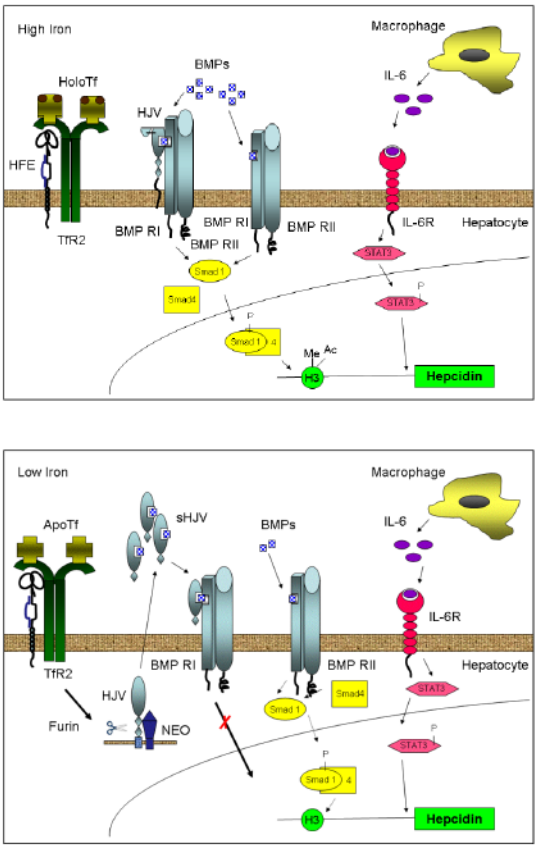
Molecular and Experimental Medicine

Schematic representation of transcriptional regulation of hepcidin by iron, bone morphogenetic proteins (BMPs), and inflammatory cytokines. High transferrin saturation conditions (HoloTf) are sensed by transferrin receptor 2 (TfR2). Hemojuvelin (HJV) acts as a co-receptor for the BMP receptors. Autocrine and paracrine activation of the BMP receptors by BMPs 2, 4, 7, and 9 result in activation of the SMAD1/4 signaling pathway, which upregulates hepcidin expression by binding to BMP-responsive elements present in both the distal and the proximal promoter. Binding of SMAD1/4 might facilitate histone 3 (H3) demethylation and deacetylation at these sites. Under low transferrin saturation conditions (ApoTf), a furinlike protease is activated, which cleaves HJV, thereby reducing the BMP-mediated regulation of hepcidin. Inflammatory cytokines, including IL-6, activate STAT3, which upregulates hepcidin by binding to a STAT-responsive element located in the proximal promoter. Work done in the laboratory of Ernest Beutler, M.D., professor.



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Chairman's Overview

On September 30, 1978 (my 50th birthday), I accepted the invitation of Frank Dixon and Charlie Edwards to serve as chairman of what was then the Department of Clinical Research. It consisted of a small and somewhat dispirited faculty, representing largely the core of clinicians who also performed research in the laboratory. Frank Dixon did not look favorably on this type of research, which he sometimes derisively referred to as "go to the lab Friday afternoon and kill a rat." Nonetheless, the group had some talented scientists, and I regarded the opportunity to build a strong department as a very worthwhile challenge. A few years later, Frank Dixon decided to consolidate the Departments of Biochemistry, Cell Biology, and Clinical Research into what we then called the Department of Basic and Clinical Research, and later the Department of Molecular and Experimental Medicine. It has been my pleasure and privilege to lead this department for the past 30 years.

Ernest Beutler, M.D.

The accomplishments of the members of the department have been a great source of pride to me, and together we have survived some difficult times. We have created outstanding leaders who have gone on to enhance scientific programs at other institutions, and their leadership reflects well on our department. Over the years, some of the faculty grew in scientific stature to the point at which they were elected to the National Academy of Sciences, including Dennis Carson, Frank Chisari, and Bernie Babior. The stature of the faculty grew so that we could even attract those already in the academy to join us: Peter Vogt and Floyd Bloom. More important, we were able to build a group of scientists with sufficient commonality of interest so that they were mutually supportive but with interests sufficiently eclectic that they were friends and colleagues—not competitors. Our interactions were strengthened by the construction of a building of sufficient size as to hold most of our faculty.

When I first joined our faculty, the retirement age, at least for administrative positions, was 65 years. This has now all changed, and when I reached the age of 70, Richard Lerner was kind enough to ask the Scripps Research Board of Directors to appoint me as a permanent chairman, a gesture that I truly appreciated. Although I felt that my age did not affect my ability either to perform research work or to manage the department as such, I was also aware of problems that might arise merely because of the age of the chairman. First of all, the younger faculty and fellows moving forward in their careers are helped by the endorsement of the chairman under whom they have worked. How long can they count on an elderly chairman? And the commitments that are made both to current members of the department and certainly to recruits must be based on mutual trust. Does not the chairman's expected longevity affect his ability to fulfill his promises?

For these reasons, it has always been my intention to retire, and I had set the age at a possibly too high 80 years. Richard Lerner has been very supportive and has resisted my earlier efforts to step down. But with the advent of my 80th birthday, we have reached an understanding that it would be best for the department and for Scripps Research to have an orderly transition to a new chairmanship for our department. It is very important to me personally that the department remain intact, and that I be allowed to continue to function as a professor with my own program in the department under the leadership of the new chairman.

After some discussions with Richard, I was delighted to learn that he had persuaded Jeffery Kelly to assume the chairmanship of the Department of Molecular and Experimental Medicine, effective September 30, 2008. Jeff and I have been good friends for many years now. In my view, Jeff is ideally suited to be the new chairman. Although trained as a chemist, his long-standing interests have been applications of problems in protein folding to human diseases, including several that represent major programs in our department. Several of our faculty,

including me, have collaborated with Jeff. I have found him to be imaginative and insightful and a collegial and generous collaborator. Moreover, Jeff's plans for expanding the department are very much along the lines that we have favored. Specifically, one of our major ongoing recruitments has been an outstanding scientist who is a leader in misfolding effects in the endoplasmic reticulum. This is one of the very areas in which Jeff is interested, and I believe that Jeff will have a much better chance of finalizing this important recruitment, than would an elderly chairman.

I am delighted that Jeff has agreed to take on this responsibility. I know that the department will flourish under his expert leadership. He will have my full support, and I trust that of the remainder of the faculty as well.

My most sincere thanks to the faculty of our department and the unstinting support they have given me and one another. It has been a great 30 years for me.

Investigators' Reports

Studies in Iron Homeostasis

E. Beutler, K. Crain, T. Gelbart, P. Lee, H. Peng, J. Truksa, J. Waalen

Iron is essential to all forms of life, but an excess of iron can be injurious to organisms, probably by facilitating the generation of free radicals. Consequently, all organisms have developed mechanisms for regulating the amount of iron that they obtain from the environment and the exchange of iron between storage depots and functional compartments (Fig. 1).

