic abnormalities. Although three of the four reports in this session use molecular tools to study nerves and muscles, they nevertheless illustrate some of the field's diversity. In his introductory remarks, Dr. Elio Raviola of HMS noted that most scientific lectures about signal transduction and other intracellular events are accompanied by slides showing circles, squares, connecting lines, and directional arrows. But none of these can be seen under the microscope, he observed to appreciative laughter. In fact, little is really know about *where* many molecular events actually take place within living cells. The lead paper in this session described a technique that can be used to track specific chemical changes in real time.

The next two presentations took a molecular look at the interface between nerves and muscles. The first considered calcium's role in the flow of information from the pre-synaptic to the post-synaptic neuron, and how this influences the activity of the synaptic cell. The second examined signal transduction pathways that link electrical signals at the muscle cell surface to transcriptional commands in its nucleus, and along the way uncovers a new role for Ras.

The narrative sweep of the final paper was unusually broad. Here the researchers had to function as social scientists in the field, making contact with members of a sprawling Italian family, before they could use the tools of molecular genetics in the laboratory. The reward was the discovery of a new mutation responsible for an unusual form of epilepsy.

Presentations

Imaging signal transduction in living cells
Tullio Pozzan, Professor
Department of Biomedical Sciences,
University of Padova

Although tyrosine kinase receptor cascades are important, they are only one part of the signal transduction story. Other important players include small molecules known as intracellular mediators or second messengers, especially Ca^{2+} and cAMP. These widely used messengers receive signals from surface receptors, and transmit signals to cellular proteins. Dr. Pozzan's lab has developed novel techniques for pinpointing second messenger activity and monitoring their dynamic interactions. His team used several fluorescent proteins, produced by the jelly fish

Aequorea victoria, to engineer specialized sensors that track the activities of Ca^{2+} or cAMP in living cells. Some of their findings challenge the conventional wisdom.

For example, the mitrochondria have traditionally been seen as the main organelle involved in Ca^{2+} handling. A sensor made with the Ca^{2+} sensitive photoprotein aequorin, which lights up and "freezes" Ca^{2+} activity in stimulated cells, made it possible to localize calcium activity in subpopulations of organelles. Studies with this luminescent probe revealed that in fact a subpopulation of mitochondria, which huddle around the endoplasmic reticulum and do not stray, carry out most of the calcium exchange.

The researchers invented a second sensor that could be used to localize cAMP signaling. This probe tags the regulatory (RII) and catalytic (Cat) subunits of protein kinase A (PKA) with either of two types of green fluorescent protein. The two GFPs were selected for their ability to generate fluorescence resonance energy transfer, or FRET. PKA is the main effector of cAMP in eukaryotic cells. When cAMP is low inside the cell, the RII and Cat subunits of PKA are in close proximity and the donor GFP can transfer energy to the nearby acceptor GFP. When cAMP levels increase, cAMP binds to the RII subunit and the active Cat subunit is released. FRET disappears as soon as the two are separated. By measuring the ratio of blue to green emissions, Dr. Pozzan's team can localize and measure cAMP fluctuations in response to selective stimulation of plasma membrane receptors.

Calcium control of transmitter release during realistic activity patterns
Wade Regehr, Assistant Professor
Department of Neurobiology,
Harvard Medical School

During the normal operation of the brain, neurons often fire in intense, high frequency bursts, separated by long silent intervals. Calcium channels open along the axon as signals travel toward the bouton where neurotransmitter is squirted into the synapse. Although synapses are known to undergo profound strength changes in response to activity, and although changing levels of calcium ions are thought to regulate the strength of the neurotransmitter message, this calcium activity has been quite difficult to observe.

Several years ago, Dr. Regehr and his colleagues developed a method for using calcium-binding fluorescent dyes to study how calcium ions govern neurotransmitter release in different types of neurons. Recently, the researchers have focused on "climbing fiber" synapses that drive the Purkinje cells in rat cerebellum. Some of their findings are surprising: they expected a cell that released a huge burst of neurotransmitter to recover more slowly than a cell that delivered a smaller amount. When presynaptic cells were rapidly and repeatedly stimulated, however, they recovered much more quickly than the researchers predicted. In general, the higher the calcium level, the faster they recuperated and fired again. When the researchers experimentally manipulated calcium levels in presynaptic cells, they found that both release and recovery could be altered. Although there is still much to learn about the dynamic regulation of synaptic strength, Dr. Regehr's interpretation is that the synapses have a complex system for filtering inputs and controlling synaptic outputs during complex activity patterns.

