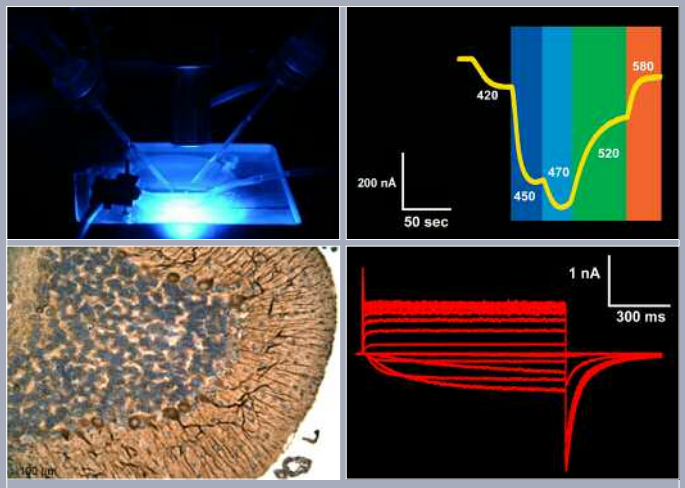
Scripps Florida

- Biochemistry
- Cancer Biology
- Infectology
- Molecular Therapeutics
- Translational Research Institute

Biochemistry



Upper left panel, Electrical signals are recorded from a *Xenopus* oocyte expressing the circadian photoreceptor melanopsin in response to illumination with blue light. Upper right panel, Photocurrent recorded from an oocyte expressing melanopsin and the TRPC3 ion channel in response to illumination at various wavelengths. Peak activation of melanopsin signaling occurs at about 470 nm, which corresponds to the peak light sensitivity of the circadian system. Lower left panel, Expression of the subthreshold potassium channel Elk2 in the soma and dendrites of cerebellar Purkinje neurons. Elk potassium channels are active at neuronal resting potentials and thus may have a strong influence on neuronal excitation. Lower right panel, Elk1 currents recorded from an inside-out patch in symmetrical potassium. Work done in the laboratory of Timothy Jegla, Ph.D., assistant professor, California campus.

Timothy J. Jegla, Ph.D.,

Assistant Professor,

Department of Biochemistry,

California Campus

DEPARTMENT OF BIOCHEMISTRY

STAFF

Steve A. Kay, Ph.D.*
Professor and Chairman
California Campus

Nicholas Gekakis, Ph.D.
Assistant Professor
California campus

Takato Imaizumi, Ph.D.
Assistant Professor of Biochemistry
California Campus

Timothy J. Jegla, Ph.D.
Assistant Professor
California Campus

Claudio A.P. Joazeiro, Ph.D.
Assistant Professor
California Campus

Malcolm A. Leissring, Ph.D.**
Assistant Professor
Florida Campus

Mathew T. Pletcher, Ph.D.***
Assistant Professor
Florida Campus

Thomas Schultz, Ph.D.
Assistant Professor of Biochemistry
California Campus

James Tam, Ph.D.
Professor
Florida Campus

Claes Wahlestedt, M.D., Ph.D.
Professor
Florida Campus

RESEARCH ASSOCIATES

CALIFORNIA CAMPUS

Hedieh Badie, Ph.D.

Antoine Baudry, Ph.D.

Mario Bengston, Ph.D.

Ghislain Breton, Ph.D.

Brenda Chow, Ph.D.

Jessie Chu, Ph.D.

Sinead Clancy, Ph.D.

Eva Farré, Ph.D.

Elizabeth Hamilton, Ph.D.

Samuel Hazen, Ph.D.

Anne Helfer, Ph.D.

Warren Lewis, Ph.D.

Andrew C.-Y. Liu, Ph.D.

Julien Mamet, Ph.D.

Brooke Miller, Ph.D.

Alessia Para, Ph.D.

Jeffery Pitman, Ph.D.

Jose Pruneda-Paz, Ph.D.

Mariko Sawa, Ph.D.

Hein Tran, Ph.D.

Axel Ulbrich, Ph.D.

David Welsh, M.D., Ph.D.

Matthew Wheeler, Ph.D.

Jason Wilkes, Ph.D.

Eric Zhang, Ph.D.

FLORIDA CAMPUS

Shaun Brothers, Ph.D.

Jia Huang, Ph.D.

Ahmad Khalil, Ph.D.

Rebecca Kocerha, Ph.D.

Kristen Clarke Ware, Ph.D.

** Joint appointments in the Department of Cell Biology and the Institute for Childhood and Neglected Diseases*

*** Joint appointment in the Department of Molecular Therapeutics*

**** Joint appointments in the Department of Molecular Therapeutics and the Translational Research Institute*

Steve A. Kay, Ph.D.

Chairman's Overview

The Department of Biochemistry at Scripps Research was recently created to recruit faculty to both the California and the Florida campuses. The overall theme of the department centers on the need to understand physiologic processes from the molecular level to the whole organism. Our faculty members are generally multidisciplinary biologists and chemists who wield cutting-edge tools of structural biology, protein dynamics, biological chemistry, genetics and genomics, pathway analysis, and computational approaches to understand how organisms maintain homeostasis and respond to stress and disease.

We have broad interests, and we seek to answer contemporary questions in neurobiology, metabolic control, immunology, and cancer biology. By taking integrative approaches to substantial problems in modern biology, we will contribute to the understanding of a wide variety of diseases such as diabetes, cancer, and CNS disorders.

Investigators' Reports

Genetics and Genomics of Circadian Clocks

S.A. Kay, A. Baudry, G. Breton, B. Chow, A. deSchöpke, E. Farré, E. Hamilton, S.P. Hazen, A. Helfer, T. Hirota, T. Imaizumi, S. Landais, W. Lewis,* C.Y. Liu,* D. Nusinow, A. Para, A. Priest, J. Pruneda, A.L. Quiroz, T.F. Schultz, D.K. Welsh,** E. Zhang*

* Genomics Institute of the Novartis Research Foundation, La Jolla, California

** University of California, San Diego, California

A vast array of cellular processes fluctuate with a 24-hour periodicity, and an endogenous circadian clock is responsible for generating these biological rhythms. Circadian rhythms are found in all kingdoms of life and control diverse events ranging from sleep-wake cycles in mammals to the overall rate of photosynthesis in plants. Many pathologic changes in humans, such as sleep disorders, most likely are due to a circadian defect, so understanding how the circadian clock operates within the cell will have significance for human health. To study how circadian clocks are built inside of cells, we use molecular, genetic, and genomic approaches in 2 model systems: mouse and *Arabidopsis*.

In mammals, the circadian clock plays an integral role in timing many physiologic rhythms, such as blood pressure, body temperature, and liver metabolism, in anticipation of dusk and dawn. The master circadian clock resides within a region of the brain that receives light information from the eyes. However, this brain region can still keep time even in the absence of light, as occurs in some who are visually blind. Mutations in the genes that encode components of the circadian clock are manifested as abnormal activity rhythms in rodents and as sleeping disorders in humans, although which photoreceptors set the clock is unclear. Thus, although marked advances have been made in understanding how the mammalian clock itself runs, little is known about how light transduces synchronizing signals to the clock.

To address this major question, we are using genetic and genomic approaches to identify new gene functions in circadian biology. We are producing a number of mouse strains with mutations in known and potential photoreceptors and are testing the mice for defects in circadian rhythm. Thus far, we have determined that one photoreceptor, melanopsin, is an important contributor in

maintaining synchrony between the clock and environmental light conditions. With the recently completed sequencing of the human and mouse genomes, we now know the sequences of more than 30,000 genes that can be investigated for potential roles in circadian function. We developed large-scale, in vitro, cell-based assays that can be used to rapidly determine if genes control clock activity. Combining this approach with genetic analysis will enable us to further dissect the connection between environmental stimuli, in the form of light, and the behavioral and physiologic events regulated by the circadian clock.

In recent years, researchers have found that intrinsic circadian clocks exist in various peripheral tissues and cell types, directly controlling local physiology and behavior. We are studying the circadian oscillators in the liver and in the vasculature. As the first step, we are investigating the heterogeneity and distinct functions of the central and peripheral oscillators. In particular, we are examining the distinct roles of the retinoid-related orphan nuclear receptors in the clock mechanism. These nuclear receptors were recently identified in our functional genomics studies.

Second, we are asking how environmental cues, mainly light-dark cycles and feeding time, entrain or synchronize peripheral oscillators. Peripheral oscillators most likely are synchronized by hormonal outputs of the suprachiasmatic nucleus or by physiologic inputs such as feeding-mediated metabolic changes. We are using transgenic mice, mice lacking certain genes, and immortalized hepatocytes and vascular smooth muscle cells in these studies along with real-time bioluminescence imaging and biochemical and genetic approaches. Furthermore, we are using high-resolution bioluminescence imaging to examine whether single neurons in the suprachiasmatic nucleus and peripheral cells are autonomous circadian clocks and to characterize the precise nature of synchronization of the molecular clockwork of individual cells.

Flowering is a major event in the life cycle of higher plants. Many plants use seasonal changes in the length of days as a signal to flower, and higher plants use their circadian clocks to perceive these changes. Recently, we defined a molecular link between the circadian clock and day length–dependent regulation of flowering. A flowering time gene known as *CONSTANS* was identified a number of years ago and is regulated by the circadian clock. We showed that clock regulation of *CONSTANS* expression is the key to seasonal control of flowering in *Arabidopsis*. We are extending these studies by comparing gene expression profiles under conditions of long days and short days to identify other components involved in perception of day length.

By combining molecular, genetic, and genomic approaches, we are beginning to define a number of molecular links between the circadian clock and rhythmic regulation of behavior and development. Analysis of circadian rhythms in multiple organisms provides a unique opportunity to define molecular controls for the behavior of whole organisms. These results will provide targets for clinical and agricultural applications to improve the quality of life.

PUBLICATIONS

Allen, T., Koustenis, A., Theodorou, G., Somers, D.E., Kay, S.A., Whitelam, G.C., Devlin, P.F. *Arabidopsis* FHY2 specifically gates phytochrome signaling to the circadian clock. Plant Cell 18:2506, 2006.

Breton, G., Kay, S.A. Circadian rhythms lit up in *Chlamydomonas*. Genome Biol. 7:215, 2006.

Faigón-Soverna, A., Harmon, F.G., Storani, L., Karayekov, E., Staneloni, R.J., Gassmann, W., Más, P., Casal, J.J., Kay, S.A., Yanovsky, M.J. A constitutive shade-avoidance mutant implicates TIR-NBS-LRR proteins in *Arabidopsis* photomorphogenic development. Plant Cell 18:2919, 2006.

Hazen, S.P., Schultz, T.F., Pruneda-Paz, J.L., Borevitz, J.O., Ecker, J.R., Kay, S.A. *LUX ARRHYTHMO* encodes a Myb domain protein essential for circadian rhythms. Proc. Natl. Acad. Sci. U. S. A. 102:10387, 2006.

Imaizumi, T., Kay, S.A. Photoperiodic control of flowering: not only by coincidence [published correction appears in Trends Plant Sci. 11:567, 2006]. Trends Plant Sci. 11:550, 2006.

Sato, T.K., Yamada, R.G., Ukai, H., Baggs, J.E. Miragila, L.J., Kobayashi, T.J., Welsh, D.K., Kay, S.A., Ueda, H.R., Hogenesch, J.B. Feedback repression is required for mammalian circadian clock function. Nat. Genet. 38:312, 2006.

Weber, F., Hung, H.-C., Maurer, C., Kay, S.A. Second messenger and Ras/MAPK signalling pathways regulate CLOCK/CYCLE-dependent transcription. J. Neurochem. 98:248, 2006.

Insulin Secretion and Action

N. Gekakis, J.J. Wilkes, J.L. Pitman, M.C. Wheeler

Type 2 diabetes is a large and growing problem across the United States and around the world. Its incidence and prevalence have increased dramatically during the past several years. In addition to being the sixth leading cause of death in the United States, type 2 diabetes is also a major contributor to heart disease, blindness, limb amputation, and kidney failure. The development of this type of diabetes is a 2-hit process, involving both resistance of peripheral tissue (liver, muscle, fat) to the action of insulin and a failure of the insulin-secreting beta cells to adequately compensate for this insulin resistance. We are using

both forward and reverse genetic approaches to study both aspects of type 2 diabetes.

A major focus of current research in diabetes is the role of inflammation in insulin resistance that results from obesity and/or high-fat feeding. We have identified a G protein–coupled receptor, Gpr43, that plays a role in inflammation-induced insulin resistance. Gpr43 is activated by short-chain fatty acids and is expressed in adipocytes and macrophages. We have shown that silencing of *Gpr43*, the gene for this receptor, improves insulin sensitivity in whole animals and that this improvement is correlated with reduced production of cytokines in macrophages and other tissue. Further, this improvement in insulin sensitivity is mediated, at least in part, by *Gpr43* expression in bone marrow–derived cells, which include macrophages. Another reverse genetic approach we are using to understand insulin resistance is screening of cDNA overexpression libraries in a functional assay of insulin action in vitro to identify those cDNAs that can inhibit insulin action.

We have also taken an innovative forward genetic approach to understanding insulin secretion and action. In this approach, mutations are induced in the mouse genome, and mutant mice are screened for diabetes or insulin resistance. Once such a family of mice is found, the gene causing the defect is mapped and cloned. In this way, we have discovered a new gene, *Sec61a1*, required for beta-cell function (Fig. 1) that highlights the susceptibility of beta cells to "ER stress." The endoplasmic reticulum is the site of synthesis of secreted proteins and as such plays an important role in quality control of those proteins, detecting and eliminating those proteins that are abnormal. ER stress is the accumulation of abnormal proteins in the endoplasmic reticulum and can lead to cellular dysfunction or cellular death. *Sec61a1* is clearly important in this quality control function, and beta cells seem especially vulnerable to ER stress–induced cell death. Because beta cells are so central to the development of diabetes and are prone to ER stress, such stress may be a fundamental contributor to type 2 diabetes in humans.

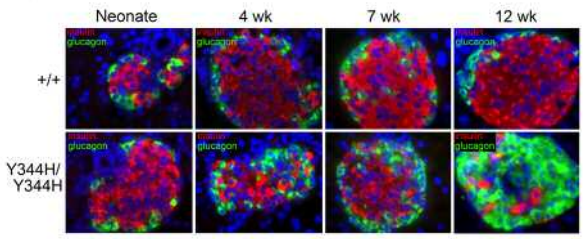


Fig. 1. Immunofluorescence detection of loss of beta cells in *Sec61a1* mutant mice. Top row, Normal distribution of insulin-secreting beta cells (red) and glucagon-secreting alpha cells (green) in wild-type (+/+) mice from neonate to 12 weeks of age. Bottom row, Mutant mice (Y344H/Y344H) begin with a normal distribution of alpha and beta cells but begin to lose beta cells between 4 and 7 weeks of age and have lost 80%–90% of their beta cells by 12 weeks of age, a time that correlates with the onset of diabetes.

PUBLICATIONS

Lloyd, D.J., Bohan, S., Gekakis, N. Obesity, hyperphagia, and increased metabolic efficiency in *Pc1* mutant mice. Hum. Mol. Genet. 15:1884, 2006.

Regulation of Neuronal Signaling by Potassium Channels

T. Jegla, H. Badie, S. Clancy, B. Chen

A major goal of our research is to understand the fundamental mechanisms through which potassium channels regulate neuronal signaling. Potassium channels have long been recognized as key determinants of neuronal excitability. The channels have diverse roles in neurons, including setting of resting potentials, regulating subthreshold excitability, repolarizing action potentials, and setting firing patterns. Recent genetic advances have provided a strong link between abnormal potassium channel activity and diseases of the human nervous system, including epilepsy, ataxia, and retinopathies. A multitude of potassium channels with distinctly different properties has been identified via molecular cloning, but large gaps remain in our understanding of how this molecular diversity corresponds to the rich physiologic diversity of potassium channels in neurons.

We are using a combination of chemical genomics, mouse genetics, biochemistry, and electrophysiology to understand how orphan classes of channels function in neurons. We are focusing on 2 major families of genes that encode potassium channels: the Elk or Kv12 channels and the so-called silent Kv channels. Elk channels contribute to subthreshold conductance and are highly expressed within many key systems within the CNS. Silent Kv channels contribute to the functional diversity of the classic delayed rectifiers that repolarize action potentials in most neurons. Key outstanding questions about these 2 types of channels include subcellular localization, modulation during neuronal signaling, and contributions to circuit activity.

We have localized the most prominently expressed Elk channel, Kv12.2, to the soma and dendrites of exci-

tatory neurons in forebrain regions such as the hippocampus, amygdala, and cortex. The activity of Kv12.2 is highly regulated by multiple factors in heterologous systems, but its function in native neurons has been difficult to define because of a lack of genetic models or defining pharmacologic characteristics. We have identified specific inhibitors of Kv12.2 and are using them to characterize Kv12.2 function in the nervous system. Genetic approaches to Kv12.2 function are also in progress.