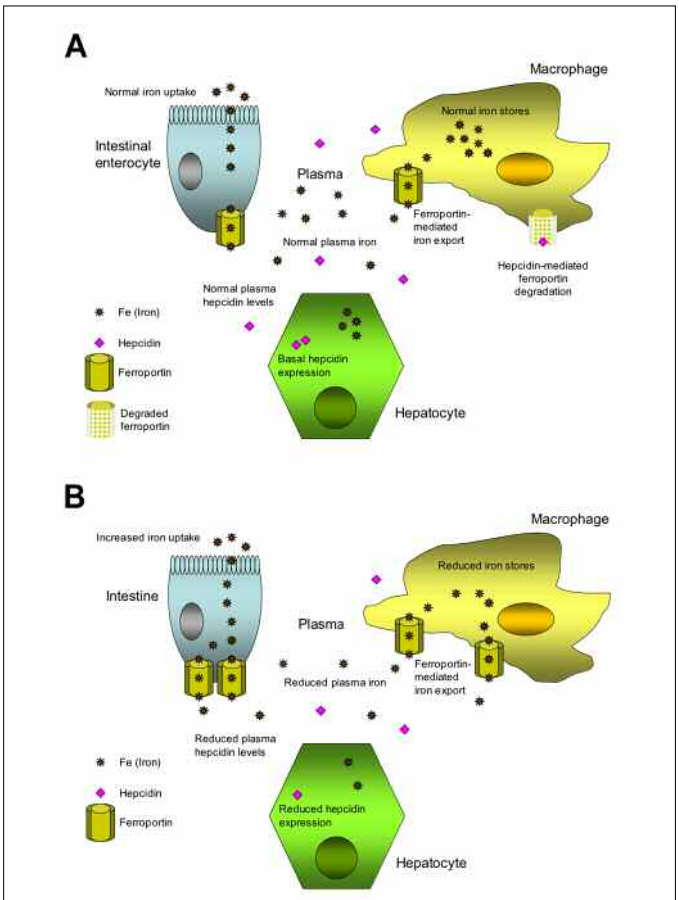

Fig. 1. Iron absorption under various conditions. A, Normal iron absorption. B, Iron deficiency. C, Iron overload. D, Anemia of inflammatory disease. E, Hemochromatosis.

In higher organisms, a 25 amino acid antimicrobial peptide, hepcidin, has emerged as the central regulator of iron homeostasis. The cognate receptor for hepcidin is ferroportin, an iron-transport protein that is required for intestinal cells to release their iron to the blood and for macrophages to release their iron stores. When hepcidin levels are high, the serum iron level decreases, and

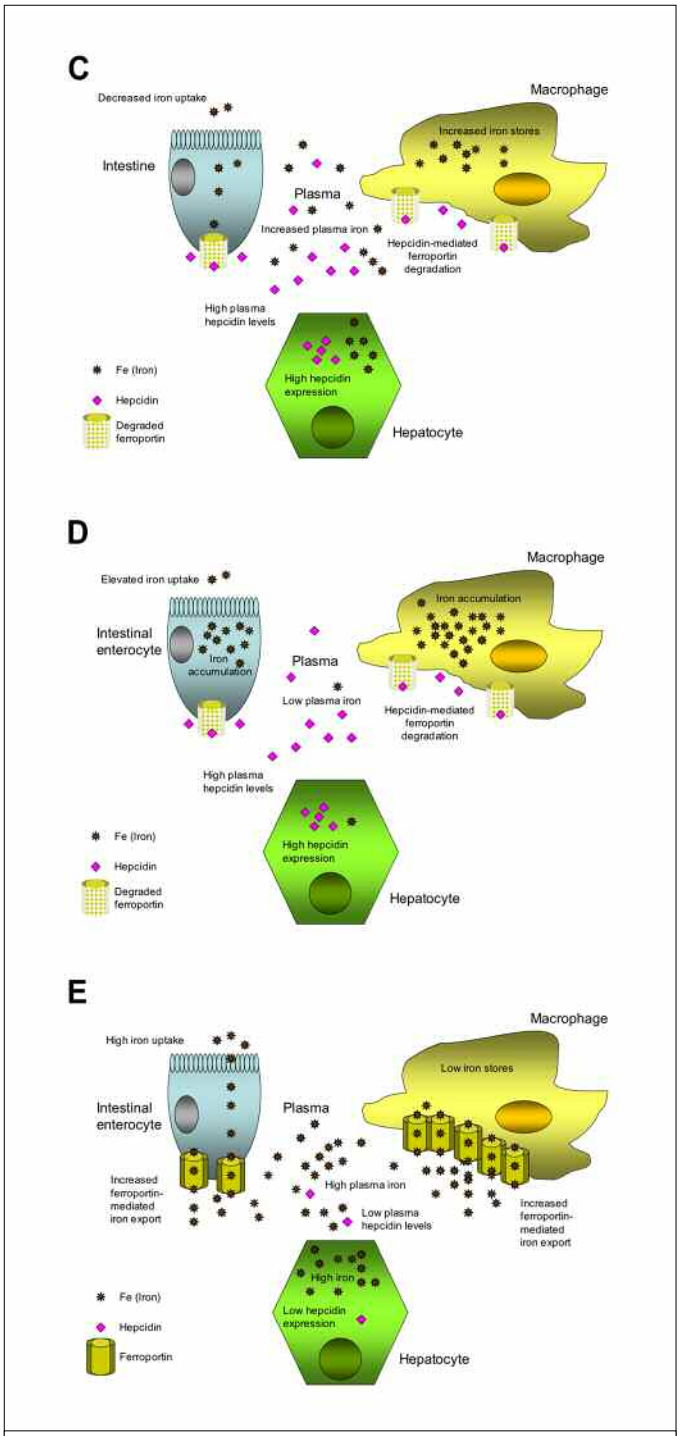

Fig. 1., continued.

intestinal iron absorption is diminished. Thus, overexpression of hepcidin results in iron deficiency. Conversely, when hepcidin levels are low, iron absorption from the gastrointestinal tract is facilitated, as is the release of iron from macrophages. This release leads to iron excess. Hepcidin is translated as a prohormone, prohepcidin, which undergoes cleavage to the active 25 amino acid peptide. Regulation of hepcidin appears to be largely or entirely transcriptional.

We have focused on the regulation of hepcidin transcription. Transcription is upregulated by the inflammatory cytokines IL-1 and IL-6, by bone morphogenetic proteins (BMPs) 2, 4, and 9, by overexpression of another iron-regulating protein, hemojuvelin, and in vivo, by iron. The regulation of hepcidin by iron is particularly important physiologically, but it is difficult to study because it does not occur in vitro. In last year's report, we described our development of an in vivo method for measuring hepcidin transcription that is based on the luminescence of a reporter in intact mice.

We are seeking to dissect the various parts of the promoter involved in the regulation of hepcidin transcription and the complex of transcription factors that are involved in this process. The transcription factors SMAD4, C/EBPα, and HNF4α have been implicated in the regulation of hepcidin. Animals that have a liver-specific deletion of the genes for SMAD4 and C/EBPα have significantly reduced hepcidin expression and hepatic iron accumulation. Animals that have a liver-specific deletion of the gene for HNF4α have significantly increased hepcidin expression. Chromosome immunoprecipitation has indicated that the transcription factor STAT3 binds the hepcidin promoter in response to IL-6. Although the STAT3-binding site has been mapped to the proximal promoter region, it was not known where SMAD4 and C/EBPα bind on the hepcidin promoter.