Nerve activity-dependent regulation of the muscle phenotype: a new role for Ras
Stefano Schiaffino, Professor
Department of Biomedical Sciences,
University of Padova

The type of nervous stimulation that a muscle receives is an established factor in both its growth and the type of fiber it comprises. Less clear are the signal transduction pathways that link depolarization at the muscle cell's surface with transcriptional changes in its nucleus. In order to identify these pathways, Dr. Schiaffino's lab uses the rat soleus muscle, in the animal's lower limb, as an *in vivo* muscle regeneration model. As a result of these studies, they have found a new role for the familiar Ras signaling pathway.

Local changes at the neuromuscular junction and broader changes in muscle phenotype occur when a muscle is deprived of nerve stimulation. If an injured rat soleus muscle is not renervated with several days, genes that produce fast-fiber myocin will quickly predominate. New connections will appear, but normal activity will not be restored. If natural healing takes place and the muscle is renervated, large quantities of slow-fiber myocin will be produced and function will return. In the laboratory, this effect can be reproduced by electrostimulation using a continuous, low-frequency pattern.

Knowing that electrical stimulation could restore normal myocin production, Dr. Schiaffino and his colleagues manipulated the Ras signal transduction pathway. They transfected regenerating muscles with either constitutively active Ras or a negative Ras mutant. An unexpected finding was that the active Ras mutant stepped up production of slow-fiber myocin and down-regulated fast myocin – even in cells that were denervated. The dominant negative Ras, in contrast, interfered with regeneration even in electrically stimulated muscles. Additional experiments showed that selective activation of different pathways downstream of Ras had differing effects on muscle growth and fiber type. RasV12S35, which activates the MAPK (ERK) pathway, was able to induce slow myocin but not muscle growth; RasV12C40, which activates the PI3K pathway, affected muscle growth but not myosin gene expression. In addition to the traditional association of Ras and inhibition of myoblast fusion and muscle cell differentiation in culture, this study identifies a new role for this pathway in the differentiation of muscle phenotype by nerve activity.

Idiopathic epilepsy: analysis of a positional candidate gene
Giorgio Casari
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San Raffaele Biomedical Science Park

Epilepsy is a label applied to a broad spectrum of seizure disorders, ranging from mild and occasional to frequent and severe, that affects as many as 1 in 200 people in the general population. Partial or focal forms account for about 60% of epilepsy cases, and there is evidence that a few percent of these are due to single gene abnormalities. Two gene loci have been linked with a Mendelian form of partial epilepsy, called autosomal dominant nocturnal frontal lobe epilepsy, or ADNFLE. The typical clinical presentation involves seizures during light sleep, which are frequently misdiagnosed as nightmares. Among affected members of the same pedigree, some rarely experience episodes and others are troubled by frequent, even nightly

seizures. Symptoms of ANDFLE, which typically begin in childhood and do not worsen over time, usually respond well to treatment with carbamazepine or other anti-seizure medications.

Dr. Casari's team learned of a large Italian family that included many members who, beginning around age 9 to 12, experienced seizures during sleep. Most had a prodrome that involved auras, shivering, or tingling sensations. The researchers used genome-wide linkage mapping to track a third locus for ADNFLE, which they call ENFL3, to a large region of chromosome 1. This region turned out to include CHRNB2, which encodes the beta-2 subunit of the neuronal nicotinic receptor (nAChR). The researchers found that family members with ADNFLE had a mis-sense mutation in this gene that resulted in this gated channel remaining open for an abnormally long time. This finding demonstrates that ADNFLE is more genetically heterogeneous than previously thought, and indicates that the cholinergic system plays a pathogenic role in this form of partial epilepsy.

Pathogens and Defense

Overview

Since its inception, the Armenise-Harvard Foundation has sponsored basic research on plants as well as animals, believing that advances in agriculture as well as medicine hold tremendous benefits for future generations. Thus, each year's symposium has featured lectures on pathogenesis and defense mechanisms in the plant world. This year, the synergy between plant and animal science was more apparent than ever before. Mammalian biologist Tomas Kirchhausen, who chaired the session, admitted that he knew little about plants before he began preparing for the symposium. And the Harvard researcher was astonished to learn that plant and animal defense systems not only resemble one another, but sometimes deploy the same genetic and molecular elements when battling their enemies.