Silent Kv subunits are so named because they do not form functional channels when expressed as homotetramers in heterologous systems; instead they modify the functional properties of classic delayed rectifiers through the formation of heteromeric channels with Kv2 family subunits. Despite the importance of the delayed rectifier to neuronal excitation and substantial evidence for delayed rectifier diversity in vivo, the native biology of these heteromers has essentially remained unexplored. We are examining the function of silent Kv channels in photoreceptors, the circadian system, and motor neurons.

Cellular Regulation by the Ubiquitin System

C. Joazeiro, M. Bengtson, W. Li, A. Ulbrich

The levels and duration of expression of intracellular proteins are determined by the proteins' rates of synthesis vs degradation. Degradation of most cellular proteins is mediated by posttranslational modification with the 8-kD protein ubiquitin, which signals recognition by the proteasome. In addition, ubiquitin can play nonproteolytic roles in signaling. Not surprisingly then, ubiquitylation is essential for most cellular processes, such as cell division, signaling, and DNA repair. In addition, deregulated ubiquitylation can cause a number of diseases, including neurodegeneration and cancer. We are interested in dissecting pathways (Fig. 1) and mechanisms that involve the ubiquitin system and in converting this information into tools for interfering with biology and disease.

PROTEIN UBIQUITYLATION IN NORMAL CELLS

E3 ubiquitin ligases mediate substrate specificity in ubiquitylation. We and others previously discovered the largest family of E3s, which is characterized by a RING finger "catalytic" domain. Our goal is to assign function to E3s and to determine their role in disease. In one approach, we annotated the hundreds of genes that

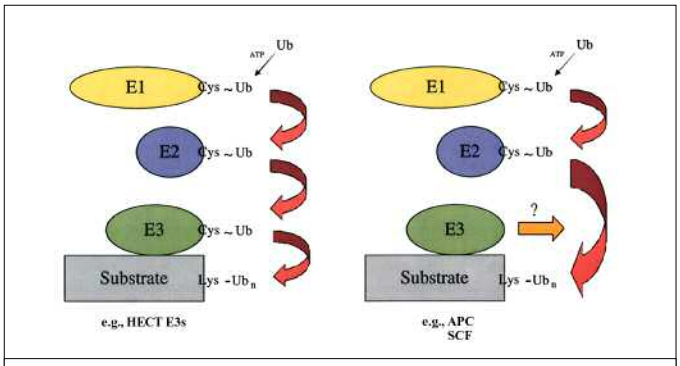


Fig. 1. Protein ubiquitination pathways.

encode E3s and E3 subunits in mammalian genomes and then built collections of short interfering RNAs and full-length cDNAs encoding most human and mouse E3s for use in functional genomic screens. For example, using an imaging-based phenotypic screen, we found a novel E3, MULAN, that localizes to mitochondria and regulates the organelle's trafficking, morphology, and signaling (Fig. 2). Currently, we are identifying the critical substrates and biological relevance of this ligase.

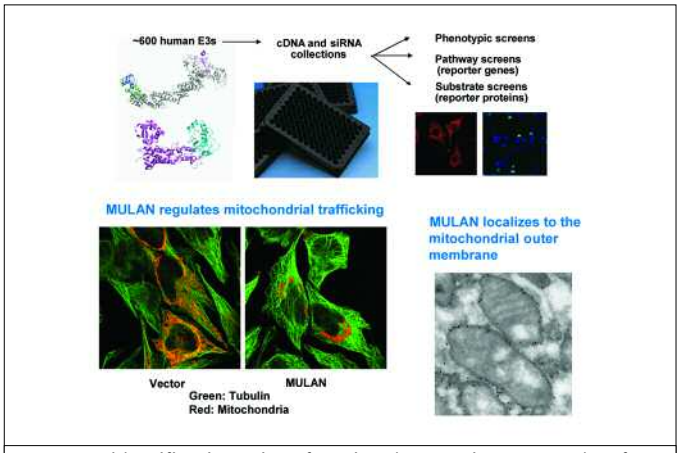


Fig. 2. Identification, via a functional genomics approach, of MULAN, a novel E3 ubiquitin ligase that regulates mitochondrial dynamics.

PROTEIN UBIQUITYLATION IN DISEASE

Although several lines of evidence implicate the ubiquitin system in neurodegeneration, the pathogenic mechanisms involved remain unclear. To shed light on this, in collaboration with S.A. Kay, Department of Biochemistry, we have been studying a new mouse model of neurodegeneration caused by mutation of a novel RING finger E3. These mice, identified by using a high-throughput phenotypic screen for recessive mutations induced by *N*-ethyl-*N*-nitrosourea, have motor neuron loss in the spinal cord and progressive paralysis. We are using biochemistry and molecular genetics to determine the process or pathway that is defective in these mutant mice.

Inspired by the success of a proteasome inhibitor in the treatment of multiple myeloma, several researchers are searching for E3 inhibitors that might have similar druglike properties. We performed a high-throughput screen for compounds that inhibit the activity of the E3 ubiquitin ligase Mdm2 and that might be useful either in the clinic by leading to stabilization of the tumor suppressor protein p53 or for manipulating p53 levels and ubiquitylation in experimental models.

PUBLICATIONS

Joazeiro, C.A.P., Anderson, K.C., Hunter, T. Proteasome inhibitor drugs on the rise. Cancer Res. 66:7840, 2006.

Sierra, J., Yoshida, T., Joazeiro, C.A.P., Jones, K.A. The APC tumor suppressor counteracts β-catenin activation and H3K4 methylation at Wnt target genes. Genes Dev. 20:586, 2006.

Zhang, Q., Liu, Y., Gao, F., Ding, Q., Cho, C., Hur, W.-Y., Jin, Y., Uno, T., Joazeiro, C.A.P., Gray, N. Discovery of EGFR-selective 4,6-disubstituted pyrimidines from a combinatorial kinase-directed heterocycle library. J. Am. Chem. Soc. 128:2182, 2006.

New Therapeutic Strategies in Alzheimer's Disease and Diabetes Mellitus

M.A. Leissring, J. Zhao, L. Li, Q. Lu

AGING AND NEURODEGENERATION

We study diseases of the nervous system that occur during aging and the fundamental mechanisms that regulate aging. Currently, we are examining a fascinating but poorly understood zinc metalloprotease known as insulin-degrading enzyme (IDE). IDE is responsible for degradation of insulin and amyloid β-protein, peptide substrates central to the pathogenesis of diabetes and Alzheimer's disease, respectively. We showed that enhancing the proteolysis of amyloid β-protein by IDE or other proteases can completely prevent Alzheimer-type pathologic changes in a mouse model of Alzheimer's disease.

We are using techniques ranging from in vitro enzymatic assays to transgenic and gene-targeted rodent models to explore the normal biology and therapeutic potential of IDE and other proteases. In parallel, we are using high-throughput screening of compounds, solid-phase peptide synthesis, and medicinal chemistry to discover and rationally design pharmacologic modulators of IDE. These pharmacologic tools promise to deepen our understanding of IDE biology and could lead to novel therapeutic interventions for Alzheimer's disease or diabetes.

RATIONAL DESIGN OF POTENT AND SELECTIVE INHIBITORS OF IDE

Despite the potentially great importance of IDE in health and disease, no useful pharmacologic tools have been available to manipulate the activity of this protease in in vivo models. On the basis of proteomic analysis of the cleavage-site specificity of IDE, we designed and synthesized peptide hydroxamates that are potent IDE inhibitors. We next used solid-phase peptide synthesis to generate a series of *retro-inverso* peptide hydroxamates, which led to the identification of unnatural amino acid substitutions that improved potency by several 100-fold. Currently, our best compounds are inhibitory in low nanomolar to high picomolar concentrations, making them almost a million times more potent than any previously described small-molecule inhibitors for IDE. These inhibitors enhance the action of insulin at multiple levels in cultured cells, and we are working to develop variants suitable for use in vivo. These new reagents promise to yield new insights into the function of IDE and could pave the way to a novel class of therapeutic agents based on slowing insulin clearance.

ANIMAL MODELING

We have made significant strides in evaluating the importance of different enzymes that degrade amyloid β-protein in vivo. Most recently, we found that deletion of cathepsin D leads to a selective increase in amyloid β-protein 42, the species that accumulates in the amyloid plaques characteristic of Alzheimer's disease.

PUBLICATIONS

Farris, W., Schütz, S.G., Cirrito, J.R., Shankar, G.M., Sun, X., George, A., Leissring, M.A., Walsh, D.M., Qiu, W.Q., Holtzman, D.M., Selkoe, D.J. Loss of neprilysin function promotes amyloid plaque formation and causes cerebral amyloid angiopathy. Am. J. Pathol. 171:241, 2007.

Kim, M., Hersh, L.B., Leissring, M.A., Ingelsson, M., Matsui, T., Farris, W., Lu, A., Hyman, B.T., Selkoe, D.J., Bertram, L., Tanzi, R.E. Decreased catalytic activity of the insulin-degrading enzyme in chromosome 10-linked Alzheimer disease families. J. Biol. Chem. 282:7825, 2007.

Leissring, M.A. Proteolytic degradation of the amyloid β-protein: the forgotten side of Alzheimer's disease. Curr. Alzheimer Res. 3:431, 2006.

Leissring, M.A., Selkoe, D.J. Structural biology: enzyme target to latch onto. Nature 443:761, 2006.

Identification of Genetic Determinants of Depression

M.T. Pletcher, K.J. Clarke, B.C. Long, B.H. Miller, L.E. Schultz, B.M. Young

Clinical depression is a mood disorder with high morbidity and mortality that is estimated to occur in more than 15% of the adult population in the United States. Depression has a wide range of symptoms, including loss of energy, changes in weight, diminished interest or pleasure in daily activities, insomnia or excessive sleep, anxiety, slowness of movement, feelings of worthlessness, difficulty concentrating, and thoughts of death.

Depression can be a one-time occurrence but is often an ongoing problem throughout a person's lifetime. A total of 15% of patients with major depressive disorders die of suicide. The onset of depression is often linked to environmental factors such as life events that greatly increase stress. Despite this association, depression has a strong genetic component. Genetics accounts for 40%–50% of the risk for depression in a person's lifetime, and the risk for members of a family does not change if the members are raised separately.

We are using cell-based screening technology and a mouse model to identify the genes and pathways that contribute to depressive behavior. Currently, we are conducting a survey in 30 strains of mice for differences in molecular, biochemical, and behavioral traits that represent endophenotypes (i.e., single well-defined symptoms or traits of a much more complex disorder) associated with depression. In cataloging the variation in quantities of neurotransmitters and stress hormones in the blood, gene expression in regions of the brain such as the hippocampus and hypothalamus, and performance in behavioral models such as the tail suspension test and open field test, we are producing the data necessary to identify the genetic controls for each of these traits. Using the in silico genetic mapping method, we can correlate the natural variation of these measurements present in the inbred lines of mice with the underlying haplotype structure of the mice, thereby pinpointing biologically important genes.

In parallel, we are developing a cell-based assay to monitor the function of the serotonin transporter, the gene target of the most common pharmaceutical treatment for depression: selective serotonin reuptake inhibitors. Using this assay, we can screen the 14,500 full-length clone cDNA library of Scripps Florida for genes that modify the function of the transporter. Additionally, introducing a selective serotonin reuptake inhibitor to the screen will enable us to identify genes that modulate the efficacy of this important class of drugs. Cumulatively, we hope to provide a better understanding of the molecular basis of depression and its treatment to allow for improved diagnosis and therapies.

PUBLICATIONS

Miller, B.H., Schultz, L.E., Gulati, A., Cameron, M.D., Pletcher, M.T. Genetic regulation of behavioral and neuronal responses to fluoxetine. Neuropsychopharmacology, *in press*.

Pinto, L.H., Vitaterna, M.H., Shimomura, K., Siepka, S.M., Balannik, V., McDearmon, E.L., Omura, C., Lumayag, S., Invergo, B.M., Glawe, B., Cantrell, D.R., Inayat, S., Olvera, M.A., Vessey, K.A., McCall, M.A., Maddox, D., Morgans, C.W., Young, B., Pletcher, M.T., Mullins, R.F., Troy, J.B., Takahashi, J.S. Generation, identification and functional characterization of the nob4 mutation of Grm6 in the mouse. Vis. Neurosci. 24:111, 2007.

Neuroscience Discovery and Pharmacogenomics

C. Wahlestedt, M.A. Faghihi, J. Huang, J. Kocerha, M. Przydzial, A. Khalil, S. Brothers, F. Modarresi, H.-Y. Zhang,* C. Scheele,* C. Dahlgren,* J.A. Timmons*

* Karolinska Institutet, Stockholm, Sweden

Our research involves aspects of Alzheimer's disease, Parkinson's disease, depression, alcohol addiction, fragile X syndrome, autism, and aging. In addition to drug discovery efforts, currently we are focusing on basic aspects of mammalian genomics, genetics, and transcriptomics.

IDENTIFICATION AND FUNCTIONAL ANALYSIS OF REGULATORY RNA TRANSCRIPTS

We are among the few neuroscientists who for a number of years have been, and continue to be, involved in high-throughput sequencing of transcriptomes (i.e., all the RNA transcripts in a cell) in humans and mice. Such efforts have provided strong evidence that in contrast to earlier understanding, the majority of the mammalian genome is transcribed in vivo. Analysis of such data sets has indicated that most mammalian RNA transcripts are noncoding.

Thus, conventional protein-coding genes appear to account for only a minority of human RNA transcripts. A substantial component of the full-length mouse and human cDNA sets that we and others have analyzed does not contain an annotated protein-coding sequence and most likely corresponds to noncoding RNA. In

addition to small RNAs, many of the noncoding RNAs constitute natural antisense RNA transcripts. We have shown that many noncoding RNAs identified to date have substantial conservation across species. We have also shown that many small noncoding RNAs and antisense transcripts have differential expression under various conditions and can affect conventional gene expression.

RNA INTERFERENCE AND DEVELOPMENT OF HIGH-THROUGHPUT GENOMICS TECHNOLOGY

RNA interference has become one of the most important gene manipulation technologies. Short interfering RNA (siRNA), the inducer of RNA interference in mammals, can be used to elucidate gene functions by rapidly silencing expression of a target gene. Today, siRNAs are widely used as research tools and have potential for becoming therapeutic agents. We have built up a powerful and versatile portfolio of siRNA technology. Finally, we have introduced the use of locked nucleic acids as components of siRNAs (and antisense oligonucleotides) and have shown a range of beneficial properties of these modified agents.

G PROTEIN–COUPLED RECEPTORS AS DRUG TARGETS FOR CNS DISORDERS

More than half of known drugs bind to G protein–coupled receptors. We have continued our long-standing work on these receptors, particularly certain neuropeptide receptors. These efforts are now part of the drug discovery program at Scripps Florida.

HUMAN GENETICS AND PHARMACOGENOMICS

We also are pursuing genotyping related to several human disorders. Our goal is to identify biomarkers associated with human disorders. We wish to understand what makes certain individuals susceptible and how their responses to drug treatment may differ (pharmacogenomics).

PUBLICATIONS

Larsson, O., Perlman, D.M., Fan, D., Reilly, C.S., Peterson, M., Dahlgren, C., Liang, Z., Li, S., Polunovsky, V.A., Wahlestedt, C., Bitterman, P.B. Apoptosis resistance downstream of eIF4E: posttranscriptional activation of an anti-apoptotic transcript carrying a consensus hairpin structure. Nucleic Acids Res. 34:4375, 2006.

Scheele, C., Nielsen, A.R., Walden, T.B., Sewell, D.A., Fischer, C.P., Brogan, R.J., Petrovic, N., Larsson, O., Tesch, P.A., Wennmalm, K., Hutchinson, D.S., Cannon, B., Wahlestedt, C., Pedersen, B.K., Timmons, J.A. Altered regulation of the *PINK1* locus: a link between type 2 diabetes and neurodegeneration? FASEB J., *in press*.

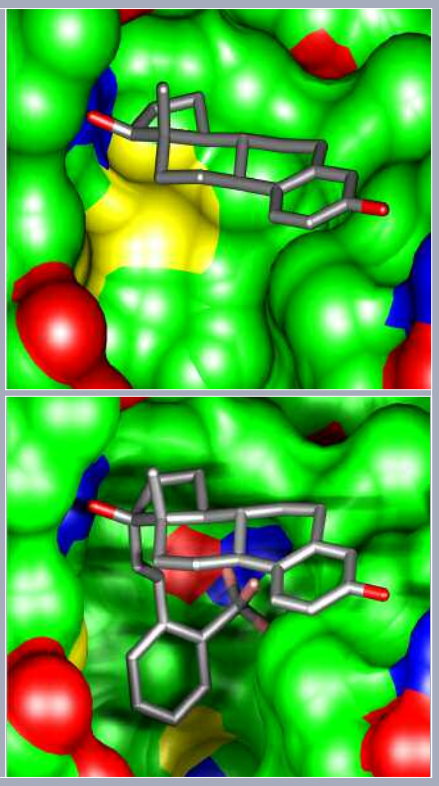
Scheele, C., Petrovic, N., Faghihi, M.A., Lassmann, T., Fredriksson, K., Rooyackers, O., Wahlestedt, C., Good, L., Timmons, J.A. The human *PINK1* locus is regulated in vivo by a non-coding natural antisense RNA during modulation of mitochondrial function. BMC Genomics 8:74, 2007.