We previously reported that the BMP- and iron-responsive region of the hepcidin promoter mapped to a region 1.6–1.8 kb upstream of the start of translation. If hemojuvelin works through the BMP–BMP receptor pathway by activating SMAD1 and SMAD4, then we would expect that the hemojuvelin- and SMAD1/4-responsive region of the hepcidin promoter to localize to the 1.6- to 1.8-kb region. Such localization was indeed the case. Using the same approach that we used to map the BMP- and iron-responsive region of the hepcidin promoter, we found that the hemojuvelin- and SMAD-responsive region localized to the 1.6- to 1.8-kb region of the distal hepcidin promoter. Nevertheless, this region had no identifiable SMAD1/4-responsive motif.

Deletion analyses narrowed the BMP-responsive element primarily to a HNF4α/COUP transcription factor binding site, although neighboring motifs seemed to also contribute to the responsiveness. Deletion of the HNF4α/COUP site or the STAT site resulted in a significantly lower basal level expression of the hepcidin promoter. Binding of recombinant transcription factors to the HNF4α/COUP site verified that HNF4α bound to a probe containing the HNF4α/COUP motif. C/EBPα bound to a probe encompassing the MEL transcription factor binding motifs. These data suggest that transcriptional activators and repressors regulating hepcidin expression might assemble into a complex with the liver-specific transcription factors HNF4α and C/EBPα at the core. This notion is consistent with the observation that hepcidin is expressed predominantly in hepatocytes.

Bruce Beutler and his group, Department of Genetics, discovered a mutant strain of mice that was designated Mask because of a phenotype in which body hair is missing but facial hair remains. This phenotype is due to a splicing mutation in the *Tmprss6* gene, a gene that encodes a membrane serine protease. These mutant mice also have a severe microcytic anemia secondary to iron deficiency. Feeding the animals a diet very rich in iron or injecting them with iron corrected not only the anemia but also the loss of body hair. We were able to show that Mask mice maintained inappropriately high levels of hepatic hepcidin mRNA despite being severely iron deficient and anemic, both conditions that independently result normally in the suppression of hepcidin transcription. Moreover, in tissue culture cells subjected to stimuli that normally increase hepcidin, overexpression of *Tmprss6* inhibited the increase. We have shown that *Tmprss6* functions far downstream in the signaling sequence that stimulates hepcidin transcription and that it does so even when only a short fragment of the promoter sequence is present. Currently, studies are directed at better understanding the mechanism by which *Tmprss6* downregulates hepcidin.

Because underexpression of hepcidin produces iron deficiency in mice, we posited that some patients with iron deficiency anemia resistant to treatment with iron might have mutations of the human ortholog. We have already detected 2 families with hereditary iron deficiency in which mutations of *TMPRSS6* are present.

We have also continued to study clinical aspects of hereditary hemochromatosis. Although our study of the Kaiser Permanente Health Appraisal Clinic population clearly showed that the penetrance of hereditary hemochromatosis was extremely low, some people want to screen for this disorder. The approach that is generally used is to either perform DNA analysis to find homozygotes for the C282Y mutation of the *HFE* gene or to measure the serum transferrin saturation. But although the screening methods indicate that most or all of the patients are homozygous, only 1% to 2% of them will need treatment. We have therefore suggested

and tested an alternative approach, viz, the measurement of serum ferritin levels. This approach seemed attractive because it is only patients with serum ferritin levels of more than 1000 ng/mL who have cirrhosis of the liver, the main clinical manifestation of hemochromatosis. We found that among 29,699 white patients participating in the study, only 59 had serum ferritin levels of more than 1000 ng/mL. Of these, 24 had homozygous mutant or compound heterozygous *HFE* genotypes. In all but 5 of the other patients, the causes of elevated ferritin were excessive alcohol intake, cancer, or liver disease. Thus, we were able to show that screening for serum ferritin levels not only detects all of the hemochromatosis patients at risk for cirrhosis but also detects patients with other medical problems that may require attention.

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Pathogenesis of Late-Onset Genetic Diseases Related to Abnormalities of Protein Conformation

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We are studying a group of hereditary and sporadic human diseases, the transthyretin amyloidoses, that are the result of age-depen-

dent protein misfolding. The misfolded molecules deposit in the heart, kidneys, and peripheral nerves, producing organ-specific disease.

CLINICAL IMPACT OF THE TRANSTHYRETIN MUTATION VAL122ILE

We have completed our analysis of the transthyretin mutation Val122Ile, an amyloidogenic allele carried by 3%–4% of African American participants, in the Cardiovascular Heart Study, a study of a demographically selected representative population of individuals more than 65 years old. We confirmed our earlier findings that African American carriers of the allele who are more than 70 years old have a higher frequency of new-onset congestive heart failure and more features of cardiac amyloidosis than do controls matched for age, sex, and ethnic background.

Our collaboration with the investigators of the Arteriosclerosis Risk in Communities study has resulted in identification of more than 100 carriers of the amyloidogenic transthyretin allele. We compared the cardiac findings in the carriers with those in noncarriers and established that no discernible differences exist between carriers and noncarriers in the population studied (in which all the participants were less than 65 years old). These data reinforce the notion that clinical manifestations of this genetic disorder are not apparent before age 65 years and that the disease behaves as an autosomal dominant with age-dependent clinical penetrance.

In collaboration with the investigators in the Jackson Heart Study, in which part of the African American cohort of the Arteriosclerosis Risk in Communities study is being followed up, we are examining the cardiac status of the carriers of the Val122Ile allele to determine the frequency of clinical penetrance and the rate of its progression to establish the baseline parameters for a clinical trial of a compound that can inhibit the formation of transthyretin amyloid.

A MURINE MODEL OF TRANSTHYRETIN AMYLOIDOSIS

Our transgenic animal model of the transthyretin amyloidoses continues to give us insights into the biology of these diseases of protein structure. We have evolved a hypothesis that we have termed "chaperoning at a distance." We propose that the capacity of the chaperone/proteasome system in the cell synthesizing systemic amyloid precursors (the hepatocyte or the plasma cell) is what determines the extent and rate of amyloid deposition and organ compromise at distant sites. In a continuing collaboration with D.R. Salomon, Department of Molecular and Experimental Medicine, we have shown that pathologic tissue deposition of transthyretin in the hearts and kidneys of the transgenic animals results in different molecular pathways of injury and response that appear to be tissue specific. The molecular chaperoning response of the liver appears to play a critical role in sparing the heart from exposure to damaging protein aggregates. In collaboration with Dr. Salomon, J.W. Kelly, Department of Chemistry, and Teresa Coelho, Hospital General de Santo António, Oporto, Portugal, we will be extending our observations to patients with the hereditary forms of transthyretin amyloidosis.

TRANSTHYRETIN STRUCTURE AND DISEASE

In collaboration with Dr. Kelly and investigators at the Burnham Institute for Medical Research, La Jolla, California, we have shown that murine transthyretin, despite having a crystal structure almost identical to that of the human protein, is much more kinetically stable. This increased stability explains why mice do not get this form of systemic amyloidosis and why development of human transthyretin amyloidosis in transgenic animals carrying the human gene depends on overexpression of the human molecule to an extent sufficient to exceed the production of the endogenous mouse protein monomer subunits.

We have completed our initial set of studies in which we found that overexpression of human transthyretin suppresses the behavioral and neuropathologic phenotype that usually occurs in transgenic mouse models of Alzheimer's disease. We have initiated experiments to examine the in vivo mechanism of the effect to determine whether the pathway might be used therapeutically in humans with Alzheimer's disease.