The first presentation in this session, for example, concerned a plant pathogen that injects harmful proteins into its leafy victims with the same syringe-like structure that salmonella uses to invade the lining of the human gut. In the second talk, the emphasis shifted to experimental methods that can be applied to either kingdom. Here it became clear that X-ray crystallography, which has provided remarkable insights into human dramas such as the binding of HIV to T cells, can also illuminate pathogen-host interactions in plants. The third paper focused on a class of proteins that act as both sentinels and warriors, recognizing certain invaders and doing battle with them. Pathogen strategies for evading host defenses were the focus of the closing talk, which made the transition from plants to animals by concentrating on how peptide antigens are presented on cell surfaces.

Presentations

Structural studies on a fungal pathogenicity factor
Benedetta Mattei and Luca Federici
Department of Plant Biology
Università di Roma La Sapienza

The first task of any plant-attacking microbe is to batter its way through the ramparts of the cell wall. A high resolution structural analysis of a fungal battering ram, done by Drs. Mattei and Federici, reveals that the best form of this weapon is also the most recognizable to plant defenses. *Fusarium moniliforme*, like other fungi, uses endopolygalacturonases (PGs) to break cell-wall proteins into pieces and dissolve them. These investigators used X-ray crystallography to determine the structure of PG from *F. moniliforme* at 1.73 Å resolution. Like other pectinolytic enzymes, this PG resembles a squared-off coil of spring, with 10 coils each made up of three or four parallel beta-helical strands, in a coil-coiled helical organization.

The researchers prepared an assortment of site-directed mutants of PG and used these to sort out which PG residues are involved in catalysis and which interact with defensive polygalacturonase inhibiting protein (PGIP). It appeared that three aspartic acids and one histidine may be involved in catalysis, but play no role in recognition by PGIP. The researchers found that amino acid substitutions at two residues (Lys269 and His188) interfered dramatically with the binding of PG and its inhibitor. These and other results led the investigators to hypothesize that inhibition mechanisms involve both competition between substrate and inhibitor and the covering of an active site cleft.

Delivery of bacterial effector proteins to plant cells specifying plant disease resistance
Brian Staskawicz, Professor
Department of Plant and Microbial Biology
University of California, Berkeley

Xanthomonas campestris pv. vesicatoria (Xcv) causes a bacterial spot disease that can devastate susceptible pepper and tomato crops. Dr. Staskawicz' laboratory studies the match between bacterial avirulence genes and the host resistance genes that recognize them, focusing mainly on the Xcv gene *avrBs2* and on *Bs2*, the corresponding plant resistance gene.

Their data suggest that *X. campestris* delivers the AvrBs2 avirulence protein via a syringe-like structure called the Hrp Type III secretion system. This mechanism is familiar to eukaryotic biologists because salmonella, shigella, and other animal pathogens use it to inject proteins into host cells. It's remarkable that plant and animal pathogens have both come up with this machinery, Dr. Staskawicz observed, and many researchers are now analyzing its evolution and genetic underpinnings.

In the meantime, Dr. Staskawicz' findings about effector proteins made by avirulence genes and the receptors that recognize them are being put to use in the field. Years ago, agriculturists found a *Bs2* resistance gene in wild peppers and successfully bred it into commercial pepper strains. As a result, many farms grow Xcv-resistant peppers. There is no indigenous *Bs2* gene in tomatoes, however, so Dr. Staskawicz and his collaborators have used the pepper resistance gene to create

transgenic tomato plants. These are now being field tested for resistance to Xcv. The environmental benefits of these plants could be substantial, as bacterial spot disease is presently controlled by spraying fields with massive amounts of copper and other toxic chemicals.

The multiple functions of the *pgip* gene family
Giulia De Lorenzo, Professor
Università di Roma La Sapienza

Although polygalacturonase-inhibiting proteins (PGIPs) were named for their ability to defend plant cells against fungal endopolygalacturonases (PGs), it turns out that they wear other hats as well. This is to be expected, Dr. De Lorenzo said, because PGIPs are made by genes in the leucine-rich repeat (LRR) family, some of which play key roles in development while others confer resistance to pathogens. In addition to PGs, PGIPs interact with macromolecules including methylated pectins and membrane-associated lipoxygenases.

Dr. De Lorenzo, in collaboration with Dr. Fred Ausubel of Harvard, has been exploring the physiologic significance of these interactions in *Phaseolus vulgaris* and *Arabidopsis*. They have found significant redundancy in *pgip* gene families, with several genes encoding the same or related products. Using knock-out and over-expression mutants, they have begun to identify differences in recognition specificity, regulation, and function for *pgip* genes. One of the most exciting findings is that a *pgip* transgene generates a more heavily methylated pectin than the type found in normal cell walls. This may be useful in helping toughen plants against pests. The researchers have also used *pgip* transgenes to grow *arabidopsis* plants that are bushier than usual.