Timmons, J.A., Scheele, C., Wahlestedt, C. Do mitochondria provide a common link between diabetes and Parkinson's disease? Pract. Diabetes Int. 24:337, 2007.

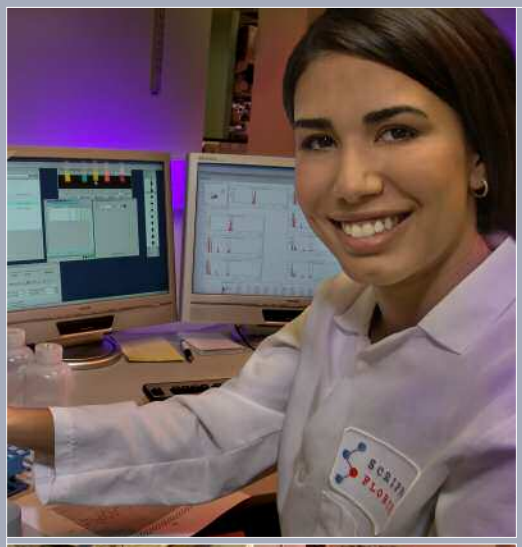
Timmons, J.A., Wennmalm, K., Larsson, O., Walden, T.B., Lassmann, T., Petrovic, N., Hamilton, D.L., Gimeno, R.E., Wahlestedt, C., Baar, K., Nedergaard, J., Cannon, B. Myogenic gene expression signature establishes that brown and white adipocytes originate from distinct cell lineages. Proc. Natl. Acad. Sci. U. S. A. 104:4401, 2007.

Zhang, H.Y., Du, Q., Wahlestedt, C., Liang, Z. RNA interference with chemically modified siRNA. Curr. Top. Med. Chem. 6:893, 2006.

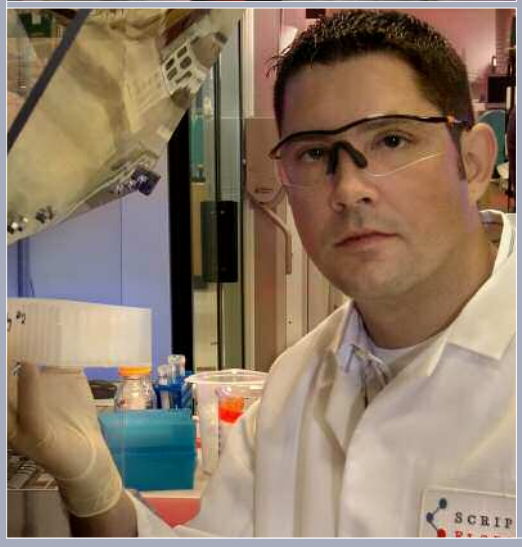
Cancer Biology



A novel binding pocket in the estrogen receptor. X-ray crystallography reveals a novel pharmacologic class of synthetic estrogens, which induce a unique restructuring of the ligand-binding pocket. Top panel, Part of the estrogen receptor ligand-binding pocket bound to estradiol. Bottom panel, The novel, extended region of the binding pocket when it is occupied by trifluoromethyl phenylvinyl estradiol, a 50-pM-affinity ligand. The 30%–40% increase in the size of the binding pocket indicates a new site for targeted chemistry, which can be used to develop improved therapeutic agents for breast cancer and other estrogen-responsive disorders. Reprinted with permission from Nettles, K.W., Bruning, J.B., Gil, G., et al. Structural plasticity in the oestrogen receptor ligand-binding domain. EMBO Rep. 8:563, 2007.



Bivian Torres, Research Technician



Jason Nowak, Research Assistant

DEPARTMENT OF CANCER BIOLOGY

STAFF

John L. Cleveland, Ph.D.
Professor and Chairman

Howard Petrie, Ph.D.
Professor

Tina Izard, Ph.D.
Associate Professor

Nagi G. Ayad, Ph.D.
Assistant Professor

Philippe R. J. Bois, Ph.D.
Assistant Professor

Michael Conkright, Ph.D.
Assistant Professor

Kendall Nettles, Ph.D.
Assistant Professor

SCIENTIFIC ASSOCIATE

Chunying Yang, M.D.

STAFF SCIENTISTS

Joanne R. Doherty, Ph.D.

Ihn Kyung Jang, Ph.D.

HaJeung Park, Ph.D.

Meredith A. Steeves, Ph.D.

RESEARCH ASSOCIATES

Antonio Amelio, Ph.D.

Mi-Ra Chang, Ph.D.

Frank C. Dorsey, Ph.D.

Rangarajan Erumbi, Ph.D.

Irina Getun, Ph.D.

German Gil, Ph.D.

Ann Griffith, Ph.D.

Mark A. Hall, Ph.D.

Jun Hyuck Lee, Ph.D.

Hiroshi Nakase, Ph.D.

Jianjun Shi, Ph.D.

Zhen Wu, Ph.D.

Bhargavakrishna Yekkala, Ph.D.

Sollepura Yogesha, Ph.D.

John L. Cleveland, Ph.D.

Chairman's Overview

The Department of Cancer Biology was launched last year at the Florida campus, with close ties to cancer researchers at the California campus.

Broadly, the goals of our research programs are to understand the molecular pathogenesis of cancer. We focus on pathways directed by oncogenes and tumor suppressors that control cell division, growth, survival, and differentiation and that modify the response to therapeutic agents. The 7 faculty members currently in the department use a battery of state-of-the-art technologies, from biochemistry and cell biology to preclinical models to x-ray crystallography, for target discovery and validation. We have developed unique models to evaluate the efficacy of new leads in cancer prevention and therapeutics.

Our goal is to understand the molecular underpinnings of all of the major human cancers, but we are also interested in cancer in infants and children, the interplay between cancer and the immune system, and the relationships between aging and cancer. One of the many strengths at the Florida campus is the access to high-throughput technologies that enable investigators to explore potential leads quickly by using both genetic and small-molecule screens. Collaborations with the major cancer centers in Florida and with cancer researchers at the California campus will enable us to convert leads into translational and clinical studies.

INVESTIGATORS' REPORTS

Myc-Mediated Pathways in Cancer and Development

J.L. Cleveland, M.A. Hall, F.C. Dorsey, R. Rounbehler, D. Sofia, J. Doherty, M. Steeves, C. Yang, T. Bratton, W. Li

Myc oncogenes (c-*Myc*, N-*Myc*, and L-*Myc*) are activated in approximately 70% of all human cancers. This activation occurs directly via gene amplification or chromosomal translocations or indirectly via alterations in signal transduction pathways or loss of tumor suppressors that normally regulate and/or harness expression of the oncoprotein Myc. Myc oncoproteins function as transcription factors, and, in part, their activation in cancer reflects their essential roles as regulators of cell growth and division; they regulate up to 10% of the genome. In addition, Myc overexpression triggers accelerated rates of cell proliferation, a situation that puts malignant cells at a considerable advantage. Myc is also essential for the tumor angiogenic response and plays key roles in tumor cell metastasis. However, in normal cells, the hyperproliferative response provoked by Myc also triggers apoptosis, and mutations in regulators of this cell death response are a hallmark of most malignant neoplasms.

We have used mouse models to dissect the contribution of key targets downstream of Myc that control tumor development. These studies have shown that Myc triggers apoptosis through the Arf-p53 tumor suppressor pathway that is inactivated in most malignant tumors and by selectively affecting the expression of members of the Bcl-2 family of proteins that directly control the intrinsic apoptotic pathway. We have shown that mutations in these pathways are a hallmark of Myc-driven cancers in mice and humans. Genetic studies have established the key roles of these pathways in controlling tumor development and metastasis and in disabling differentiation programs. In addition, we have shown that Myc itself also often acquires somatic mutations during tumor progression that selectively disable its ability to activate some apoptotic pathways.

Although apoptotic regulators clearly act as guardians against Myc-induced tumorigenesis, we have found that the ability of Myc to provoke accelerated cell growth is also critical for tumorigenesis. First, Myc coordinately regulates the expression of cytokines that direct tumor angiogenesis, and this regulation occurs through a circuitous route: Myc regulates the transcription of regulatory factors that in turn control the half-life of mRNAs that encode angiogenic cytokines. Second, Myc suppresses the expression of the universal cyclin-dependent kinase inhibitor $p27^{Kip1}$ by inducing the expression of the Cks1 component of the SCF^{Skp2} E3 ubiquitin ligase complex that targets $p27^{Kip1}$ for destruction by the 26S proteasome. Accordingly, loss of *Cks1*, the gene for Cks1, disables the ability of Myc to suppress $p27^{Kip1}$ and markedly impairs Myc-induced proliferation and tumorigenesis, whereas loss of the gene for $p27^{Kip1}$ accelerates Myc-induced tumorigenesis. Remarkably Cks1 overexpression is a hallmark of all lymphomas with Myc involvement, and this pathway is apparently not bypassed during lymphoma development (high levels of nuclear, and thus presumably functional, $p27^{Kip1}$ are expressed in Myc-induced, Cks1-deficient lymphomas). Finally, our recent studies indicate that Myc also induces the expression of components of other SCF complexes, suggesting this pathway is a general route by which Myc coordinates cell growth and division.

Because Myc regulates such a large number of genes and is essential for cell growth and division, agents that directly target the transcription functions of Myc are likely to also effect the growth of normal cells. Therefore, we have focused on key transcription targets that might be suitable therapeutic targets. Indeed, we have shown that inhibiting ornithine decarboxylase, a direct transcription target of Myc and the rate-limiting enzyme of polyamine biosynthesis, impairs Myc-induced proliferation and transformation. These results were underscored by our findings that heterozygosity in the gene for ornithine decarboxylase, a condition that only reduces the enzyme activity of ornithine decarboxylase and the generation of its product by half, triples the life span of tumor-prone mice. Thus, agents that target the polyamine pathway have promise in both the prevention and treatment of cancer. Currently, we are defining the mechanism by which targeting ornithine decarboxylase disables the proliferative response of Myc.

We recently discovered that additional Myc transcription targets that can be exploited in cancer therapy include components of the autophagy pathway, an ancient survival pathway that directs the digestion of bulk cytoplasmic material and organelles when cells are faced with nutrient- or oxygen-deprived conditions, a scenario that certainly applies to the tumor microenvironment. We have shown that agents that disable

autophagy have tremendous potential in cancer prevention and treatment. Currently, we are defining the mechanism by which Myc regulates the expression of genes associated with the autophagy pathway and the potential of these genes as targets for cancer therapeutic agents.

PUBLICATIONS

Carew, J.S., Nawrocki, S.T., Cleveland, J.L. Modulating autophagy for therapeutic benefit. Autophagy 3:464, 2007.

Carew, J.S., Nawrocki, S.T., Kahue, C.N., Zhang, H., Yang, C., Chung, L., Houghton, J.A., Huang, P., Giles, F.J., Cleveland, J.L. Targeting autophagy augments the anticancer activity of the histone deacetylase inhibitor SAHA to overcome Bcr-Abl-mediated drug resistance. Blood 110:313, 2007.

Hall, M.A., Cleveland, J.L. Clearing the TRAIL for cancer therapy. Cancer Cell 12:4, 2007.

Keller, U.B., Old, J.B., Dorsey, F.C., Nilsson, J.A., Nilsson, L., MacLean, K.H., Chung, L., Yang, C., Spruck, C., Boyd, K., Reed, S.I., Cleveland, J.L. Myc targets Cks1 to provoke the suppression of $p27^{Kip1}$, proliferation and lymphomagenesis. EMBO J. 26:2562, 2007.

Maclean, K.H., Kastan, M.B., Cleveland, J.L. *Atm* deficiency affects both apoptosis and proliferation to augment Myc-induced lymphomagenesis. Mol. Cancer Res. 5:705, 2007.

Nilsson, L.M., Keller, U.B., Yang, C., Nilsson, J.A., Cleveland, J.L., Roussel, M.F. *Ink4c* is dispensable for tumor suppression in Myc-induced B-cell lymphomagenesis. Oncogene 26:2833, 2007.

Wen, R., Chen, Y., Bai, L., Fu, G., Schuman, J., Dai, X., Zeng, H., Yang, C., Stephan, R.P., Cleveland, J.L., Wang, D. Essential role of phospholipase C-γ2 in early B-cell development and Myc-mediated lymphomagenesis. Mol. Cell. Biol. 26:9364, 2006.

Steady-State T-Cell Lymphopoiesis

H.T. Petrie, A. Griffith, H. Nakase, M. Chang, J. Shi, M. Fallahi, K. McKevitt, B. Torres

Our main area of research is identification of intrathymic processes that induce bone marrow stem and/or progenitor cells to differentiate into cells of the T-lymphocyte lineage. Like most hematopoietic cells, T cells must be made not only during embryonic development but also throughout life; they are lost because of a variety of causes, including bleeding, cellular senescence, and immune activation. Deficiencies in the production of new T lymphocytes lead to a marked increase in susceptibility to infectious diseases, particularly viral diseases. The causes of such deficiencies may be congenital or acquired (as a consequence of exposure to retroviruses, chemotherapeutic drugs, or environmental agents) or may occur naturally as a consequence of aging. By understanding the production of T lymphocytes in normal circumstances, we hope to define the underlying causes of diseases and autoimmune disorders due to T-cell deficiencies and to develop strategies that can be used to correct these abnormalities.

Both in the embryo and after birth, the main site of T-cell production is the thymus. One of the unique features of T-cell production by the thymus after birth is that the stem cells that perpetuate this process do not reside within the organ. Instead, new T lymphocytes are derived from bone marrow–derived stem cells circulating in the bloodstream. Consequently, the ability of the thymus to attract circulating stem cells out of the bloodstream is a crucial and obligatory first step in T-cell production. Remarkably, nearly nothing is known about the signals that induce stem cells to leave the blood and enter the thymus. We think that defining such signals is critical for developing new strategies to correct deficiencies in T-cell production. To this end, we first sought to define the anatomic sites where bone marrow–derived stem cells enter the postnatal thymus under normal conditions, because these sites are where such signals must be found.

We found that blood-borne cells enter the thymus through a particular type of blood vessels, postcapillary venules, that are found only in the perimedullary cortex, a very restricted tissue region. Using laser-based microdissection, we have developed a method to isolate these specific blood vessels, and we are using microarray screening to reveal the homing mechanism, including chemoattractive stimuli, ligands for adhesion of progenitor cells, and various other related factors required for the recruitment process. In parallel, we are using microarray technology to characterize cells that home to the thymus to evaluate expression of the appropriate receptors, counterreceptors, or adhesion molecules and to determine the lineage potential and proliferative capacity of the cells. This double-sided approach of homing sites and homing cells permits both greater scrutiny and built-in validation, and it has allowed us to rapidly move forward in an area that has long remained enigmatic.

Once blood-borne stem cells enter the thymus, T-lymphocyte production begins. Each stem cell is induced to proliferate and give rise to approximately 1 million immature thymocytes. Like the stem cells from which they are derived, the earliest intrathymic cells have the potential to give rise to numerous other types of blood cells. However, the environment provided by the thymus causes these multipotential precursors to undergo a series of developmental changes

that result in commitment to the T-lymphocyte lineage. Again, the signals that the thymus provides to induce this commitment and the subsequent proliferation of T cells are poorly understood. To date, only 3 critical interactions between the thymus and developing lymphoid cells have been defined (Notch, c-kit, and IL-7R), and even in these cases, the precise function of these signals is not clear.

To identify novel signals, we first determined the positional context of each stage of development of early progenitor cells. We found that the different developmental stages could be mapped to identifiable subregions of the cortex or the medulla of the thymus. By correlating these locations with the known functions at each developmental stage, we were able to generate a functional map of stromal signaling microenvironments in the thymus. Using this functional map, we are determining how each stromal region differs from the other regions and what unique signals stromal cells in each region deliver to developing lymphocytes. For these studies, we are using microdissection of each mapped region and then in silico subtraction of those genes expressed by the lymphoid cells. This method has allowed us to define, for the first time, global transcription signatures for stromal cells from the thymic cortex and medulla and from functionally defined subregions of each compartment. We are now correlating molecules secreted by stromal cells or found on the surface of stromal cells with the corresponding receptors on developing lymphocytes to identify new, region-specific signals for T-cell development. Ultimately, we wish to evaluate the roles of these signals in various immunodeficient states, including age-related immune disorders, and to develop strategies for correcting these immunodeficiencies through transplantation and/or gene therapy. Further, this unique stromal database has allowed us to understand the biology of thymic stromal cells at an unprecedented level and to define region-specific stromal promoters that can be used for region-specific overexpression or conditional gene deletion. We expect that these topics will provide a strong base for studies for many years to come.