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Exocytosis of Neutrophil Granules: Regulation by Rab27a Effectors

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Neutrophil granules contain secretory molecules that contribute to the implementation of all neutrophil functions. The molecular components that regulate the exocytosis of neutrophil granules have not been characterized. Using short interfering RNA gene targeting approaches and granulocytes from genetically modified mice, we characterized the Rab27a effectors JFC1/Slp1 and Munc13-4 as components of the exocytic machinery of granulocytes. Using total internal reflection fluorescence microscopy analysis, we showed that Rab27a and JFC1 colocalize in predocked and docked vesicles in granulocytes. Next, we showed that JFC1-downregulated granulocytes have impaired myeloperoxidase secretion. Using immunologic interference, we confirmed that JFC1 plays an important role in azurophilic granule exocytosis in human neutrophils. Interference with Rab27a but not JFC1 impaired secretion of matrix metalloproteinase 9 in neutrophils, suggesting that a different Rab27a effector modulates this process.

In similar studies, we confirmed that Munc13-4 regulates matrix metalloproteinase 9 secretion. Immunofluorescence analysis indicated that Munc13-4 localizes at secretory organelles in neutrophils. Using neutrophils from mice deficient in Munc13-4, we found that Munc13-4 plays a central role in the regulation of exocytosis of various sets of secretory organelles. However, mobilization of CD11b was not affected in neutrophils deficient in Munc13-4, indicating that secretory defects in these cells are limited to a selective group of exocytosable organelles.

Signaling Mechanisms for Neutrophil Exocytosis Activated by Toll-Like Receptors

S.D. Catz, A.A. Brzezinska, D.B. Munafo, B.A. Ellis, J.L. Johnson

The signaling mechanisms involved in the regulation of neutrophil exocytosis triggered by lipopolysaccharide are currently unknown. Using neutrophils from mice deficient in interleukin-1 receptor–associated kinase-4 (IRAK-4) and Toll–IL-1 receptor domain–containing adapter-inducing IFN-β (TRIF), we dissected the signaling pathways that control exocytosis. Neutrophils from mice deficient in IRAK-4 had marked defects in exocytosis of both peroxidase-negative and azurophilic granules in response to lipopolysaccharide. Contrarily, the exocytic response to lipopolysaccharide of neutrophils from mice deficient in TRIF did not differ from that of wild-type cells. The exocytosis of secretory organelles in neutrophils from mice deficient in IRAK-4 did not differ from that in neutrophils from wild-type mice when the 2 groups were stimulated with phorbol 12-myristate 13-acetate. Electron microscopy showed no morphologic abnormalities in the granules of neutrophils from mice deficient in IRAK-4, suggesting that the lack of an exocytic response to lipopolysaccharide is not due to developmental abnormalities.

Using pharmacologic inhibitors, we found that phosphatidylinositol-3′-kinase is essential for exocytosis of azurophilic granules but not for the mobilization of other neutrophil granules in response to lipopolysaccharide. Exocytosis of azurophilic granules in response to *Listeria monocytogenes* depended on phosphatidylinositol-3′-kinase but not on IRAK-4 activity, suggesting that alternative signaling pathways are activated when neutrophils from mice deficient in IRAK-4 are exposed to whole gram-positive bacteria. Our results indicate that IRAK-4 and phosphatidylinositol-3′-kinase are important regulatory components with different roles in the signaling pathways that control neutrophil exocytosis triggered by ligands for Toll-like receptors.

Regulation of Immune Responses to Transplants by Immunoregulatory Tissue Alloantigens and Vascular Cells

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Class I and class II MHC antigens are the primary barrier to acceptance of allografts. However, certain class I MHC antigens may also trigger regulatory immune responses. Thus, in humans, HLA-G, a nonpolymorphic class Ib HLA molecule, may mediate immunologic tolerance at sites of immune privilege, such as the anterior chamber of the eye, the testis, the thymus, and the cytotrophoblast.

Several explanations for the immunoregulatory functions of HLA-G have been considered. The limited polymorphism of HLA-G in humans may allow the recognition of tissues expressing high levels of this molecule as "self," thereby preventing the activation of autoreactive or alloreactive T cells and natural killer cells. Alternatively, HLA-G may foster the development of specific immunoregulatory lymphocytes capable of downregulating alloreactivity. Our previous finding that HLA-G is expressed in the human thymic medullary epithelium strongly supports both possibilities. Thus, the purpose of HLA-G expression in the thymic medulla may be to both (1) educate developing T cells to recognize HLA-G as self, and (2) induce the selection of HLA-G–specific immunoregulatory T-cell populations.

We are investigating the immune responses elicited by HLA-G in human thymocytes and peripheral T cells. Our goals are to dissect the molecular mechanisms of HLA-G immune functions and then use this information to bioengineer HLA-G expression in tissues suitable for transplantation. Particular emphasis is given to models of pancreatic islet transplantation for the treatment of diabetes. For this purpose, we have generated lines of human pancreatic cells expressing either low or high levels of membrane-bound or soluble recombinant HLA-G. These HLA-G^{low} and HLA-G^{high} cell lines are useful tools for studies of HLA-G functions both in vitro and in vivo in models of cell transplantation.

Another promising line of research for the bioengineering of cells for transplantation was provided by our recent work on the identification of endothelial cell progenitors in human cord blood. While studying human thymopoiesis in a chimeric mouse model in which mice are reconstituted with human cord blood, we discovered that cord blood hematopoietic stem cells engrafted in these mice not only reconstituted the bone marrow and repopulated the human thymic grafts but also contributed to the formation of new blood vessels at sites of wound healing.

We are characterizing this population of putative endothelial progenitors to be used as another target-cell type for transplantation. Specifically, we have defined some of the growth and differentiation signals required for the expansion ex vivo of human bone marrow–derived endothelial progenitors. Currently, using a mouse model of bone marrow–derived vasculogenesis, we are characterizing immunologic and angiogenic properties of bone marrow–derived vascular cells. Furthermore, in collaboration with B.E. Torbett, Department of Molecular and Experimental Medicine, and M. Friedlander, Department of Cell Biology, we are using mice with severe combined immunodeficiency transplanted with human cells to test the functional impact of human bone marrow–derived vasculogenic cells on angiogenic processes in models of retinal neovascularization.

Ultimately, cotransplantation of HLA-G–transduced allogeneic tissue along with HLA-G–bioengineered endothelial cell progenitors and/or enhancing recruitment of bone marrow–derived endothelium with intrinsic immunomodulatory properties may endow tissue grafts with an additional level of immunoprotection. In addition, proangiogenic functions associated with bone marrow–derived vasculogenic cells may be used to regulate tissue repair during neovascularization. These approaches may be useful in developing novel strategies for accelerating tissue healing and inducing immunologic tolerance after transplantation.