Proteolysis and the biology of antigen presentation
Hidde Ploegh, Professor
Department of Pathology,
Harvard Medical School

Dr. Ploegh made the transition from plants to animals, describing an ingenious strategy that human cytomegalovirus (CMV) uses to escape detection by the immune system. Killer T cells are usually alerted to the presence of a viral invader when they spot viral antigens, bound to the MHC Class I complex, on the surface of infected cells. This display prompts CD8+ T cells to destroy infected cells. CMV sabotages this by making two proteins, US2 and US11, that keep the MHC Class I complex from reaching the cell surface.

Experiments in Dr. Ploegh's lab recently showed how these two proteins disrupt antigen presentation. US2 and US11 appear to grab newly synthesized MHC Class I products by their tails, which protrude from the endoplasmic reticulum (ER), and drag them into the cytosol. Being ripped from the ER in an untimely fashion, these complexes are viewed by the cell as mis-folded proteins; ubiquitin marks them for destruction and they are whisked off to the proteasome and shredded. There are some other actors in this plot, such as unknown proteins that strip off ubiquitin immediately before proteolysis, and the researchers are still looking for them.

CMV's wiles may help explain why this virus can infect such a wide range of cell types, especially in patients with AIDS or other forms of immune suppression. In the long term,

experiments such as these may contribute to the design of superior gene therapy vectors, which might be able to evade the immune system en route to their targets.

Cell Cycle, Senescence, Programmed Cell Death

Overview

Nothing goes on forever, including the capacity of a normal, well-nourished cell to keep dividing. When a cell becomes senescent, it stops dividing but remains metabolically active for a time, so that it gradually fades away. Programmed cell death is a dramatically different scenario, in which healthy cells act decisively to commit suicide. When senescence and programmed cell death occur at the right time and place, they are an integral part of the life and death of a normal organism. When they occur inappropriately, however, developmental abnormalities or disease can result.

Senescence and programmed cell death are complex phenomena that require a veritable symphony of intracellular signals and processes. Some of the individual contributors were examined by presentations in this session. The first paper described how temperature-sensitive small molecules can be used to selectively block the transport of materials from one organelle to the next. The second concerned programmed death in plant cells, which turns out to bear a surprising resemblance to apoptosis in animal cells. The genetic control of premature senescence in acute promyelocytic leukemia was the focus of the third, and the session ended with an update on the dynamics of LDL-receptor binding.

Presentations

Chemical genetics of membrane traffic
Tomas Kirchhausen, Professor
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Harvard Medical School

Dr. Kirchhausen's laboratory specializes in clathrin, a protein involved in the formation of vesicles that sort and transport materials from the membrane to intracellular organelles. Several years ago, he and his colleagues used X-ray crystallography to solve the structure of clathrin. But they've had less success using yeast knock-out models to analyze its interactions with certain proteins. Chemical genetics offered them a new way to identify substances that can selectively block clathrin's biosynthetic pathway.

Chemical genetics is a novel method for identifying small molecules that disrupt gene or protein function. A fluorescent microscope is used to detect activity in a sample tray with nearly 400 wells, each containing whole cells to which candidate molecules and markers have been added. Dr. Kirchhausen used a temperature-sensitive glycoprotein whose migration from ER to golgi, then from golgi to membrane, can be controlled by temperature manipulation. This enabled the researchers to visualize the activity of different chemicals at selected points in biosynthesis.

Dr. Kirchhausen's team screened approximately 10,000 chemicals, and identified more than two dozen that disrupt the clathrin pathway. They found 2 chemicals that block the exit of newly-synthesized proteins from the ER, and 6 more that block exits from the golgi to the membrane. Eight others altered the structure of the golgi in various ways. The investigators were surprised to find 10 agents that could induce the formation of vacuoles in human cells, and to see that this process could be reversed without damaging the cells.

PML regulates *p53* acetylation and premature senescence induced by oncogenic ras

Mark Pearson
European Institute of Oncology

When the *p53* gene functions normally as a tumor suppressor, it induces cellular senescence in response to oncogenic signals. Although the activity of the P53 protein is modulated by protein stability and post-translational modification, including phosphorylation and acetylation, exactly how the *p53* gene is activated by oncogenes in the first place has been a mystery.