The same methods that enabled us to characterize stromal signaling regions in the thymus have also provided tools uniquely suited for studies of the enigmatic process of thymic involution with age. Progressive deterioration of the thymus, beginning around the time of puberty, is primarily a consequence of aging thymic stroma. During the past few years, it has become increasingly clear that age-related decreases in the production of naive T cells leads to the selective accumulation of memory T cells and, ultimately, to accumulation of "holes" in the immune repertoire. These immune insufficiencies result in increased susceptibility to infectious disease and in a decreased ability to resolve infections. Further, deterioration of thymic stroma, and the deceased efficiency in negative selection that results, probably plays a role in the advent of age-related autoimmune diseases, including diabetes, arthritis, and atherosclerosis. Of note, the thymus can be induced to completely regrow, although the best means for doing so—castration—is somewhat undesirable.

Using microdissection, microarrays, and in silico subtraction techniques, we can now analyze the differences between thymic stromal cells from young or old mice and during the initiation, log growth, and termination phases of thymic regeneration after induction of regrowth by castration. These methods provide us a unique opportunity to clearly characterize the molecular biology of aging in thymic stromal cells and the requirements for induction, sustenance, and termination of thymic regrowth. In a collaborative effort with scientists at StromaCyte Corporation, Jupiter, Florida, we will use the data generated by these studies to identify therapeutic targets that can be used to induce regrowth of the thymus without the need for castration.

Finally, we are interested in the use of T-cell reconstitution after bone marrow transplantation to treat hematopoietic or lymphoid malignant neoplasms. Particularly in adults, reconstitution of the pool of the naive T cells is poor, and, consequently, transplant recipients may succumb to otherwise trivial infections such as those caused by cytomegalovirus or Epstein-Barr virus. In most cases of transplantation in adults, the T cells that do eventually reconstitute the host are donor T cells that are neither tolerant nor naive. Rather, they are homeostatically expanded, donor-derived T cells with a memory phenotype.

Using a novel mouse model for thymic reconstitution, we found that the almost complete inability of the thymus in adults to make new T cells after bone marrow transplantation is due to the exquisite sensitivity of thymic stromal cells to chemotherapeutic or myeloablative drugs and radiation. We are now identifying the changes induced by such treatments in thymic stromal cells. Using these data and a better understanding of normal stromal cell biology and metabolism, we hope to define strategies that can be used either prophylactically to protect thymic tissue during chemotherapy or afterward to help regenerate it.

PUBLICATIONS

Petrie, H.T., Zuniga-Pflucker, J.C. Zoned out: functional mapping of stromal signaling microenvironments in the thymus. Annu. Rev. Immunol. 25:6649, 2007.

Schwartz, R., Engel, I., Fallahi-Sichani, M., Petrie, H.T., Murre, C. Gene expression patterns define novel roles for E47 in cell cycle progression, cytokine-mediated signaling, and T lineage development. Proc. Natl. Acad. Sci. U. S. A. 103:9976, 2006.

Umland, O., Mwangi, W.N., Anderson, B.M., Walker, J.C., Petrie. H.T. The blood contains multiple distinct progenitor populations with clonogenic B and T lineage potential. J. Immunol. 178:4147, 2007.

Novel Regulators of the Anaphase-Promoting Complex

N. Ayad, D. Harmey, S. Simanksi, L. Owens

The anaphase-promoting complex (APC) is an essential regulator of the eukaryotic cell cycle. New findings suggest that the APC integrates signals from multiple pathways to induce particular cell-cycle transitions. However, no unbiased genome-wide approach has been used to determine the signaling pathways that regulate APC activity. We used a cell-based screening method to identify APC regulators that are potential mediators of signaling pathways. We cotransfected 14,000 cDNAs with the N terminus of the APC substrate cyclin B fused with luciferase and identified several novel regulators of the APC (Fig. 1).

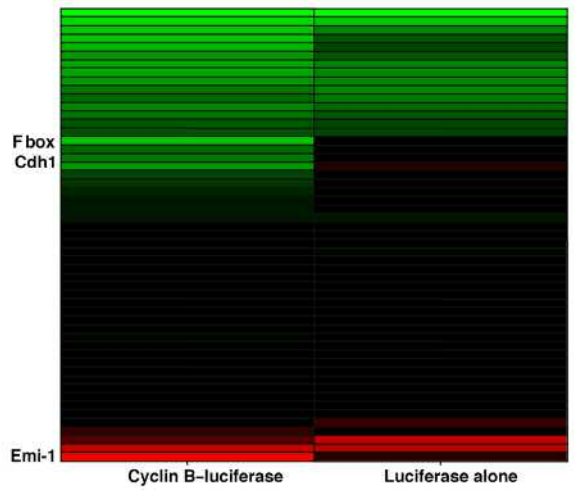


Fig. 1. The N terminus of the APC substrate cyclin B was fused to luciferase and used as a measure of APC activity. Cotransfection of 14,000 cDNAs with the fusion protein or luciferase alone allowed us to identify APC regulators. The known APC regulator Cdh1 was identified as a protein that reduced the steady-state levels of the fusion protein relative to luciferase alone. An uncharacterized F box protein also reduced levels of the fusion protein specifically. Heat map key: green is 2-fold lower than control plasmid transfection (Sport6); red is 2-fold or higher than control plasmid. The known APC inhibitor Emi-1 was used as a control on each plate.

One protein we have extensively characterized is an F box protein that stimulates APC activity. Expression of this protein is highest during exit from mitosis, suggesting that the protein may play a role in regulating APC activity at that time. Furthermore, depletion of this protein by transfection of short interfering RNA induced a delay in mitotic exit. Currently, we are analyzing the mechanism by which this protein regulates APC activity in vitro and in vivo. These studies suggest that cell-based screening is an effective tool for determining the signaling networks that regulate ubiquitin ligases and cell-cycle transitions.

PUBLICATIONS

Smith, A., Simanski, S., Fallahi, M., Ayad, N.G. Redundant ubiquitin ligase activities regulate wee1 degradation and mitotic entry. Cell Cycle, *in press*.

Molecular Mechanisms of cAMP-Mediated Transcription

M.D. Conkright, B.A. Mercer, A.L. Amelio

A variety of biological functions depend on the cAMP signaling cascade, including long-term memory, survival of beta cells, glucose metabolism, cardiomyopathy, and chondrocyte proliferation. We study the molecular mechanisms involved in the conversion of cAMP signals into transcriptional events. Increases in cellular levels of cAMP stimulate the expression of numerous genes by liberating the catalytic subunits of protein kinase A, which phosphorylates the transcription factor cAMP-responsive element binding protein (CREB). Phosphorylation of CREB promotes the recruitment of the coactivators CREB-binding protein/p300 and the initiation of transcription.

The diversity of biological functions associated with CREB and the cAMP pathway will be impossible to fully understand until all of the components involved in the pathway have been identified and characterized. Currently, we are using high-throughput cell-based screening technologies available at the Scripps Florida campus, including cDNA expression libraries, short interfering RNA libraries, and small-molecule libraries, to identify all of the proteins that make up and regulate the cAMP pathway. Using this technology, we have identified proteins called transducers of regulated CREB activity, a novel family of CREB coactivators. Ascertaining the factors involved in the cAMP signaling pathway will be paramount in delineating why the biological function of CREB differs so drastically between tissues.

Signaling Specificity Through the Nuclear Receptor Ligand-Binding Domain

K.W. Nettles, J. Bruning, G. Gil, J. Nowak

Our overall goal is to understand how small-molecule ligands control specific physiologic outcomes through the chemical-structural interface of the ligand with specific nuclear receptors.

We have developed a technique that speeds our ability to obtain x-ray crystal structures of the nuclear receptor ligand-binding domain by several orders of magnitude, allowing us to obtain structural information on entire classes of receptor-ligand complexes in a short time. We are currently using this approach to define the chemical and structural features that determine NF-κB–selective signaling through the estrogen receptor, the mechanisms of partial agonist activity through peroxisome proliferator-activated receptor γ, and allosteric signaling across the retinoid X receptor heterodimer interface.

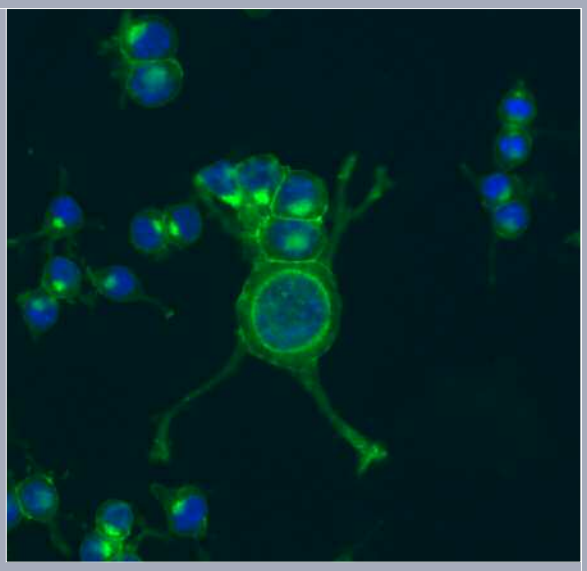
PUBLICATIONS

Nettles, K.W., Bruning, J.B., Gil, G., O'Neill, E.E., Nowak, J., Hughs, A., Kim, Y., DeSombre, E.R., Dilis, R., Hanson, R.N., Joachimiak, A.J., Greene, G.L. Structural plasticity in the oestrogen receptor ligand-binding domain. EMBO Rep. 8:563, 2007.

Zhou, H.B., Nettles, K.W., Bruning, J.B., Kim, Y., Joachimiak, A., Sharma, S., Carlson, K.E., Stossi, F., Katzenellenbogen, B.S., Greene, G.L., Katzenellenbogen, J.A. Elemental isomerism: a boron-nitrogen surrogate for a carbon-carbon double bond increases the chemical diversity of estrogen receptor ligands. Chem. Biol. 14:659, 2007.

Zhou, H.B., Sheng, S., Compton, D.R., Kim, Y., Joachimiak, A., Sharma, S., Carlson, K.E., Katzenellenbogen, B.S., Nettles, K.W., Greene, G.L., Katzenellenbogen, J.A. Structure-guided optimization of estrogen receptor binding affinity and antagonist potency of pyrazolopyrimidines with basic side chains. J. Med. Chem. 50:399, 2007.

Infectology



The prion protein (green) is expressed at the surface and inside cultured neurons, near the nucleus of the cell (blue) but also, interestingly, at the extremity of the neurite. The latter is best seen on the large central cell but also on smaller bipolar cells. This observation suggests that the normal prion protein could be involved in synaptic transmission. In prion diseases, the prion protein is converted into a misfolded form and causes the death of the neurons. These cultured neuronal cells are not infected, but can be infected, with prions. The cells were labeled with DAPI for the nuclei and with a specific antibody coupled to Alexa 488. They were then examined under objective x20 with a Zeiss microscope, and the images were analyzed by using Zeiss AxioView software. Work done by Nicole Sales, Ph.D., senior staff scientist, in the laboratory of Corinne Lasmézas, D.V.M., Ph.D., professor.

Tim Tellinghuisen, Ph.D., Assistant Professor, and Ross Einsteder, Summer Research Intern

DEPARTMENT OF INFECTOLOGY

STAFF

Charles Weissmann, M.D., Ph.D.
Professor and Chairman

Corinne Lasmézas, D.V.M., Ph.D.
Professor

Donny Strosberg, Ph.D.
Professor

Tim Tellinghuisen, Ph.D.
Assistant Professor

Joaquin Castilla, Ph.D.
Assistant Professor

SENIOR STAFF SCIENTIST

Nicole Salès, Ph.D.

STAFF SCIENTIST

Sukhvir Mahal, Ph.D.

SENIOR RESEARCH ASSOCIATES

Chris Baker, Ph.D.

Carlos Coito, Ph.D.

RESEARCH ASSOCIATES

Shawn Browning, Ph.D.

Altan Ercan, Ph.D.

Natalia Fernandez-Borges, Ph.D.

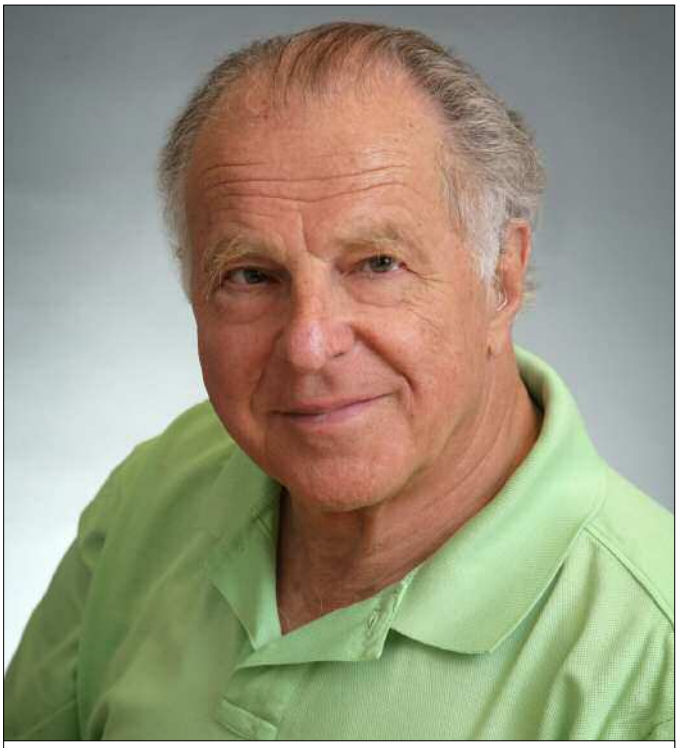
Yervand Karapetyan, M.D.

Jiali Li, Ph.D.

Paula Saá Prieto, Ph.D.

Prem Subramaniam, Ph.D.

Minghai Zhou, Ph.D.



Charles Weissmann, M.D., Ph.D.

Chairman's Overview

Researchers in the Department of Infectology currently focus on 2 main areas: prion diseases and hepatitis C. Three groups, those of Corinne Lasmézas and Joaquin Castilla and my own, study the molecular biology of prions and the pathogenesis of prion diseases.

Prion diseases affect animals as well as humans and are of particular interest to us because of the unusual properties of the infectious agent. Prions consist of a multimeric assembly of PrP^{Sc}, a conformer of a normal monomeric host protein, PrP^{C}, and perhaps other as yet unidentified components. The seeding hypothesis posits that prion replication comes about by recruitment of host PrP^{C} into the PrP^{Sc} assembly, a process that entails conformational rearrangement of the PrP^{C} and occasionally cleavage of the assembly. Many strains of prions can be associated with the same protein sequence, and it is thought that different strains are encoded by different PrP^{Sc} conformations. Interestingly, the various strains not only target distinct brain areas but also differ in their capacity to infect disparate cell lines. The question of how cells can distinguish between different prion strains is being addressed by Shawn Browning, and the processes involved in maintaining prion infection are being investigated by Chris Baker and Jiali Li.

Important resources established in our laboratory by Sukhvir Mahal are the semiautomated, cell-based assay for prions (scrapie cell assay), which replaces the slow, expensive, and less accurate mouse-based bioassay, and the cell panel assay, which allows rapid distinction between different prion strains. Dr. Castilla has perfected the protein misfolding cyclic amplification procedure that allows the cell-free replication of prions. He

made the amazing discovery that prions that are infectious to animals can be generated spontaneously. He is also investigating the fidelity of prion replication in vitro. Dr. Lasmézas and her group are investigating the therapeutic possibilities of using antibodies to PrP and PrP-binding aptamers to curb prion diseases.

Our second field of endeavor is hepatitis C. Donny Strosberg and his group are studying interactions between hepatitis C virus proteins and are screening chemical libraries for compounds that disrupt such interactions, in a search for drug candidates. Tim Tellinghuisen and his colleagues are using x-ray crystallography and site-directed mutagenesis to elucidate the role of the hepatitis C virus protein NS5A in viral replication and the interaction between NS5A and NS5B.

The project of screening for drugs against leishmaniasis, specifically against J-binding protein, has been terminated because the scientist involved moved to a different department at Scripps Florida.

Investigators' Reports

Generation and Transmission of Prions

C. Weissmann, C.A. Baker, S.P. Mahal, S. Browning, J. Li, C. Demczyk, A. Sherman, E. Smith, D. Ercan

Prions, the agents that cause transmissible spongiform encephalopathies, consist mainly or entirely of PrP^{Sc}, an abnormal conformer of a normal host protein, PrP^{C}, and propagate by a PrP^{Sc}-catalyzed conversion of PrP^{C}. Intriguingly, distinct prion strains, which generate different disease phenotypes, are associated with the same PrP sequence, suggesting that the phenotypes are encoded by different PrP conformations. Our major interests are the mechanism of prion replication, the structural basis of strain specificity, and the mechanism of strain recognition by cells.