Essential Pathways in Tumor Progression

T.F. Deuel, Y. Chang, P. Perez-Pinera

We previously identified and cloned pleiotrophin, an 18-kD cytokine with diverse roles in development, inflammation, and the development and progression of malignant tumors. Pleiotrophin signals by inactivating the receptor protein tyrosine

phosphatase (RPTP) β/ζ. Inactivation of RPTPβ/ζ leads to increased levels of tyrosine phosphorylation of the substrates of RPTPβ/ζ by tyrosine kinases that phosphorylate the same sites that normally are dephosphorylated by RPTPβ/ζ in cells not stimulated with pleiotrophin. Known substrates of RPTPβ/ζ include β-catenin, β-adducin, Fyn, histone deacetylase 2, anaplastic lymphoma kinase (ALK), and TrkA, the receptor of nerve growth factor. Through this unique signaling mechanism, pleiotrophin regulates levels of tyrosine phosphorylation of important proteins in different cellular systems, including tyrosine kinases known to be "drivers" essential to growth and survival of cancer cells.

Pleiotrophin is an oncogene. Inhibition of the pleiotrophin-RPTPβ/ζ signaling pathway in different human cancer cells can prevent the growth of xenografts of the cells in nude mice. Thus, the pleiotrophin-RPTPβ/ζ signaling pathway is essential to the growth and survival of the cancer cells. These results indicate that the pleiotrophin-RPTPβ/ζ signaling pathway may be an important therapeutic target in those cancers in which the pathway is essential to the growth and survival of the tumor cells. We are using genomic and proteomic screens to identify the proteins in downstream pathways activated by pleiotrophin that are essential to tumor cell growth and survival.

ACTIVATED ALK AND EPITHELIAL-MESENCHYMAL TRANSITION

We found that ALK activated through the pleiotrophin RPTPβ/ζ signaling pathway phosphorylates β-catenin. This phosphorylation is associated with loss of association of β-catenin with E-cadherin, loss of cell-cell adhesion, and initiation of an epithelial-mesenchymal transition, suggesting a critical role for ALK in the regulation of cytoskeletal structure and function. Mass spectrometry indicated that the tyrosine phosphorylated in β-catenin in pleiotrophin-stimulated cells is the tyrosine at position 333. Phosphotyrosine 333 phosphorylated by ALK is dephosphorylated by RPTPβ/ζ and thus is a substrate of RPTPβ/ζ. The crystal structure of the β-catenin–E-cadherin complex previously published indicates that the phosphate group on phosphoserine 692 in E-cadherin forms hydrogen bonds with the hydroxyl group of tyrosine 333 and establishes charge interactions with 2 flanking lysine residues of β-catenin; phosphorylation of tyrosine 333 destroys this interaction. The juxtaposition of the alternative β-catenin tyrosine 333 and E-cadherin serine 692 thus establishes a "switch" termed "mutually excluding phosphates" that functions as an important mechanism to regulate cell-cell adhesion.

These results show that the pleiotrophin-RPTPβ/ζ signaling pathway is a critical regulator of cell-cell adhesion and that an activated pleiotrophin-RPTPβ/ζ signaling pathway induces an epithelial-mesenchymal transition. The findings suggest that the activated pleiotrophin-RPTPβ/ζ signaling pathway in cancer cells mediates cell-cell adhesion and an epithelial-mesenchymal transition in the cells. These 2 functions are vital to cancer cells and are needed for the aggressive phenotype.

EXPRESSION OF ALK AND RPTPβ/ζ IN HUMAN BREAST CANCER CELLS

We showed that ALK is strongly expressed in different histologic subtypes of human breast cancer. Furthermore, ALK is expressed in both nuclei and cytoplasm and in the "dotted" pattern characteristic of the ALK fusion proteins in anaplastic large cell lymphoma. RPTPβ/ζ is also expressed in each of the 63 human breast cancers that express ALK and in 10 additional samples of human breast cancer. These results clearly indicate that the pleiotrophin-RPTPβ/ζ signaling pathway is expressed in human breast cancers and support the possibility that activated ALK may be important in these cancers. The data suggest ALK is potentially activated through the pleiotrophin-RPTPβ/ζ signaling pathway or, alternatively, is an activated fusion protein that stimulates progression of breast cancer in humans.

REVERSAL OF THE MALIGNANT PHENOTYPE OF GLIOBLASTOMA CELLS BY BLOCKADE OF ANGIOTENSIN II SIGNALING

Angiotensin is the principal vasoactive peptide of the renin-angiotensin system. Angiotensin signals through binding to 2 major receptors: AT1 and AT2. The components of the renin-angiotensin system are expressed in human glioblastomas, leading to the hypothesis that inhibition of the system might be a way to treat glioblastomas. We showed that U87MG glioblastoma cells express the angiotensin II receptor AT1, angiotensinogen, and angiotensin-converting enzyme. We also examined the impact of pharmacologic inhibition of the renin-angiotensin signaling pathway. Captopril markedly impaired growth of U87MG cell xenografts in vitro and in vivo and tumor angiogenesis in nude mice. We also found that the pleiotrophin-RPTPβ/ζ signaling pathway also is activated in these glioblastoma cells. Blockade of the pleiotrophin-RPTPβ/ζ signaling pathway in these human glioblastoma cells equally impaired growth of U87MG cell xenografts in vitro and in vivo and tumor angiogenesis in nude mice. The data establish that constitutive endogenous angiotensin signaling and signal-

ing through the pleiotrophin-RPTPβ/ζ signaling pathway are critical factors in the progression of U87MG glioblastoma cells to the highly malignant phenotype. Our findings support the possibility that both the angiotensin II signaling pathway and the pleiotrophin-RPTPβ/ζ signaling pathway are essential to the growth and survival of glioblastoma cells and are potential targets for interventional therapy of glioblastoma.

BLOCKADE OF ENDOGENOUS PLEIOTROPHIN SIGNALING AND PROGRESSION OF BREAST CANCER TO MORE AGGRESSIVE PHENOTYPES

Previously, we found that constitutive expression of *Ptn*, the gene for pleiotrophin, introduced by mouse mammary tumor virus (MMTV) into polyoma virus middle T (PyMT)-*Ptn* double transgenic mice stimulates progression of breast cancers to the aggressive "scirrhous" carcinoma phenotype through remodeling of the tumor microenvironment; marked increases in new collagens, elastin, and blood vessels; and striking increases in stromal fibroblasts associated with the more aggressive breast cancer phenotypes. Surprisingly, we found that MMTV-PyMT mouse breast cancer cells expressed significant levels of endogenous *Ptn*, suggesting the endogenous *Ptn* also contributed to progression of breast cancer. We also introduced a dominant-negative *Ptn* driven by MMTV (MMTV-PTN 1-40) into MMTV-PyMT mice. No foci of scirrhous carcinoma were found in breast cancers from MMTV-PyMT-PTN 1-40 mice, suggesting that constitutive *Ptn* expression is required for this aggressive breast cancer phenotype in mice. Progression of early-stage to late-stage carcinomas also was delayed. Tumor angiogenesis was significantly lacking, and the angiogenic foci were not associated with the foci of breast cancer. The number of stromal fibroblasts in MMTV-PyMT-PTN 1-40 mouse breast cancers was markedly reduced, and new collagens and elastin were limited and not associated with the cancer cells.

These data further support the conclusion that constitutive *Ptn* expression stimulates progression of breast cancers in mice to a more aggressive phenotype. Deregulated *Ptn* expression is probably a critical determinant of the more aggressive phenotype. The findings indicate that the pleiotrophin-RPTPβ/ζ signaling pathway is essential for tumor progression in transgenic mouse breast cancer models.