Now Dr. Pearson and his colleagues report that a tumor suppressor gene called *PML*, first identified in a mouse model for acute promylocytic leukemia, acts in concert with *p53* to induce senescence. This gene appears to regulate *p53*'s response to oncogenic signals from Ras. Expression of this oncogene causes *p53* to accumulate and *PML* expression to increase, *PML* over-expression acetylates *p53* at lysine-382, and this makes *p53* biologically active. The outcome is senescence.

The researchers have also shown that Ras stimulation causes *p53* and the acetyltransferase CBP to form a trimeric *p53*-*PML*-CBP complex within the nuclear bodies, a site where *PML* occurs even in normal cells. Further evidence for *PML*'s role comes from knock-out experiments, which showed that *PML*-/- fibroblasts lose Ras-induced *p53* acetylation, *p53*-CBP complex stability, and senescence. These data establish a link between *PML* and *p53* and indicate that unless *PML* is on hand, signals from Ras will go unheard by the cell.

A system to explore programmed cell death in plant-pathogen interactions

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The hypersensitive response is a classic defense strategy of plants, in which resistance genes trigger programmed cell death (PCD) at the site of a pathogen invasion. In order to study signal transduction in this form of cell suicide, Dr. Stone and her colleagues developed a pathogen-free system they could use to trigger focal cell death in *Arabidopsis*. When they treated *Arabidopsis* with fumonisin B1 (FB1), a fungal toxin, the resulting lesions had the hallmarks of the hypersensitive response, including accumulation of phenolics, callose, and camalexin, production of reactive oxygen intermediates, and induction of pathogenesis-related gene expression. Although this model yielded cleaner data than they could have gotten using a whole pathogen, Dr. Stone's team thought they could do better still if they used an even simpler model.

They switched to using protoplasts, which are plant cells stripped of their walls, grown in culture. When these cells were challenged with FB1, the resulting cell death was consistent with PCD in *Arabidopsis*: it was dependent on *de novo* transcription, translation, and protein phosphorylation. Dr. Stone also observed that salicylate-, jasmonate- and ethylene-dependent pathways contribute to FB1-induced PCD, as indicated by FB1 susceptibility of mutants.

To identify other factors contributing to FB1-induced PCD, they selected FB1-resistant (*fbr*) mutants by sowing seeds on FB1-containing agar media. In this hostile environment, two resistant mutants, *fbr1* and *fbr2*, were able to grow. When these mutants were challenged with a different bacterial pathogen, a type of *Pseudomonas syringae* pv. *Maculicola* that expresses the avirulence gene *avrRpt2*, they exhibited no resistance. However, *fbr1* and *fbr2* displayed enhanced resistance to an isogenic strain that did not express *avrRpt2*. These results indicate that this protoplast system can be used to reveal mutants with pathogen phenotypes, and suggest that triggering host PCD is a common feature of compatible plant-pathogen interactions.

Structure of modular elements of cell-surface receptors
Stephen C. Blacklow, Assistant Professor
Department of Pathology,
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The LDL receptor (LDLR) is the primary mechanism that animal cells use to take up particles of low-density lipoproteins from the blood. Healthy cells make LDL receptors and insert them into the plasma membrane when they need cholesterol for membrane synthesis. This normal process is disrupted in people with familial hypercholesterolemia (FH), who inherit defective genes for the LDLR. Because these mutated receptors cannot bind circulating cholesterol, abnormally high levels of lipoproteins accumulate in the blood and these individuals are at high risk for coronary artery disease.

Many researchers have studied the amino-terminal domain of the receptor, which is responsible for binding LDL and consists of seven tandemly repeated LDL-A modules. Each LDL-A module is ~40 residues long, and contains six cysteine residues engaged in three disulfide bonds. The fifth of these modules is regarded as being most critical for LDL binding, but the specific contact points between LDL and its receptor were unknown.

Dr. Blacklow's lab used nuclear magnetic resonance (NMR) imaging to take a closer look at the five-six pair (LR5-LR6) of the LDL-A module. Comparison of proton and multidimensional heteronuclear NMR spectra of individual modules to those of the module pair indicates that most of the significant spectroscopic changes lie within the linker region of the molecules, and that the cores of modules 5 and 6 have scant interaction with one another. The four-residue linker that separates the two modules is highly flexible, and its shape may be mediated by pH-dependent calcium binding. This, in turn, may involve mutations in the epidermal growth factor receptor. What this work-in-progress shows, Dr. Blacklow said, is that normal LDL binding can probably be disrupted by mutations in any of several transmembrane receptors.