We have developed a cell panel assay (the strain discrimination panel) and a cell-based prion assay (the scrapie cell assay). Used in conjunction, these 2 assays allow rapid discrimination between prion strains in cell culture, largely obviating the mouse bioassay, which requires large numbers of animals and about a year to complete. The cell panel assay is based on the finding that some cell lines can be infected by one or a few prion strains but not by others. Thus, for example, the prion strain Me7 can infect the cell line LD9 but not the cell lines PK1 or R33, strain RML can infect LD9 and PK1 cells but not R33 cells, and strain 22L can infect all 3 cell lines. The cell panel assay has been expanded to discriminate between 5 prion strains.

We found that in some instances the characteristics of a prion strain change after passaging in cell lines. We are investigating whether this change is due to a "strain switch" or to a modification imparted by the cell, such as different N-glycosylation, that is reversed when the prions are again passaged in mice (a phenomenon for which we propose the designation "type switch"). We are also determining, in collaboration with M.B.A. Oldstone, Molecular and Integrative Neurosciences, whether various prion strains passaged through transgenic mice that express PrP devoid of a glycosylphosphatidylinositol anchor retain strain specificity. Initial results suggested that strain specificity is not retained. In addition, in collaboration with J. Castilla, Department of Infectology, we are using protein misfolding cyclic amplification to determine whether prions propagated in a cell-free system maintain strain specificity.

N2a-PK1, a cell line highly susceptible to RML and 22L prions, was selected as a rare variant from an N2a neuroblastoma cell population. We have isolated a set of subclones from a cloned N2a-PK1 population. We found that the subclones are extremely variable in their susceptibility to RML and 22L prion strains. This variability was not correlated with either the PrP expression level (PrP expression is essential but not sufficient) or the doubling time of the cells and thus reflects some other property that remains to be identified. Array analysis of PK1 and its subclones revealed overexpression of 2 genes that was absolutely correlated with susceptibility to RML. The functional significance of these genes is currently being examined. We are also using comparative chromosome hybridization to correlate susceptibility with the genome-wide copy number of loci of these highly aneuploid cells. Our working hypothesis is that a mutation, such as a gene amplification event, on a single copy of a chromosome is responsible for susceptibility and that high variability in the population is due to unequal chromosome partitioning among daughter cells.

To test the hypothesis that a particular chromosome is required to maintain a high susceptibility, we devel-

oped subclones of RML-infected N2a-PK1 cells in which individual chromosomes were tagged with a lentiviral insert containing an antibiotic resistance gene as a selection marker and a coding sequence for green fluorescent protein. Currently, we are growing these clones in the presence and absence of antibiotic to select a clone that maintains infection under selection but not in the absence of selection. So far, the responses of the 22 clones tested have not been promising.

In other studies, we are investigating how cell lines persistently infected with prions maintain infection. Previously, we found that cloned RML-infected neuroblastoma cell populations are heterogeneous, consisting of infected and noninfected cells, probably because noninfected cells are continuously generated as cells replicate faster than RML prions do. The cell population remains persistently infected because noninfected cells are continually reinfected by prions secreted by the infected cells. However, neuroblastoma cell populations infected with strain 22L consist entirely of infected cells, presumably because the doubling rate of prions exceeds the doubling rate of the cells.

We also discovered that an inhibitor that abrogates maturation of N-linked glycans prevents infection of the neuroblastoma-derived cell line PK1 by certain prion strains, such as RML, but not by others, such as 22L. However, once infection is established, prion replication is not affected by the inhibitor. These results suggest that the inhibitor prevents uptake and/or transport of prions to an intracellular "replication compartment," that a glycosylated cell protein other than PrP is required for this process, and that improper glycosylation of this protein abrogates its function or prevents its formation.

PUBLICATIONS

Grover, R.K., Pond, S.J., Cui, Q., Subramaniam, P., Case, D.A., Millar, D.P., Wentworth, P., Jr. O-glycoside orientation is an essential aspect of base J recognition by the kinetoplastid DNA-binding protein JBP1. Angew. Chem. Int. Ed. 46:2839, 2007.

Mahal, S.P., Demczyk, C.A., Smith, E.W., Jr., Klohn, P.-C., Weissmann, C. Assaying prions in cell culture: the standard scrapie cell assay (SSCA) and the scrapie cell assay in end point format (SCEPA). Methods Mol. Biol., *in press*.

Nishina, K.A., Deleault, N.R., Mahal, S.P., Baskakov, I., Luhrs, T., Riek, R., Supattapone, S. The stoichiometry of host PrP^{C} glycoforms modulates the efficiency of PrP^{Sc} formation in vitro. Biochemistry 45:14129, 2006.

Therapy for Prion Diseases

C.I. Lasmézas, N. Salès, P. Saá Prieto, M. Zhou, A. Sturny, G. Sferrazza, Y. Karapetyan

Prions, the transmissible agents responsible for prion diseases, are thought to consist mainly or entirely of PrP^{Sc}, an abnormally folded isoform of the ubiquitous prion protein PrP. Prions are thought to replicate by an autocatalytic process of template-induced conformational change.

Determining targets for therapeutic intervention requires understanding the mechanisms of the neurotoxic effects elicited by the accumulation of PrP^{Sc} in the brain. We found that oligomeric assemblies of recombinant prion protein (provided by H. Rezaei, National Institute for Agricultural Research, Paris, France, and H. Schätzl, Technical University of Munich, Munich, Germany) are toxic to primary cultures of cortical neurons, whether or not the cells express PrP. This toxic effect could be prevented by antibodies that block the hydrophobic domain of the prion protein, located between amino acids 106 and 126. Antibodies to other PrP domains did not prevent oligomer-induced toxic effects.

Because the requirement of endogenous PrP^{C} expression for neuronal toxic effects has been a matter of unresolved debate, we verified in vivo the significance of our findings obtained with cultured neurons. Using stereotactic procedures, we injected oligomeric and fibrillar PrP preparations as well as monomeric PrP and an inert control medium alone subcortically into wild-type and $PrP^{0/0}$ mice of the same genetic background. Histologic examination of brain samples obtained 24 hours after injection revealed that oligomeric PrP was highly toxic to the pyramidal neurons of the hippocampus adjacent to the injection site. Fibrillar PrP was moderately toxic; neither the control medium nor the monomeric PrP had any adverse effect on neuronal survival or morphology. These findings indicate that as in other amyloidotic neurodegenerative diseases (e.g., Alzheimer's disease, Parkinson's disease, systemic amyloidosis), oligomers of misfolded protein, rather than large molecular weight aggregates, are responsible for the toxic effects on neurons. On the basis of our data, new prion intervention strategies can be devised, aimed at blocking PrP oligomers, especially the hydrophobic domain responsible for toxic effects.

In an alternative approach to therapy of prion diseases, we are studying RNA aptamers that bind spe-

cifically to PrP. Aptamers are small nucleic acids (DNA or RNA) selected from a pool of random DNA or RNA sequences for the ability to bind to a given molecular target. The in vitro procedure involves several rounds of selection and amplification. We focused on RNA aptamers because RNAs adopt specific, sometimes complex, 3-dimensional structures. In collaboration with scientists at the Gene Center, Munich, Germany, we are assessing the capacity of 2 PrP-binding aptamers to inhibit prion replication and PrP^{Sc} formation in chronically infected cells. A major hurdle to effective therapy is delivering the aptamer into the proper cell compartment. We are evaluating various promoters in a lentiviral delivery system, which would enable in vivo therapeutical assays once the proper strategy has been determined in cell culture.

In collaboration with C. Weissmann, Department of Infectology, we are searching for factors that might play an important role in prion susceptibility and strain specificity. Because studies of the cellular pathogenesis of prion infection are hampered by the difficulty in distinguishing between PrP and PrP^{Sc}, we are working to improve current immunocytochemistry protocols and to develop strategies to detect PrP^{Sc} in infected cells.

PUBLICATIONS

Simoneau, S., Rezaei, H., Salès, N., Kaiser-Schulz, G., Lefebvre-Roque, M., Vidal, C., Fournier, J.-G., Comte, J., Wopfner, F., Grosclaude, J., Schätzl, H., Lasmézas, C. In vitro and in vivo neurotoxicity of prion protein oligomers. PLoS Pathog. 3:e125, 2007.

Protein-Protein Interactions in Hepatitis C

A.D. Strosberg, A. Ercan, C. Coito, S. Kota, M. Gosink,* C. Nahmias**

* Informatics, Scripps Florida

** Cochin Institute, Paris, France

INTERACTIONS BETWEEN HEPATITIS C VIRUS PROTEINS

The need to discover novel drugs for treatment of hepatitis C has not diminished. Although no vaccine has been developed, several inhibitors of a viral protease and the viral polymerase are in advanced clinical trials. However, because of its high mutability, hepatitis C virus (HCV) most likely will become resistant to these types of drugs. Therefore, identifying novel viral targets is desirable. For this purpose, we have continued our studies of protein interactions involving HCV proteins. Our goals are to better understand the respective roles of the proteins and to identify small-molecule inhibitors that might affect HCV replication.

We have now prepared 4 pairs of interacting HCV protein domains that we identified by using a high-throughput protein-interaction mapping technology. cDNA constructs encoding these HCV protein domains tagged with oligonucleotides encoding epitopes such as the octapeptide Flag or glutathione-*S*-transferase (GST), were expressed in *Escherichia coli* and affinity-purified by using nickel-containing agarose, which recognizes the 6-histidine–containing epitope attached at the C terminus of the interacting domains.

Core-core interactions were first assayed by binding a GST-tagged core domain (the first 106 residues of the HCV core protein) to a glutathione-coated microtiter plate, exposing the plate to a Flag-tagged core domain, and using an antibody to Flag to determine the amount of bound Flag. We then developed a 384 well–based sensitive high-throughput homogenous time-resolved fluorescence assay with fluorescent antibodies to Flag and GST. We used the assay to screen a set of 18mer peptides covering the first 109 residues of the core domain and discovered 2 peptides that inhibit core 106 dimerization. We also screened a library of 1280 pharmacologically active compounds. We will now screen larger and chemically diversified collections of molecules in order to identify novel inhibitors of HCV.

A second pair of HCV protein domains tagged with GST and Flag, respectively, consisted of the protease part of the NS3 protein and the soluble part of its cofactor, NS4A. This cofactor is essential for the correct folding, membrane insertion, and activity of the protease. The NS4A dependence of NS3 has been confirmed with the proteins expressed in *E coli*, and we are now developing both the enzymatic screening assay and the homogenous time-resolved fluorescence assay to identify novel inhibitors of the NS3 protease.

INTERACTIONS BETWEEN HCV AND HOST PROTEINS IN HUMANS

Cyclosporine A, a well-known immunosuppressive agent, can block replication of both HIV and HCV. In HCV, cyclophilin B, one of the receptors for cyclosporine A, promotes the viral NS5B polymerase activity. Binding of cyclosporine A to cyclophilin B blocks the rotamase activity of cyclophilin B and thereby prevents its interaction with NS5B, resulting in inhibition of viral replication. Cyclosporine A thus is an example of a small-molecule inhibitor of a protein-protein interaction involving viral and host cell proteins. We will clone

and express the domains from NS5B, reported to be responsible for the interaction with cyclophilin B. We are currently developing a homogenous time-resolved fluorescence assay to identify compounds that would mimic cyclosporine A but would not have its immunosuppressive activity or, if possible, its effects on the rotamase activity of cyclophilin B.

HEPATOMA CELL CULTURE SYSTEM FOR HCV GENOTYPE 1B

To further evaluate potential inhibitors of interactions between HCV proteins or between HCV and human host proteins, we have developed a culture system in which HCV genotype 1b CG strain replicates at low levels in hepatoma cells (Fig. 1). The supernatant of the

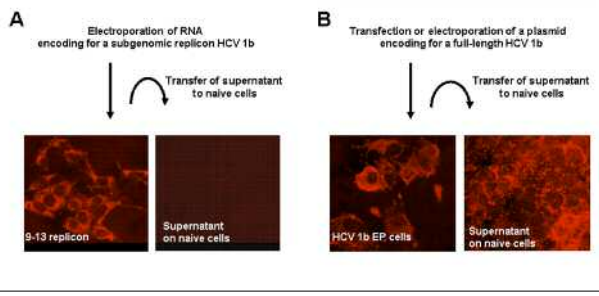


Fig. 1. Transfer of infectious HCV 1b. A and B, upper panels, Schema of the experiment. A and B, lower panels, Immunofluorescence detection of HCV NS5A in cells infected with subgenomic replicon HCV 1b or full-length HCV 1b. A, Supernatant of subgenomic replicon HCV 1b 9-13 was added to naive Huh7.5 cells. B, Supernatant of transfected or electroporated (EP) Huh7.5 by a plasmid that encoded a full-length HCV 1b was added to naive Huh7.5 cells. A replicon is a replicating part of the HCV genome devoid of the structural proteins and thus is noninfectious.

cells is infectious for naive cells. This system, which would still benefit from improvements, seems comparable to the genotype 2a JFH strain infectious system developed recently in part by our Scripps colleagues, F.V. Chisari, Department of Molecular and Experimental Medicine, and T.L. Tellinghuisen, Department of Infectology. We have confirmed the comparable inhibitory effect of cyclosporine A on the 2 infectious HCV culture systems.

The advantage of using a 1b HCV strain rather than the 2a strain is the wider prevalence of the 1b strain in the Western world. In addition, the 2a strain was isolated from a patient with a rare form of fulminant hepatitis C.

PUTATIVE TUMOR SUPPRESSOR GENE IN HEPATOCELLULAR CARCINOMA

The end-stage of HCV infection is hepatocellular carcinoma. Despite its rare occurrence, HCV infection is nevertheless the leading cause of liver cancer in the United States. Recently, in collaboration with C. Nahmias, CNRS, Paris, France, we found that more than 10% of patients with hepatocellular carcinoma have mutant forms of the gene that encodes ATIP, a protein that specifically interacts with the C-terminal region of the AT2 receptor for angiotensin II. This putative tumor suppressor gene is underexpressed in other forms of cancer. A study of more than 90 primary hepatocellular carcinomas or cell lines indicated that the gene for ATIP is indeed consistently repressed more often in the most aggressive class of hepatocellular carcinoma than in the 3 other classes, thus supporting our previous findings for a specific role of ATIP in hepatocellular carcinoma.

Hepatitis C Virus RNA Replication

T.L. Tellinghuisen, J.C. Treadaway, K.L. Foss

Hepatitis C virus (HCV) is a human pathogen of global importance; according to some estimates, nearly 3% of the world's population is chronically infected with this virus. Long-term viral replication in these individuals leads to severe liver disease, including cirrhosis and often hepatocellular carcinoma. The current treatment regimen with agents nonspecific for HCV is poorly tolerated and is ineffective in about half of the patients, emphasizing the need for effective antiviral drugs specific for the virus.

The HCV replicase, the multicomponent machine that replicates the viral RNA, is an ideal drug target. The core replicase consists of 5 HCV proteins associated with well-characterized polymerase, protease, and helicase activities. Some HCV replicase proteins, such as NS5A, are essential for HCV replication; however, their specific functions remain enigmatic.

We have been characterizing NS5A. Our goal is to understand the role of this protein in replication and, more generally, the replicase itself. We have defined NS5A as an absolutely required, 3-domain metalloprotein component of the replicase. Our recent crystal structure of domain I of NS5A has provided a glimpse of the potential interactions of NS5A in the viral replicase and has suggested potential antiviral targets.

Using genetic methods, we have identified all of the amino acids in the poorly understood domains II and III that are required for HCV RNA replication. Additionally,

we recently discovered an interaction between the membrane anchor of NS5A and the protein NS4B, another component of the replicase. This interaction appears to localize NS5A to the replicase and is essential for RNA replication. We are identifying regions of NS5A whose functions are required for the production of infectious virus but not for RNA replication. Our findings suggest that NS5A may function as a regulator of the switch between RNA replication and virus production. We are conducting biochemical, genetic, and structural experiments to evaluate the potential interaction surfaces and activities of the NS5A observed in our previous structural and genetic research. We have also begun applying high-throughput genetic screens to identify required host components of the replicase.

Our ultimate goal is to understand, at the molecular level, the assembly, activity, and regulation of the HCV RNA replication machinery. Greater insight into the poorly understood replicase components, such as the NS5A protein, will provide a more complete view of the replicase complex and will fuel new drug design.

PUBLICATIONS

Randall, G., Panis, M., Cooper, J.D., Tellinghuisen, T.L., Soukhodolets, K.E., Pfeffer, S., Landthaler, M., Landgraf, P., Kan, S., Lindenbach, B.D., Chien, M., Weir, D.B., Russo, J.J., Ju, J., Brownstein, M.J., Sheridan, R., Sander, C., Zavolan, M., Tuschl, T., Rice, C.M. Cellular cofactors affecting hepatitis C virus infection and replication. Proc. Natl. Acad. Sci. U. S. A. 104:12884, 2007.

Tellinghuisen, T.L., Foss, K.L, Treadaway, J., Rice, C.M. Deletion analysis of the domain II and III regions of the hepatitis C virus NS5A protein: identification of residues required for RNA replication. J. Virol., *in press*.