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Mechanisms of Brain Metastasis

B. Felding-Habermann, J.S. Krueger, D. O'Sullivan, M. Lorger, K. Staflin, A. Fernandez-Santidrian, J.S. Forsyth, M. O'Neal, K.D. Janda, R. Jandial,* J.F. Kroener**

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Cancers of the breast and lung as well as melanoma often metastasize to the brain, leading to a poor prognosis and a median survival of less than 6 months. The difficulty of treating patients with brain metastases is exacerbated because single initial lesions can spread throughout the brain and CNS. Our goal is to develop molecular and functional profiles of the events that underlie entry of tumor cells into the brain and the survival and dissemination of these cells within the brain tissue. Information from this project will help identify targets for effective prevention and treatment of brain metastasis.

MODEL OF THE HUMAN BLOOD-BRAIN BARRIER

After establishing a model of the human blood-brain barrier, we identified variants of a metastatic human tumor cell line that either penetrate the barrier easily but grow slowly once inside the brain or that penetrate the barrier poorly but grow extremely rapidly within the brain tissue. After generating cDNA expression libraries from these cells and using reciprocal transduction, we will screen for clones with increased transmigration across the blood-brain barrier or increased intracranial growth to help us identify genes and pathways that promote tumor metastasis to the brain.

CONTROL OF METASTATIC GROWTH IN THE BRAIN BY INTEGRIN ACTIVATION AND CHANGES IN TUMOR CELL METABOLISM

Selection of brain-homing human breast cancer and melanoma cell variants in immunodeficient mice served as a basis for functional analyses and proteomic profiling. The results revealed that expression of the

integrin $\alpha_v\beta_3$, an adhesion receptor, in a high-affinity state and alterations in cellular energy metabolism can critically promote intracranial growth of brain metastatic cells (Fig. 1). We are exploring autocrine and paracrine

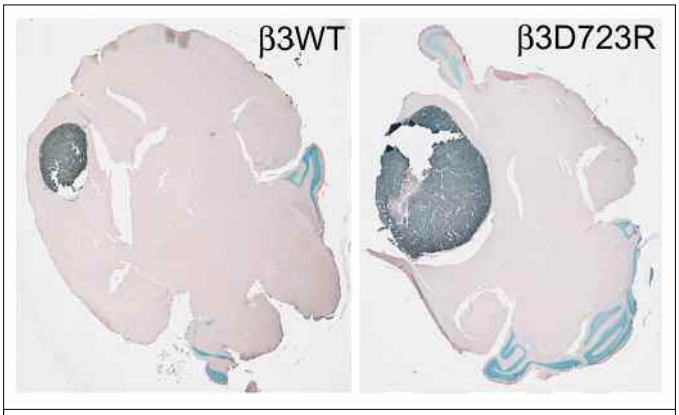

Fig. 1. Metastatic tumor cell growth in the brain can be controlled by the activation state of integrin $\alpha_v\beta_3$. Metastatic lesions developing from tumor cells expressing constitutively activated $\alpha_v\beta_3$ (β3D723R; right) grow much more rapidly than do cells expressing the nonactivated receptor (β3WT; left).

mechanisms through which the activated form of $\alpha_v\beta_3$ confers a survival and growth advantage to tumor cells within the brain microenvironment. Regulation of apoptosis and proliferation of brain metastatic cells, through deviation from the "classical" tumor cell metabolism of enhanced glycolysis and suppressed oxidative phosphorylation (Warburg effect) toward intensified rates of glucose utilization in oxidative pathways, distinguish the brain-homing tumor cells from their extracranial counterparts and may reflect an adaptation, or a predisposition, for survival in the brain microenvironment.

HYALURONIDASE 1 AS A MEDIATOR OF METASTATIC DISSEMINATION WITHIN THE CNS

The unique extracellular matrix of the brain is incompletely understood, but its structural organization is based on long stands of very high molecular weight hyaluronic acid decorated with aggregated proteins and proteoglycans to form networks of cross-linked fibers. Using gene expression profiling, we found that metastatic variants derived from lung, bone, and brain metastases from orthotopically implanted human tumor cells in mice with severe combined immunodeficiency have a significant upregulation of hyaluronidase 1, the enzyme that degrades hyaluronic acid. Silencing the gene for hyaluronidase 1 strongly diminished the ability of intracranially implanted tumor cells to disseminate within the brain tissue and CNS. These data imply that tumor-derived hyaluronidase 1 can degrade hyaluronic acid within the brain extracellular matrix and mediate intracranial migration of cancer cells. Hyaluronidase 1 may be a potential target for inhibiting tumor cell invasion of the brain.

The results from our engineered cell models are compared with the results of experiments done with cell lines derived from fresh surgical specimens of metastatic lesions from breast cancer patients. A clinical relevance of molecular pathways, validated in these systems, would help identify targets for new therapies.

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Protein Oxidation, Oxidative Stress, and Disease

J.S. Friedman, F.M. Martin, A.C. Takeda, K. Soldau

We study protein carbonylation, a type of oxidative damage that is increased in inflammatory disorders, neurodegenerative disease, and aging. Carbonyls arise when lysine or arginine residues in a protein interact with reactive oxygen species. We have developed a mouse model in which loss of the antioxidant protein superoxide dismutase 2 (SOD2) causes protein oxidative damage within developing and mature red blood cells. As a consequence, mice with blood cells lacking SOD2 are anemic. This mouse model has a human disease counterpart, sideroblastic anemia, that occurs most often in patients with the bone marrow disorder myelodysplastic syndrome. To link the murine model with the human disease, we have analyzed bone marrow samples provided by several collaborators at Scripps Clinic and around the United States.

As shown in Figure 1, we devised a method for purifying diseased cells (called sideroblasts) from bone marrow by taking advantage of the iron content of sideroblasts. As shown in the figure, when a suspension of bone marrow cells is passed over a magnetic column, iron-containing cells are retained, providing a simple method for purification. Characterization of purified sideroblasts indicated that cells from both SOD2-deficient mice and patients with sideroblastic anemia produce high levels of reactive oxygen species and contain high levels of carbonylated protein. This finding suggests that oxidative stress plays an important role in the development of sideroblastic anemia.

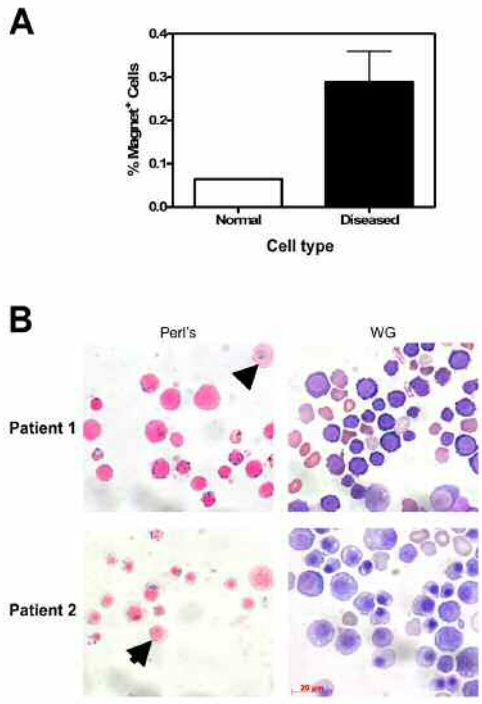

Fig. 1. Magnetic purification of bone marrow cells from patients with ringed sideroblasts allowed purification of iron-laden erythroid precursors. A, Bar graph shows the percentage of magnetically purified cells in samples of normal bone marrow cells (0.08%; n = 2) and samples of bone marrow cells from patients with sideroblastic anemia (0.29%; n = 8). B, Microscopic analysis showed that purified cells were predominantly sideroblasts, with occasional siderocytes. Magnetically purified cells were stained by using Perl's iron technique (left) or the Wright-Giemsa technique (right). Representative samples from 2 patients are shown. The arrow points to a ringed sideroblast; the arrowhead, to a siderocyte. The purified cell populations were overwhelmingly erythroid progenitors.

To better understand the role of protein carbonylation in disease processes, we have developed a novel approach to quantify and identify oxidized proteins. We use multiple fluorophores (e.g., Cy-2, Cy-3, and Cy-5) that can form derivatives of carbonylated proteins via a hydrazide moiety. Individual samples are labeled with distinct fluorophores, and then samples are combined in a multiplex fashion for comparative 2-dimensional gel analysis. This method is similar to the comparative proteomic method termed difference gel electrophoresis, or DIGE, and thus we coined the term oxo-DIGE.

With support from the National Center for Research Resources, during the past year we have purchased equipment and set up a new capability within the Proteomics Core that includes DIGE imaging, software for analysis of differential protein expression, a robotic spot picker, and an automatic digester to interface with existing mass spectrometry capabilities. Using this integrated facility, we are identifying and quantitatively comparing oxidized proteins by using blood cells from normal and SOD2-deficient mice and purified bone marrow cells from patients with sideroblastic anemia. Our goal is to define the oxidized proteome. We are investigating whether oxidant-labile proteins can be used as biomarkers of disease, indices of response to therapy, or targets for therapeutic intervention.

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The Antithrombotic, Anti-inflammatory and Antiapoptotic Protein C Pathway

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Plasma proteins involving the coagulation pathways, the fibrinolysis pathways, and multiple anticoagulant mechanisms prevent bleeding while avoiding harmful blood clots. We are studying the roles of various plasma proteins in preventing bleeding and avoiding blood clots. We are also investigating the protein C pathways, which provide beneficial antithrombotic, anti-inflammatory, and cytoprotective activities.

ANTIAPOPTOTIC AND CYTOPROTECTIVE EFFECTS OF ACTIVATED PROTEIN C

Protein C is a vitamin K–dependent plasma protein zymogen. Genetic mild or severe deficiencies in protein C are linked with risk for venous thrombosis or neonatal purpura fulminans, respectively. Studies in past decades showed that activated protein C (APC) inactivates coagulation factors Va and VIIIa to downregulate the generation of thrombin. In more recent basic and preclinical research, scientists have characterized the direct cytoprotective effects of APC that involve

alterations in gene expression profiles, anti-inflammatory and antiapoptotic activities, and stabilization of the endothelial barrier. These actions generally require the endothelial cell protein C receptor and protease-activated receptor 1. Because of these direct cytoprotective actions, APC reduces mortality in murine models of endotoxemia and severe sepsis and provides neuroprotective benefits in murine models of ischemic stroke. Furthermore, APC reduces mortality in patients with severe sepsis.

Although much remains to be clarified about the mechanisms of the direct effects of APC on various cell types, we have shown that the molecular features of APC that determine its antithrombotic action are partially distinct from those that provide cytoprotection. We have engineered recombinant APC variants with selective reduction or retention of either anticoagulant or cytoprotective activities. Such variants can provide relatively enhanced levels of either cytoprotective or anticoagulant activities for various therapeutic applications and for mechanistic studies in various animal models of injury. We speculate that APC variants with reduced anticoagulant action but normal cytoprotective actions might reduce the risk for bleeding (because of the attenuated anticoagulant activity) while reducing mortality via direct cytoprotective effects on cells.

In collaboration with H. Weiler, Milwaukee Blood Center, we showed that mortality reduction in lipopolysaccharide-induced endotoxemia in mice requires the enzymatic active site of APC, endothelial cell protein C receptor, and protease-activated receptor 1, highlighting a key role for the cytoprotective actions of APC. A recombinant APC variant with normal signaling but less than 10% anticoagulant activity, 5Ala-APC, was as effective as wild-type APC in reducing mortality after lipopolysaccharide challenge, and 5Ala-APC enhanced the survival of mice with peritonitis induced by gram-positive or gram-negative bacteria or polymicrobial peritoneal sepsis triggered by implanting a stent in the ascending colon. These findings showed that the efficacy of APC in severe sepsis is predominantly based on cell signaling dependent on endothelial cell protein C receptor and protease-activated receptor 1 and that APC variants with normal cell signaling but reduced anticoagulant activities retain efficacy while reducing the risk of bleeding.

NEUROPROTECTIVE ACTIVITIES OF APC

Stroke is a major cause of morbidity and mortality, and few therapeutic options are available for ischemic stroke. Thrombolytic therapy with tissue plasminogen activator (tPA) is an option, but use of tPA is problematic because of its neurotoxic effects, including induction of bleeding. The results of our collaboration with B. Zlokovic, University of Rochester, in studies of the multiple neuroprotective activities of APC led the National Institutes of Health to fund a new clinical trial of APC for ischemic stroke.

Studies with murine stroke models indicated that APC reduces bleeding induced by tPa, which is a thrombolytic agent. More recent studies indicated that APC stimulates both the growth of new blood vessels (angiogenesis) and the mobilization of neuronal cells to promote repair after ischemic stroke. We are now deciphering the mechanisms of these neuroprotective effects of APC.

INFLUENCE OF LIPIDS ON BLOOD COAGULATION

In other studies, we are focusing on how lipoproteins and lipids of minor abundance in the blood might regulate blood clotting. Dyslipoproteinemia involving low levels of high-density lipoprotein is linked to venous thrombosis in young men and to recurrence of venous thrombosis in patients who have experienced a previous episode of unprovoked venous thrombosis. Plasma cholesteryl ester transfer protein (CETP) modulates metabolism of high-density lipoprotein, and some lipoproteins can affect blood coagulation by having either procoagulant or anticoagulant effects.

We evaluated relationships between the mass of CETP and blood coagulability in plasma samples from 39 healthy adults. For clotting initiated by dilute tissue factor or factor XIa, clotting times were significantly correlated with CETP antigen levels. Thus, coagulation initiated by either the extrinsic or the intrinsic coagulation pathway is positively correlated with CETP plasma levels. When added to plasma, a recombinant CETP preparation dose dependently shortened factor Xa–1-stage clotting times, indicating that the preparation augmented procoagulant activity in plasma. In reaction mixtures containing purified factors Xa and Va and prothrombin, the recombinant CETP preparation dose dependently increased prothrombin activation, suggesting that CETP specifically enhances prothrombinase activity. Thus, our data highlight a previously unknown positive relationship between CETP plasma levels and blood coagulability that might be related to risks for thrombotic events.