Spontaneous Generation of Prions in a Cell-Free System

J. Castilla, N. Fernández-Borges, G.M. Cosseddu, J. de Castro, M. Márquez

Transmissible spongiform encephalopathies are a group of fatal neurodegenerative disorders that affect both humans and animals. The disorders can be classified as genetic, sporadic (putatively spontaneous), or infectious. The infectious agent associated with these encephalopathies, the prion, appears to consist of the single protein PrP^{Sc}, an abnormal conformer of the natural host protein PrP^{C}. PrP^{Sc} propagates by converting host PrP^{C} into PrP^{Sc}. Interestingly, prions occur in the form of different strains with distinct biological and physicochemical properties, even though all the strains are encoded by PrP with the same amino acid sequence, albeit in presumably different conformations.

The occurrence of spontaneous cases of prion diseases in humans (sporadic Creutzfelt-Jakob disease) and in other species, such as bovine spongiform encephalopathy in cattle in Europe and the United States and the recent cases of atypical scrapie in sheep, suggest that de novo generation of prions may be taking place infrequently but ubiquitously. However, no cases of spontaneous prion disease in experimental wild-type rodent models have been reported. We have used a novel technique, protein misfolding cyclic amplification, with homogenates of normal brain as substrate, to rapidly propagate prions in the test tube. Prions propagated in vitro are infectious in vivo and maintain their prion strain specificity. Protein misfolding cyclic amplification has been used to efficiently amplify a variety of prion strains from mice, hamsters, bank voles, deer, cattle, sheep, and humans.

Because previously infectivity could not be generated de novo by starting with normal PrP^{C}, scientists thought that spontaneous generation of prions was an infrequent in vivo phenomenon that occurred in only a few species. Therefore, mimicking spontaneous generation of infectivity in vitro became one of the most important challenges in the study of prions. We recently, for the first time, generated infectious prions from 2 different rodent species (including wild animals) by starting with noninfectious brain homogenates. Several biochemically different prion strains have been generated, and we are characterizing their biological behavior in different rodent models. We will also determine if the in vitro spontaneous phenomenon takes place in other more relevant species.

PUBLICATIONS

Brun, A., Gutiérrez-Adán, A., Castilla, J., Pintado, B., Díaz San-Segundo, F., Cano, M.J., Parra, B., Espinosa, J.C., Torres, J.M. Reduced susceptibility to bovine spongiform encephalopathy prions in transgenic mice expressing a bovine PrP with five octapeptide repeats. J. Gen. Virol. 88(Pt. 6):1842, 2007.

Castilla, J., Saá, P., Morales, R., Abid, K., Maundrell, K., Soto, C. Protein misfolding cyclic amplification for diagnosis and prion propagation studies. Methods Enzymol. 412:3, 2006.

Espinosa, J.C., Andréoletti, O., Castilla, J., Herva, M.E., Morales, M., Alamillo, E., Díaz San-Segundo, F., Lacroux, C., Lugan, S., Salguero, F.J., Langeveld, J., Torres. J.M. Sheep-passaged bovine spongiform encephalopathy agent exhibits altered pathobiological properties in bovine-PrP transgenic mice. J. Virol. 81:835, 2007.

Espinosa, J.C., Morales, M., Castilla, J., Rogers, M., Torres, J.M. Progression of prion infectivity in asymptomatic cattle after oral bovine spongiform encephalopathy challenge. J. Gen. Virol. 88:1379, 2007.

Hetz, C., Castilla, J., Soto, C. Perturbation of endoplasmic reticulum homeostasis facilitates prion replication. J. Biol. Chem. 282:12725, 2007.

Martín, S.F., Burón, I., Espinosa, J.C., Castilla, J., Villalba, J.M., Torres, J.M. Coenzyme Q and protein/lipid oxidation in a BSE-infected transgenic mouse model. Free Radic. Biol. Med. 42:1723, 2007.

Martín, S.F., Parra, B., Herva, M.E., Espinosa, J.C., Castilla, J., Brun, A., Torres, J.M. Cell expression of a four extra octarepeat mutated PrP^{C} modifies cell structure and cell cycle regulation. FEBS Lett. 580:4097, 2006.

Morales, R., González, D., Soto, C., Castilla, J. Advances in prion detection. *In:* Microbial Food Contamination, 2nd ed. Wilson, C.L. (Ed.). CRC Press, Boca Raton, FL, *in press*.

Pastrana, M.A., Sajnani, G., Onisko, B., Castilla, J., Morales, R., Soto, C., Requena, J.R. Isolation and characterization of a proteinase K-sensitive PrP^{Sc} fraction. Biochemistry 45:15710, 2006.

Richt, J.A., Kasinathan, P., Hamir, A.N., Castilla, J., Sathiyaseelan, T., Vargas, F., Sathiyaseelan, J., Wu, H., Matsushita, H., Koster, J., Kato, S., Ishida, I., Soto, C., Robl, J.M., Kuroiwa, Y. Production of cattle lacking prion protein. Nat. Biotechnol. 25:132, 2007.

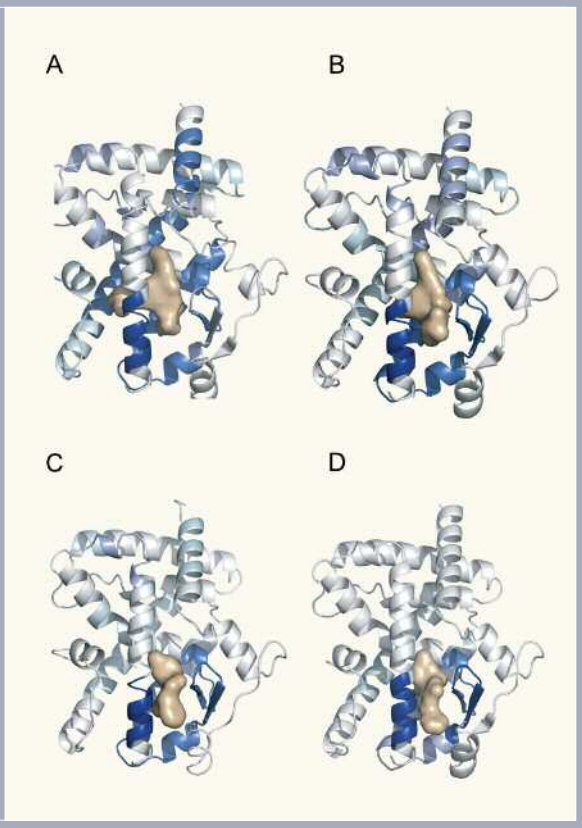
Saá, P., Castilla, J., Soto, C. Presymptomatic detection of prions in blood. Science 313:92, 2006.

Saá, P., Castilla, J., Soto, C. Ultra-efficient replication of infectious prions by automated protein misfolding cyclic amplification. J. Biol. Chem. 281:35245, 2006.

Soto, C., Estrada, L., Castilla, J. Amyloids, prions and the inherent infectious nature of misfolded protein aggregates. Trends Biochem. Sci. 31:150, 2006.

Molecular Therapeutics

Ribbon diagram of the ligand-binding domain of peroxisome proliferator-activated receptor γ (PPARγ) overlaid with differential hydrogen-deuterium exchange data. Ligand density is displayed as a surface representation. The 4 ligand-receptor complexes are PPAR ± MRL-20 (full agonist; A), MRL-24 (partial agonist; B), nTZDpa (partial agonist; C), and BVT.13 (intermediate agonist; D). The degree of stabilization of receptor dynamics as determined by hydrogen-deuterium exchange is shown in color; dark blue indicates the largest magnitude of stabilization. Cocrystal structures were obtained by John Bruning, Ph.D. Hydrogen-deuterium exchange data was obtained by Michael Chalmers, Ph.D., who also generated the figure. These data refute the previous model of for ligand activation of PPAR, in which H12 alone acts as the molecular switch, and suggest a secondary coactivator epitope in the β-sheet region of the receptor. Work done in the laboratory of Pat Griffin, Ph.D., professor.



Paul J. Kenny, Ph.D., Assistant Professor,

and Qun Lu, Research Assistant

DEPARTMENT OF MOLECULAR THERAPEUTICS

STAFF

Patrick Griffin, Ph.D.
Professor and Chairman
Director, Translational Research Institute

Jennifer Caldwell-Busby, Ph.D.*
Assistant Professor

Gregg Fields, Ph.D.
Adjunct Professor

Paul J. Kenny, Ph.D.
Assistant Professor

Malcolm A. Leissring, Ph.D.**
Assistant Professor

Philip LoGrasso, Ph.D.*
Associate Professor

Matthew T. Pletcher, Ph.D.***
Assistant Professor

SENIOR SCIENTISTS

Scott Busby, Ph.D.

Michael Chalmers, Ph.D.

STAFF SCIENTIST

Lisa Cherry, Ph.D.

RESEARCH ASSOCIATES

Yuan Dai, Ph.D.

Brian Ember, Ph.D.

Kevin Hayes, Ph.D.

Jonathan Hollander, Ph.D.

** Joint appointment in the Translational Research Institute*

*** Joint appointment in the Department of Biochemistry*

**** Joint appontments in the Department of Biochemistry and the Translational Research Institute*

Patrick R. Griffin, Ph.D.

Chairman's Overview

The Department of Molecular Therapeutics was established at Scripps Florida in 2007. Faculty in the department use chemical biology approaches to dissect signaling pathways and transcriptional programs. We rely on state-of-the-art multidisciplinary technology and methods and a variety of model systems for target identification, validation, and preclinical studies. Currently, the department has 5 tenure-track faculty members and several non–tenure track members who oversee key functional cores within Scripps Florida. These investigators have created strong research programs that take advantage of the unique high-throughput core facilities of Scripps Florida, including genomics, cell-based screening, proteomics, x-ray crystallography, informatics, and lead optimization.

Research activities include discovery and development of therapeutic agents for unmet medical needs in neurodegeneration, Parkinson's disease, acute respiratory distress syndrome, spinal cord injury, cardiovascular disease, cancer, addiction, and metabolic disorders such as insulin resistance and type 2 diabetes. Therapeutic areas and targets include G protein–coupled receptors, proteases, channels, and kinases.

Members of the department interact with researchers in the Translational Research Institute (TRI), which merges drug discovery expertise with advanced technology. The lead identification and high-throughput screening operation of TRI is headed by Peter Hodder of our department. Dr. Hodder and his group focus on technology and assay development and novel chemical approaches to expand compound libraries. The TRI discovery biology group is headed by Phil LoGrasso of our department. Dr. LoGrasso and members of his group focus on small-molecule therapeutic agents as neuroprotective agents in diseases such as Parkinson's. The TRI drug metabo-

lism and pharmacokinetics group is headed by Mike Cameron of our department. Dr. Cameron and his group are involved in mechanistic studies of P450s and drug biotransformation mechanisms.

Other research interests in the Department of Molecular Therapeutics include the neuropharmacology of addiction. Paul Kenny is establishing the role of several G protein–coupled receptors in addiction. Malcolm Leissring is investigating insulin-degrading enzyme as a drug target for treatment of diabetes and, perhaps, Alzheimer's disease.

Other faculty members in the department oversee technology cores. The genomics platform is headed by Mathew Pletcher. He and members of his laboratory use a systems biology approach to identify the determinants of antidepressant efficacy. Researchers in the genomics core oversee genotyping and gene expression profiling technology platforms. The services provided by this core allow Scripps Research investigators to query the genome at both the genetic and the transcriptional levels for the genes that underlie common diseases. In collaboration with Scripps Florida colleagues, members of the genomics core have been involved in projects to identify the genes responsible for pathologic states such as addiction and alcoholism, systemic lupus erythematosus, autism, obsessive-compulsive disorder, diabetes, obesity, and prion diseases.

The cell-based screening platform is headed by Julie Conkright. The faculty advisor to this core is Michael Conkright, Department of Cancer Biology. The cell-based screening platform leverages high-throughput technologies toward a systematic description of the function of genes encoded by the human genome and a more comprehensive understanding of the genetic basis for human disease. The cell-based screening group provides investigators with access to genome-wide collections of cDNAs and short interfering RNAs that can be used to examine cellular models of signal transduction pathways and phenotypes.

The proteomics platform is headed by Jennifer Caldwell-Busby. Liquid chromatography and state-of-the-art mass spectrometry are used to identify, quantify, and characterize proteins and protein modifications. Scientists in this core are involved in scientific collaborations in which novel technologies are used to identify biologically important proteins and protein modifications. Large-scale differential analysis is being used to map the pathways related to insulin sensitization and adipogenesis. In other projects, chromatographic enrichment techniques are used to identify sites of phosphorylation and other posttranslational modifications. Researchers in the proteomics core collaborate with other scientists to create experiments that will provide meaningful mass spectrometric results.

INVESTIGATORS' REPORTS

Mechanism of Activation of Nuclear Receptors

P.R. Griffin, S.A. Busby, M.J. Chalmers, S. Prasad, S.Y. Dai

We use a wide range of technologies to study ligand activation of nuclear receptors. Previously, we focused heavily on peroxisome proliferator-activated receptor γ (PPARγ; see following), but we are beginning to study several orphan nuclear receptors that are implicated in a variety of disorders. Of particular interest are the orphan receptors liver receptor homolog-1 and steroidogenic factor-1. We are building the appropriate tools to begin comprehensive mechanistic studies of these 2 receptors.

MECHANISTIC STUDIES OF LIGAND ACTIVATION OF PPARγ

PPARγ is a multidomain ligand-dependent transcription factor. Activation of PPARγ is regulated by binding of ligands to the receptor's ligand-binding domain, which induces a change in the conformational dynamics of the domain-mediated dissociation of corepressor molecules and forms suitable neoepitopes for binding coactivator molecules. Ligands of PPARγ are characterized as full or partial agonists on the basis of their ability to transactivate PPARγ target genes. Full agonists have maximal transcriptional activity, whereas partial agonists have moderate activity. Although the structural and molecular determinants of full-agonist regulation of PPARγ have been studied in detail, the determinants for partial agonists have not been completely characterized. We are using structural, biochemical, and cell-based techniques to examine the mechanism of regulation of PPARγ transcriptional activity by partial agonists.

Hydrogen-deuterium exchange (HDX) coupled with mass spectrometry was used to characterize ligand-dependent structural and dynamic changes in PPARγ. Ligand-induced protection of amide exchange in a transcriptional complex composed of PPARγ, retinoic X receptor α, and either steroid receptor coactivator-1 or steroid receptor coactivator-3 were probed by using automated solution-phase HDX. We used fluorescence-based assays to determine recruitment of coactivators and to measure the affinity of the receptor heterodimer composed of PPARγ and retinoic X receptor α.

A mammalian 2-hybrid genetic screen was used to probe the molecular determinants of ligand-dependent coactivator selectivity.

We found that the magnitude of PPARγ agonism is regulated by coactivator recruitment selectivity of p160 coactivators. The functional relevance of the genetic screen was further confirmed in a 3T3-L1 preadipocyte differentiation assay as an in vitro model of adipogenesis. We found that conversion of the full-agonist phenotype to the partial-agonist phenotype and vice versa was a function of the availability of specific p160 coactivators.

Using combined proteomic and genomic differential analysis, we have extended these studies to examine the molecular components of PPARγ-mediated insulin sensitization and adipogenesis after treatment of preadipocyte cells with full and partial agonists of PPARγ. Our goal is to determine unique components of the insulin sensitivity pathway and dissociate them from components of the proadipogenic pathways that lead to the adverse side effects common after therapy with PPARγ full agonists. To complete this study, we are developing new strategies to improve the detection of membrane- and lipid-associated proteins, and we are using new mass spectrometry methods to measure the samples.

In other studies, we are using coactivators as chemical tools to generate desired functional responses and distinguish beneficial functions from adverse functions, a novel therapeutic avenue for treating insulin resistance that has not yet been exploited. Our goals are to determine the structure-activity relationships between PPARγ ligands and their coactivator recruitment selectivity and to obtain PPARγ ligands with preferences for specific coactivators. To this end, we have developed a validated homogenous time-resolved fluorescence assay for ligand-dependent recruitment of the coactivator to PPARγ for a large-scale high-throughput screen to identify coactivator-selective agonists of the receptor. We will do 3 different primary screenings of the National Institutes of Health small-molecule chemical library. In each screen, we will target PPARγ association with a different coactivator to obtain coactivator-specific agonists. The results obtained from this research will provide molecular insight into how recruitment of coactivators modulates activation of PPARγ and will shed light on the role of specific coactivators in the pharmacologic behavior of PPARγ modulators.

MECHANISTIC STUDIES OF LIGAND ACTIVATION

OF THE ESTROGEN RECEPTOR

In collaboration with scientists at Eli Lilly and Company, Indianapolis, Indiana, we used HDX to characterize the estrogen receptor. As drug targets, estrogen receptors play important roles in the treatment of multiple diseases, including breast cancer and osteoporosis. Tamoxifen and raloxifene, modulators of estrogen receptors approved by the Food and Drug Administration, have intriguing mixed agonism and antagonism effects depending on the target tissue. Structural studies have revealed differences between complexes consisting of the estrogen receptor and an agonist and complexes consisting of the receptor and an antagonist and have provided insight into how these ligands interact with the receptor. We have used HDX to examine an array of chemical compounds that have different degrees of agonism/antagonism. We found an excellent correlation between HDX profiles and pharmacologic properties, and we were able to classify estrogen receptor ligands on the basis of HDX signatures. Discoveries derived from this study will help in understanding tissue-specific activities of drugs targeted to estrogen receptors and will facilitate future drug development programs.

PUBLICATIONS

Busby, S.A., Chalmers, M.J., Griffin, P.R. Improving digestion efficiency under H/D exchange conditions with activated pepsinogen columns. Int. J. Mass Spectrom. 259:130, 2007.

Pascal, B.D., Chalmers, M.J., Busby, S.A., Mader, C.C., Southern, M.R., Tsinoremas, N.F., Griffin, P.R. The Deuterator: software for the determination of backbone amide deuterium levels from H/D exchange MS data. BMC Bioinformatics 8:156, 2007.

Quint, P., Ayala, I., Busby, S.A., Chalmers, M.J., Griffin, P.R., Rocca, J., Nick, H.S., Silverman, D.N. Structural mobility in human manganese superoxide dismutase. Biochemistry 45:8209, 2006.

Mass Spectrometry for Analysis of Proteins

J.A. Caldwell Busby, V. Cavett

We work to develop methods compatible with mass spectrometry for the analysis of proteins. Our areas of interest are wide-ranging and include methods for subcellular compartmentalization for large-scale proteomics profiling and identification of proteins involved in gene regulation. Gene regulation, or epigenomics, is of particular interest. To date, the focus of epigenomics research has been patterns of DNA methylation and posttranslational modifications of the histone N termini. However, many other proteins are associated with chromatin, including transcription factors, enzymes, and scaffolding proteins. We are developing methods for isolating and analyzing the supermolecular structure of chromatin, particularly the regulatory protein machineries, and we are using cutting-edge mass spectrometric techniques to characterize the proteins involved in gene regulation.

Study of the interactions of proteins and DNA biopolymers is not new; much work has been done on transcription and gene regulation. However, most of this research has relied on the analysis of DNA methylation patterns and chromatin immunoprecipitation methods. Although informative, these studies ignore the extensive protein components of the system, potentially a rich source of information about how epigenetic patterns are established, maintained, and modified in healthy tissue. Such studies also cannot be used to assess how changes in these protein components contribute to disease states, aging, or cell death.

Mass spectrometry is a widely used technique in proteomics. Powerful, high-resolution instruments, coupled with new fragmentation methods, including electron capture dissociation, are driving the identification of novel proteins and new sites of protein identification. This identification is something for which neither traditional molecular biology nor modern RNA/DNA analysis is well suited. The development of a mass spectrometry–based process to identify components of the chromatin regulatory network will yield a broadly applicable method that will contribute to a wide range of biological investigations, including chromatin regulation of gene expression.

PUBLICATIONS

Godeny, M.D., Sayyah, J., VonDerLinden, D., Johns, M., Ostrov, D.A., Caldwell-Busby, J., Sayeski, P.P. The N-terminal SH2 domain of the tyrosine phosphatase, SHP-2, is essential for Jak2-dependent signaling via angiotensin II type AT_1 receptor. Cell Signal. 19:600, 2007.

Sloley, S., Smith, S., Algeciras, M., Cavett, V., Caldwell Busby, J.A., London, S., Clayton, D.F., Bhattacharya, S.K. Proteomic analyses of songbird (zebra finch; *Taeniopygia guttata*) retina. J. Proteome Res. 6:1093, 2007.

Sloley, S., Smith, S., Gandhi, S., Caldwell Busby, J.A., London, S., Luksch, H., Clayton, D.F., Bhattacharya, S.K. Proteomic analyses of zebra finch optic tectum and comparative histochemistry. J. Proteome Res. 6:2341, 2007.

Neurobiology of Compulsive Behaviors

P.J. Kenny, J.A. Hollander, P. Johnson, Q. Lu, D. Pham*

* Florida Atlantic University, Jupiter, Florida

We focus on elucidating the neurobiological mechanisms of compulsive behaviors, in particular, the motivational processes that drive compulsive drug consumption in addiction and overeating of palatable foods in obesity. Our findings will be used to facilitate the development of novel therapeutic agents for treatment of substance abuse disorders and obesity.

Recently, we investigated the role of *N*-methyl-D-aspartate (NMDA) receptors in the rewarding properties of nicotine. We found that pharmacologic blockade of NMDA receptors reversed the "hedonic valence" of nicotine in rats; animals were averse to previously rewarding doses of nicotine after treatment with an NMDA receptor antagonist. In addition, we found that nicotine increased NMDA receptor–mediated transmission throughout brain reward circuitries, and chronic intake of nicotine was associated with profound alterations in the expression of NMDA receptors throughout the brain. Taken together, these data support a critical role for NMDA receptors in nicotine reward.

In ongoing studies, we are investigating the subtypes of nicotinic acetylcholine receptors at which nicotine acts in the brain to elicit its rewarding properties. Specifically, we are assessing the rewarding effects of nicotine in mice with mutations in various subunits of the receptors. To compliment this genetic approach, we are also using a lentiviral-based short interfering RNA approach to silence the expression of the genes of targeted subunits of the nicotinic acetylcholine receptors in the brains of rats in a spatially and temporally controlled manner. Subsequently, we will assess the effects of the gene silencing on nicotine reward. These studies should provide important new insights into the neurobiological mechanisms of nicotine addiction, with direct relevance for the treatment of the tobacco habit in humans.

In collaborations with scientists in the Translational Research Institute, Scripps Florida, we are also developing small-molecule drugs that may be useful as novel therapeutic agents for treatment of substance abuse disorders. The targets for these drugs are G protein–coupled receptors that we previously showed play a role in drug dependence.

Finally, we have started a new program to investigate the possibility that neurobiological mechanisms similar to those that drive compulsive drug seeking in addicted rats may also contribute to overeating in obese rats.

PUBLICATIONS

Kenny, P.J. Brain reward systems and compulsive drug use. Trends Pharmacol. Sci. 28:35, 2007.

Kenny, P.J., Chen, S.A., Kitamura, O., Markou, A., Koob, G.F. Conditioned withdrawal drives heroin consumption and decreases reward sensitivity. J. Neurosci. 26:5894, 2006.

Kenny, P.J., Markou, A. Nicotine self-administration acutely activates brain reward systems and induces a long-lasting increase in reward sensitivity. Neuropsychopharmacology 31:1203, 2006.

Inhibition of Jun N-Terminal Kinase 2/3 for the Treatment of Parkinson's Disease

P. LoGrasso, M. Cameron, W. Chen, D. Duckett, J. Habel, R. Jiang, T. Kamenecka, S. Khan, L. Lin, Y.-Y. Ling, M. Lopez, C. Ruiz, Y. Shin, L. Smith, X. Song, T. Vojkovsky, D. Zadory

Apoptosis, or programmed cell death, plays a vital role in the normal development of the nervous system and is also thought to contribute to the aberrant neuronal cell death that characterizes many neurodegenerative diseases. Therefore, blocking neuronal apoptosis could be an approach for treating neurodegenerative diseases. A major pathway implicated in neuronal cell death and survival is the MAP kinase pathway, which controls cell proliferation and cell death in response to many extracellular stimuli. Recent studies have linked Jun N-terminal kinase (JNK) activity with the cell death associated with Parkinson's disease and Alzheimer's disease.

JNK is linked to many of the hallmark pathophysiologic components of Parkinson's disease, such as oxidative stress, programmed cell death, and microglial activation. Many pieces of evidence support JNK as a target for treatment of the pathologic changes that underlie Parkinson's disease. One attractive feature of JNK3 as a selective drug target is that this kinase is almost exclusively expressed in the brain; levels expressed in the kidney and testis are extremely low. In contrast, JNK1 and JNK2 are ubiquitously expressed. Despite the ubiquitous expression of JNK2, we are developing a therapy to prevent degeneration of dopaminergic neurons and halt the progression of Parkinson's disease by targeting JNK2/3.

Our strategy for inhibition JNK2/3 is based on the results of experiments with mice in which the gene for JNK3 or JNK2 was deleted and mice in which the genes for both JNK2 and JNK3 or both JNK1 and JNK2 were deleted. In contrast to mice lacking the gene for JNK1 alone, which had defective T-cell differentiation, mice lacking the gene for JNK2 alone had normal T- and B-cell development and normal T-cell proliferation. Moreover, mice lacking the gene for JNK2 alone and mice lacking the gene for JNK3 alone were protected against the effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a compound used to induce parkinsonian signs in animal models of Parkinson's disease, whereas both wild-type and mice lacking the gene for JNK1 were not. In other research, compared with wild-type mice, mice lacking the genes for both JNK2 and JNK3 were dramatically protected against acute MPTP-induced injury of the nigrostriatal pathway. This protective effect resulted in a 3-fold increase in the number of neurons positive for tyrosine hydroxylase, an indication of the increase in survival of dopaminergic neurons.

On the basis of these in vitro and in vivo data, we are synthesizing potent, selective JNK 2/3 inhibitors that are efficacious in MPTP animal models of Parkinson's disease. We have established homogenous time-resolved fluorescence biochemical assays for JNK3 and counterscreens for JNK1 and p38. We have generated more than 500 compounds from 3 different structural classes; many of the compounds are inhibitory for JNK3 in nanomolar concentrations. Some of the compounds have a cellular potency of 40–60 nM and in vitro efficacy in promoting primary survival of dopaminergic neurons. We have tested compounds in vivo in rats and mice for drug metabolism and pharmacokinetic properties. Many of the JNK3 inhibitors have had good oral absorption, good brain penetration, and good pharmacokinetic properties that enable efficacy studies.

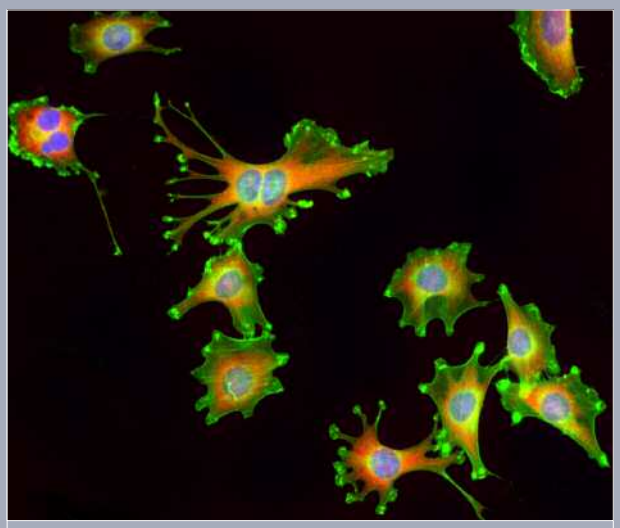
We have also developed an in vivo target modulation assay to monitor inhibition of c-Jun phosphorylation and an in vivo efficacy model with MPTP to create lesions in the substantia nigra. Moreover, we have solved the crystal structure of 10 complexes of JNK3 with inhibitor at approximately 2.2-Å resolution. This information is being used in structure-based drug design to help guide medicinal chemistry studies and optimize compounds for potency, selectivity, brain penetration, oral absorption, half-life, clearance, and efficacy.

PUBLICATIONS

Feng, Y., Cameron, M.D., Frackowiak, B., Griffin, E., Lin, L., Ruiz, C., Schröter, T., LoGrasso, P. Structure-activity relationships and drug metabolism and pharmacokinetic properties for indazole piperazine and indazole piperidine inhibitors of ROCK-II. Bioorg. Med. Chem. Lett. 17:2355, 2007.

Rech, J.C., Yato, M., Duckett, D., Ember, B., LoGrasso, P.V., Bergman, R.G., Ellman, J.A. Synthesis of potent bicyclic bisarylimidazole c-Jun N-terminal kinase inhibitors by catalytic C-H bond activation. J. Am Chem Soc. 129:490, 2007.

Translational Research Institute



Src overexpression in NIH-3T3 fibroblasts. Overexpression results in actin relocalization toward structures known as "podosomes," which are thought to be involved in cell motility. In order to examine actin localization and apoptosis activity after treatment with src inhibitors, the cells were stained with Alexa Fluor 488 phalloidin, Bcl-2, and Alexa Fluor 647 $F(ab')_2$ goat antirabbit fragment of IgG. The nuclei were stained with Hoechst dye 33342. The cells were plated in a 96-well plate and visualized with the IN Cell 1000. The image was analyzed for granularity by using Developer Toolbox. Further processing was done with Adobe Photoshop. Work done by Evelyn Griffin, research assistant, in the laboratory of Thomas Schroeter, Ph.D., senior scientist.

Philip LoGrasso, Ph.D., Senior Director, Discovery Biology, and Brian Ember, Ph.D., Research Associate

TRANSLATIONAL RESEARCH INSTITUTE

STAFF

Patrick Griffin, Ph.D.*
Director

Thomas D. Bannister, Ph.D.
Associate Scientific Director, Medicinal Chemistry

Jennifer Caldwell-Busby, Ph.D.*
Associate Scientific Director, Proteomics

Michael Cameron, Ph.D.*
Associate Scientific Director, Drug Metabolism and Pharmacokinetics

Derek R. Duckett, Ph.D.
Associate Scientific Director, Discovery Biology

Yangbo Feng, Ph.D.
Associate Scientific Director, Medicinal Chemistry

Peter Hodder, Ph.D.
Scientific Director, Lead Identification

Ted Kamenecka, Ph.D.
Associate Scientific Director, Medicinal Chemistry

Congxin Liang, Ph.D.
Scientific Director, Medicinal Chemistry

Philip LoGrasso, Ph.D.*
Senior Director, Discovery Biology

Patricia McDonald, Ph.D.
Associate Scientific Director, Discovery Biology

Mathew T. Pletcher, Ph.D.**
Assistant Professor, Genomics Core

William Roush, Ph.D.***
Executive Director, Medicinal Chemistry
Associate Dean, Kellogg School of Science and Technology

Layton Smith, Ph.D.
Associate Scientific Director, Discovery Biology

SENIOR SCIENTISTS

Yenting Chen, Ph.D.

Marcel Koenig, Ph.D.

Jiuxiang Ni, Ph.D.

Alok Pachori, Ph.D.

Louis Scampavia, Ph.D.

Thomas Schroeter, Ph.D.

Youseung Shin, Ph.D.

Tomas Vojkovsky, Ph.D.

STAFF SCIENTISTS

Juliana Conkright, Ph.D.

Dympna Harmey, Ph.D.

Sahba Tabrizifard, Ph.D.

RESEARCH ASSOCIATES

Xingang Fang, Ph.D.

Jeffrey Habel, Ph.D.

Yuanjun He, Ph.D.

Par Holmberg, Ph.D.

Rong Jiang, Ph.D.

Franck Madoux, Ph.D.

Sanjay Saldanha, Ph.D.

E. Hamp Sessions, Ph.D.

Anthony Smith, Ph.D.

Michael Smolinski, Ph.D.

Xinyi Song, Ph.D.

Prem Subramaniam, Ph.D.

Bo Wang, Ph.D.

Lei Yao, Ph.D.

Yan Yin, Ph.D.

SCIENTIFIC ASSOCIATES

Dmitriy Minond, Ph.D.

Timothy Spicer

HTS ROBOTICS ENGINEERS

Pierre Baillargeon

Peter Chase

Lina Deluca

INFORMATICS STAFF

Kashif Hoda

Chris Mader

Bruce Pascal

Mark Southern

Joel Zysman

** Joint appointment in the Department of Molecular Therapeutics*

*** Joint appointments in the Departments of Biochemistry and Molecular Therapeutics*

**** Joint appointment in the Department of Chemistry*

Patrick R. Griffin, Ph.D.

Director's Overview

The Translational Research Institute merges drug discovery efforts at Scripps Florida with advanced technology platforms to rapidly identify and validate biological pathways that can be targeted for therapeutic intervention. The technology platforms are grouped into genomics, cell-based screening, and proteomics cores.

The goal of the drug discovery operation is to discover and develop small-molecule therapeutic agents for unmet medical needs in neurodegeneration, Parkinson's disease, acute respiratory distress syndrome, spinal cord injury, cardiovascular disease, cancer, and metabolic disorders such as insulin resistance and type 2 diabetes. Therapeutic areas and targets, which include G protein–coupled receptors, proteases, channels, and kinases, are selected on the basis of unmet needs and the ability to attract external funding. The drug discovery operation is fully integrated with the following groups: Lead Identification and High-Throughput Screening, headed by Peter Hodder, Department of Molecular Therapeutics; Medicinal Chemistry, headed by William Roush, Department of Chemistry; Discovery Biology, headed by Phil LoGrasso, Department of Molecular Therapeutics; and Drug Metabolism and Pharmacokinetics, headed by Mike Cameron, Department of Molecular Therapeutics.

The Lead Identification department enables drug-target lead identification via ultra-high-throughput screening technology. Using state-of-the-art automation and instrumentation, members in this department are responsible for developing and executing biochemical or cell-based high-throughput screening assays in a miniaturized 1536-well microtiter plate format. In addition to its support of internal Scripps Research objectives, the group participates in the National Institutes of Health Molecular Libraries Screening Centers Network, in which qualified assays are screened against the network's high-throughput screening compound library. Several internal and external investigators have accessed the department's expertise via collaborative or core-charge mechanisms.

The genomics core is headed by Mathew Pletcher. Scientists in this core oversee genotyping and gene expression profiling. The services provided by the core allow Scripps Research investigators to examine the genome at both the genetic and the transcriptional levels for the genes that underlie common diseases. In collaboration with Scripps Florida colleagues, members of the core have been involved in projects to identify the genes responsible for pathologic conditions, such as addiction and alcoholism, systemic lupus erythematosus, autism, obsessive-compulsive disorder, diabetes, obesity, and prion diseases.

The cell-based screening platform is headed by Julie Conkright, Department of Molecular Therapeutics. The faculty advisor to the core is Michael Conkright, Department of Cancer Biology. In this group, high-throughput technologies are used to provide a systematic description of the function of genes encoded by the human genome and a more comprehensive understanding of the genetic basis for human disease. Members of the group provide investigators access to genome-wide collections of cDNAs and short interfering RNAs that can be used to examine cellular models of signal transduction pathways and phenotypes.

The proteomics platform is headed by Jennifer Caldwell-Busby, Department of Molecular Therapeutics. The focus of this core is using liquid chromatography and state-of-the-art mass spectrometry technology to identify, quantify, and characterize proteins and protein modifications. Researchers in the core are involved in scientific collaborations in which novel technologies are used to identify biologically important proteins and protein modifications. Large-scale differential analysis is

being used to map the pathways related to insulin sensitization and adipogenesis. In other projects, chromatographic enrichment techniques are used to identify sites of phosphorylation and other posttranslational modifications. Researchers in the proteomics core collaborate with other scientists to create experiments that will provide meaningful mass spectrometric results.

INVESTIGATORS' REPORTS

Drug Metabolism and Pharmacokinetics Laboratory

M.D. Cameron, L. Lin, C. Ruiz, S. Khan

The Drug Metabolism and Pharmacokinetics Laboratory at Scripps Florida provides in vitro and in vivo evaluation of the pharmacokinetic and pharmacodynamic properties of new chemical entities. We work on project teams within the Drug Discovery group of the Department of Molecular Therapeutics and support chemistry efforts within the Scripps Research Institute Molecular Screening Center. We help bridge medicinal chemistry and pharmacology by evaluating the metabolic fate and identifying the liabilities of compounds. Pharmacokinetic studies provide basic parameters, including peak plasma concentration, bioavailability, exposure, half-life, clearance, volume of distribution, and tissue distribution. Research interests include P450 structure-function relationships and the formation of reactive intermediates during metabolism. The laboratory is equipped with liquid chromatography–tandem mass spectrometry system and a Q-trap hybrid triple quadrupole/linear ion-trap mass spectrometer.

PUBLICATIONS

Cameron, M.D., Wright, J., Black, C.B., Ye, N. In vitro prediction and in vivo verification of enantioselective human tofisopam metabolite profiles. Drug Metab. Dispos., *in press*.

Feng, Y., Cameron, M.D., Frackowiak, B., Griffin, E., Lin, L., Ruiz, C., Schröter, T., LoGrasso, P. Structure-activity relationships, and drug metabolism and pharmacokinetic properties for indazole piperazine and indazole piperidine inhibitors of ROCK-II. Bioorg. Med. Chem. Lett. 17:2355, 2007.

Proteomics Laboratory

J.A. Caldwell-Busby, V. Cavett

The Proteomics Laboratory at Scripps Florida provides proteomics services and expertise to scientific collaborators at Scripps Research facilities in both Florida and California, universities within the state of Florida, and other educational institutions. We use cutting-edge mass spectrometry technology to identify proteins, map modifications that occur after translation, and do relative quantitation experiments with a variety of samples.

In its lifetime, a protein it can have several locations and functions within a cell. Location, function, and 3-dimensional structures of proteins are all influenced by static and dynamic chemical modifications that occur after translation. These modifications vary from small methyl and acetyl groups, which are a part of the histone codes, to large lipid and glycosylation modifications, which act as cellular markers and signaling molecules. With mass spectrometry, we can detect both the small and the large changes in mass that occur in proteins because of these modifications, and we can identify the specific amino acids modified.

Relative changes in protein levels between multiple samples provide biologically relevant information about cellular pathways and proteins of interest. Large-scale studies of this type require rigorous sample preparation and highly tuned algorithms for comparing different mass spectrometric analyses. We are currently validating methods for both sample fractionation and data analysis for these types of large-scale differential protein experiments.

Mass spectrometers at the facility include an ion-trap spectrometer, which is used mostly to identify proteins and peptides, and a triple quadrupole mass spectrometer, which is used for relative quantitation experiments. A new addition is a mass spectrometer that can be used to perform accurate mass and high-resolution experiments. Each mass spectrometer is interfaced to nanoflow electrospray ionization sources and capillary high-performance liquid chromatography columns.

Data analysis is performed primarily via automated workflow on a cluster maintained by the bioinformatics group. Automation of the front-end processing allows for a more thorough review of the resultant data and more time for development of innovative software in collaboration with information technology groups at Scripps Research and beyond.

PUBLICATIONS

Godeny, M.D., Sayyah, J., VonDerLinden, D., Johns, M., Ostrov, D.A., Caldwell-Busby, J., Sayeski, P.P. The N-terminal SH2 domain of the tyrosine phosphatase, SHP-2, is essential for Jak2-dependent signaling via angiotensin II type AT_1 receptor. Cell Signal. 19:600, 2007.

Sloley, S., Smith, S., Algeciras, M., Cavett, V., Caldwell Busby, J.A., London, S., Clayton, D.F., Bhattacharya, S.K. Proteomic analyses of songbird (zebra finch; *Taeniopygia guttata*) retina. J. Proteome Res. 6:1093, 2007.

Sloley, S., Smith, S., Gandhi, S., Caldwell Busby, J.A., London, S., Luksch, H., Clayton, D.F., Bhattacharya, S.K. Proteomic analyses of zebra finch optic tectum and comparative histochemistry. J. Proteome Res. 6:2341, 2007.

Probe and Drug Discovery: The Lead Identification Department

P. Hodder, P. Baillargeon, P. Chase, L. DeLuca, F. Madoux, B. Mercer, D. Minond, S. Saldanha, L. Scampavia, T. Spicer, P. Subramaniam, L. Sullivan

The Lead Identification Department is responsible for developing and executing high-throughput screening (HTS) assays and for supporting downstream medicinal chemistry and probe development efforts. The anchors of the department are 2 fully automated robotic platforms (Fig. 1). One supports screening of 384- and 1536-well microtiter plates in a variety of biochemical and cell-based assay formats. The other is used to manage and characterize the screening library of more than 600,000 compounds used for drug discovery at Scripps Research. The facility also contains an assay development laboratory with equipment for tissue culture and semiautomated liquid handling and detection. Supporting this operation is an integrated laboratory information management system, which is used to track HTS data and compound usage and quality. Additionally, we are involved in developing metallo-β-lactamase chemical probes.

Fig. 1. The Scripps Research uHTS platform. A, An industrial anthropomorphic robotic arm moves assay and compound microtiter plates. B, A pin tool is used to transfer test compounds rapidly from compound plates to assay plates. C, Liquid handlers capable of dispensing up to 32 different reagents and of washing plates are integrated into the platform. D, Incubators, each capable of holding approximately 700,000 samples in 1536-well format, are used to store microtiter plates at a variety of temperatures and gas concentrations. E, A multimode plate reader measures absorbance, luminescence, fluorescence, or fluorescence resonance energy transfer (with time resolution) from microtiter plates. F, A kinetic imaging plate reader allows measurement of second-messenger or ion channel activity in live cells. Not shown is a compound management robot capable of storing and retrieving desirable compounds from the Scripps Research screening file.

THE SCRIPPS RESEARCH INSTITUTE MOLECULAR SCREENING CENTER

Established in 2005, the Scripps Research Institute Molecular Screening Center is a national resource for small-molecule screening and the development of chemical probes. It is 1 of 10 members in the Molecular Libraries Screening Centers Network, a translational research initiative sponsored by the National Institutes of Health (NIH) and part of the NIH Roadmap Initiative. The mission of the Scripps center is to screen the NIH library of more than 150,000 individual compounds against peer-reviewed targets; the goal is to discover proof-of-concept probes. The results are available to the scientific community through the PubChem Web site of the National Center for Biotechnology Information: http://pubchem.ncbi.nlm.nih.gov. Currently, the Lead Identification Department serves as the HTS core within the Scripps screening center; our responsibilities are to develop biological and biochemical assays, perform HTS campaigns, manage the resulting data, steward the NIH screening library, and provide assay support for the development of probes.

OTHER SCREENING ACTIVITIES

Since the inauguration of the ultra-HTS (uHTS) operation in November of 2005, we have also been actively screening the Scripps collection of compounds against drug discovery targets not only from the MLSCN but also from scientists at Scripps Research and from outside partners. So far, members of the department have initiated and successfully completed more than 30 uHTS-related collaborations (Table 1).

DISCOVERY AND DEVELOPMENT OF CLASS B METALLO-β-LACTAMASE INHIBITORS

The diversity of bacterial β-lactamases continues to outpace the development of useful β-lactam–based antibiotics. Although the development of class B β-lactamase inhibitors has been an active area of research, an array of potent, class-specific small-molecule inhibitors has yet to be fully characterized in the clinically relevant VIM-2 metallo-β-lactamase system. Additionally, VIM-2 inhibitors that are effective inhibitors of other class B β-lactamases will be of great interest. Such compounds

Table 1. Summary of HTS and HTS assay development collaborations

Target class	Target name (abbreviation)	Collaboration	Collaborator
G protein–coupled receptor	$5HT_{1a}$	β-Lactamase reporter gene uHTS campaign	M. Teitler[a]
	$5HT_{1e}$		
	GalR2		S. Brown[b]
	$S1P_1$		H. Rosen[b]
	$S1P_2$		
	$S1P_3$		
	AGTRL-1 (APJ)	FLIPR/TETRA HTS assay development	L. Smith[c]
	GLP-1		P. LoGrasso[d]
	GPR119		P. McDonald[d]
	μ-Opiod heterodimers		L. Devi[e]
	NPY-Y1	FLIPR/TETRA uHTS campaign	C. Wahlestedt[d]
	NPY-Y2		
Kinase	JNK3	TR-FRET uHTS campaign	P. LoGrasso[d]
	ROCK2		
	PKA	Luminescence uHTS campaign	T. Schroeter[d]
	FAK	TR-FRET uHTS campaign	P. Hodder[d]
Transcription factor	PPARγ/Src1	TR-FRET uHTS campaign	P. Griffin[d]
	PPARγ/Src2	TR-FRET HTS assay development	
	PPARγ/Src3	TR-FRET uHTS campaign	
	NF-κB	Luciferase reporter gene uHTS campaign	J. Reed[f]
	STAT3	Luciferase reporter gene uHTS campaign	D. Frank[g]
	STAT5		
Protein/protein	EphB4-ephrinB2	FP uHTS campaign	P. Kuhn[b]
	HCV core homodimer	TR-FRET HTS assay development	D. Strosberg[d]
	NS5B/CYPB		
Protein/RNA	HIV Rev-RRE RNA	FRET uHTS campaign	J. Williamson[b]
Nuclear receptor	Estrogen receptor	Luciferase reporter gene HTS assay development	K. Nettles[d]
Orphan nuclear receptor	SF1 (NR5A1)	Luciferase (transient-transfection) reporter gene uHTS campaign	X. Li[h]
	RORα (NR1F1)		
Metalloproteinase	ADAMTS4	QFRET HTS assay development	G. Fields[i]
	MMP13	QFRET uHTS campaign	
β-Lactamase	VIM-2	Absorbance HTS assay development	P. Hodder[d]
	IMP-1		
Protein misfolding	Hsp70	Luciferase reporter gene HTS assay development	R. Morimoto[j]
	AL-09	QFRET HTS assay development	M. Ramirez-Alvarado[k]
Ubiquitin proteolysis	WEE1	Luciferase reporter gene HTS assay development	N. Ayad[d]
Proliferation/viability	Jurkat E6.1Cells	Luminescence uHTS campaign	P. Hodder[d]
Reductase	msr A	Absorbance HTS assay development	H. Weissbach[i]
Ion channel	Aquaporins (AQP)	Yeast-spheroplast HTS assay development	M. Yeager[b]
Stem cell proliferation	Notch	Luminescence (cell-based) HTS assay development	H. Petrie[d]

Abbreviations: FLIPR, fluorescence imaging plate reader; FP, fluorescence polarization; FRET, fluorescence resonance energy transfer; QFRET, quenched FRET; TETRA, brand name for a FLIPR system; TR-FRET, time-resolved FRET.

[a] Albany Medical College, Albany, New York
[b] Scripps Research
[c] Burnham Institute, Orlando, Florida
[d] Scripps Florida
[e] Mt. Sinai School of Medicine, New York, New York
[f] Burnham Institute, La Jolla, California
[g] Dana-Farber Cancer Institute, Boston, Massachusetts
[h] Orphagen Pharmaceuticals, San Diego, California
[i] Florida Atlantic University, Boca Raton, Florida
[j] Northwestern University, Chicago, Illinois
[k] Mayo Clinic, Rochester, Minnesota

will be useful as tools for characterizing gram-negative pathogens or as adjuvants in antibiotic therapy.

One of our goals is to develop HTS-ready assays suitable for rapid identification of compounds that modulate the activity of Ambler molecular class B (Bush-Jakoby-Medeiros group 3) metallo-β-lactamases, specifically the VIM-2 and IMP-1 enzymes. In preliminary research efforts, we have developed HTS-ready

fluorescence- and absorbance-based VIM-2 and IMP-1 inhibition assays. In collaboration with K.B. Sharpless, Department of Chemistry, we have screened a diverse click-chemistry library of compounds designed specifically to inhibit metallo-β-lactamases. Currently, we are developing several novel scaffolds that appear to be specific inhibitors.

PUBLICATIONS

Lauer-Fields, J.L., Spicer, T.P., Cudic, M., Burstein, G.D., Nagase, H., Hodder, P., Fields, G.B. Screening of potential ADAMTS-4 inhibitors utilizing a collagen-model FRET substrate. Anal. Biochem., *in press*.

Schröter, T., Minond, D., Weiser, A., Dao, C., Habel, J., Spicer, T., Chase, P., Baillargeon, P., Scampavia, L., Schürer, S., Chung, C., Mader, C., Southern, M., Tsinoremas, N., LoGrasso, P., Hodder, P. Comparison of miniaturized time-resolved fluorescence energy transfer and enzyme-coupled luciferase high-throughput screening assays to discover inhibitors of Rho-kinase II (ROCK-II). J. Biomol. Screen., *in press.*

Development of Protein Kinase Inhibitors

C. Liang, M. Koenig, P. Holmberg, Y. He

Our goal is to discover protein kinase inhibitors that can be used as therapeutic agents for the treatment of human diseases such as cancer and arthritis. Protein kinases are a class of enzymes that catalyze the transfer of the γ-phosphate from ATP to protein substrates. These enzymes play critical roles in signal transduction for a number of cellular functions. In particular, they regulate most of the hallmarks of cancer: cell proliferation, cell survival, cell motility/metastasis, cell cycle/division, and angiogenesis. Protein kinases are also implicated in inflammatory diseases such as arthritis and asthma. For these reasons, protein kinases are being investigated as valuable therapeutic targets by almost every pharmaceutical company, and according to estimates, about 25% of all current pharmaceutical research is devoted to these targets.

In the past year, we successfully completed the first drug discovery project in collaboration with Poniard Pharmaceuticals (formerly NeoRx Corporation), Seattle, Washington. The collaboration was then expanded to include D.D. Schlaepfer, Department of Immunology. Our goal was to identify lead compounds that would inhibit focal adhesion kinase. By April, we had exceeded the original goal; we have identified novel compounds with excellent potency and oral bioavailability. Preclinical studies are under way to identify potential clinical candidates. Provisional patent applications have been filed.

Pharmacology Services

L.H. Smith, A. Pachori, S. Khan, M. Lopez, D. Zadory

We provide comprehensive pharmacology services and expertise in support of drug discovery efforts for Scripps Research at both the Florida and California sites. We use cutting-edge technology and established models of disease to validate and confirm the efficacy of new chemical compounds and to assess the druglike characteristics of the compounds. Our disease models include tumor xenografts, hypertension, angiogenesis, hyperlipidemia, obesity induced via diet or genetic manipulation, diabetes, and Parkinson's disease. Studies of this kind require expertise in many scientific disciplines. Thus, we work with researchers in Medicinal Chemistry, Drug Metabolism and Pharmacokinetics, and Cell Biology and the leaders of each project team to move drug discovery projects from the test tube to in vivo models and, ultimately, to clinical trials.