Antithrombotic Mechanisms

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We focus on anticoagulant plasma proteins that regulate blood coagulation and prevent thrombosis. Protein S, protein Z, and protein Z–dependent protease inhibitor are all inhibitors of factor Xa and other targets. Heterozygous deficiency of protein S or protein Z is associated with increased risk for stroke and venous thrombosis, illustrating the importance of these proteins. Knowledge of the molecular mechanisms of the anticoagulant activities of these proteins may lead to new antithrombotic therapies.

Protein S is a cofactor for the anticoagulant activated protein C (APC). However, we found that protein S also has APC-independent, direct anticoagulant (PS-direct) activity via inhibition of factors Xa and Va. We validated PS-direct activity in a baboon model of thrombosis, in which protein S effectively curbed platelet and fibrin deposition and formation of thrombin-antithrombin complexes. These effects occurred even when protein C activation was blocked, indicating APC independence. Thus, protein S has promise as an antithrombotic drug.

We found that protein S isolated by commonly used conventional methods had much weaker PS-direct activity than did immunoaffinity-purified protein S in the baboon thrombosis model. In clotting assays measuring endogenous thrombin potential, plasma depleted of protein S had a shorter lag time before thrombin generation than did normal plasma containing protein S. Immunoaffinity-purified protein S reconstituted protein S–depleted plasma and increased the lag in thrombin generation, but conventionally purified protein S did not. We investigated the source of variability in PS-direct activity of protein S to reveal a feature of the protein that is crucial for PS-direct activity.

Immunoaffinity-purified protein S contained 1 zinc atom per molecule, whereas conventionally purified protein S contained 0–0.3 atoms of zinc per molecule. Zinc content correlated with PS-direct activity. Removal of zinc from active protein S by treatment with the zinc chelator phenanthroline resulted in loss of PS-direct activity. Most PS-direct activity was regained after subsequent incubation with zinc at pH 2.7. Conversely, inactive protein S gained PS-direct activity and zinc content when incubated with zinc at pH 2.7 or in 6 M urea.

We postulated a zinc-binding site at the interface of 2 laminin G–type domains at the C-terminal region of protein S that are homologous to sex hormone binding globulin domains. Zinc may be essential for interdomain interactions required for PS-direct activity, and site-directed mutagenesis may confirm this location. An antibody directed against a site at the interdomain interface efficiently recognized only protein S that contained zinc, suggesting differences in conformation between zinc-deficient and zinc-containing protein S.

We are defining which molecular interactions of protein S are affected by zinc occupancy. Factor Xa binds to zinc-containing protein S with 20 times greater affinity than it does to zinc-deficient protein S. Factor Va also binds with greater affinity to zinc-containing protein S. However, tissue factor pathway inhibitor binds almost equally well to either type of protein S, suggesting a binding site for the inhibitor on protein S outside the zinc-containing region. APC cofactor activity of protein S is also unaffected by zinc content. The N-terminal region of protein S is largely responsible for APC cofactor activity, further suggesting that zinc is located in the C-terminal region. Detailed study of the activities and binding of protein S to these several molecules as a function of the zinc content is under way. Zinc may partially regulate PS-direct activity.

On a related topic, 10 neonates with life-threatening thrombosis have been reported who had compound heterozygous protein S deficiency. Remarkably, we identified a young adult with compound heterozygous protein S deficiency who experienced recurrent thrombosis that did not begin until he was 10 years old. His protein S levels were 10% of normal. All protein S was in the form of complexes composed of protein S and C4b-binding protein, a form with poor APC cofactor activity. Thus, PS-direct activity may have been partially protective in this young man.

Structure and Function of Coagulation Cofactors

A.J. Gale, T. Cramer, J. Cruz, D. Rozenshteyn

Coagulation factors Va and VIIIa are highly homologous cofactors of the serine proteases factor Xa and factor IXa, respectively. These cofactors are the primary targets of activated protein C (APC) in its downregulation of the procoagulant pathway. To

investigate mechanisms of cofactor function and regulation, we have used 3-dimensional models to direct mutagenesis of these cofactors and APC. For example, we engineered disulfide bonds between domains in both factor Va and factor VIIIa. In factor Va, the disulfide bond facilitated investigation of the mechanisms of inactivation of factor Va by APC cleavage.

Factor VIIIa, however, is inactivated by 2 mechanisms. Thrombin activation of factor VIII results in a heterotrimer that consists of the A1 subunit, the A2 subunit, and the light chain. Both spontaneous dissociation of the A2 subunit and proteolytic cleavage of factor VIIIa by APC inactivate factor VIIIa. Hemophilia A, a deficiency of factor VIII, is treated by infusions of recombinant factor VIII. But the usefulness of factor VIII is limited because it is unstable after activation by thrombin as a result of the spontaneous dissociation of the A2 subunit.

We generated 2 factor VIII mutants in which 2 newly introduced cysteine residues form a de novo disulfide bridge that cross-links the A2 and A3 domains. These interdomain disulfides prevent the spontaneous dissociation of the A2 subunit. These variants may provide an improved therapy for hemophilia A. We are using both in vivo assays in mice and ex vivo assays in whole blood and plasma to evaluate the therapeutic potential of these stabilized variants. One disulfide variant clearly has improved functional properties both ex vivo and in vivo.

We are also studying the mechanisms of inactivation of factor VIIIa and factor Va by APC. We are investigating the APC-cofactor activity of factor V during APC proteolysis of factor VIIIa and factor Va. To investigate mechanisms of factor VIIIa inactivation, we are using disulfide-stabilized variants as tools alone and in combination with mutants of APC cleavage sites. We are also using these tools to investigate the roles of factor VIIIa dissociation and APC proteolysis in the in vivo inactivation of factor VIIIa in mice. In other studies, we are investigating modulation of the functions of factor VIII and factor V by the intrinsic pathway of coagulation. Additionally, we are interested in the role that *N*-linked glycosylation plays in factor VIII function and metabolism. A goal of all these studies is to use lessons learned about factor VIII stability, inactivation, and metabolism to improve recombinant factor VIII as a therapeutic agent for treatment of hemophilia.

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Preserving Vision in Patients With Macular Degeneration

A. Hanneken, J. Johnson

We are developing new treatments for patients with the dry form of macular degeneration. The focus of our research is maintaining the health of aging retinal pigment epithelial (RPE) cells and preserving the health of retinal photoreceptors. We have identified these targets to preserve and improve visual function in patients with this condition.

We are searching for new compounds that protect the retina from the type of injury thought to initiate the death of RPE cells in macular degeneration, identifying neuroprotective agents that preserve and maintain normal retinal function in patients with macular degeneration, and identifying new models for how visual function is reduced in macular degeneration and how function can be restored.

Macular degeneration is a multifactorial disease that is linked to both environmental and genetic risk

factors. It is associated with an injury known as oxidative stress, which is caused by an overproduction of reactive oxygen species (ROS), or byproducts of oxygen metabolism. High concentrations of these compounds can overwhelm the body's natural defense mechanisms and lead to cell injury and death. We have developed a screening assay to identify compounds that can protect RPE cells from the damage induced by ROS. In this assay, RPE cells are exposed to bright white or blue light in the presence of various photosensitizers, including lipofuscin, which accumulates in RPE cells with age. The levels of ROS in the RPE cells are measured before and after exposure to various compounds of interest. Typically, the concentration of ROS in RPE cells is high, and the concentration decreases if the compounds tested are protective. Figure 1 shows the reduction of ROS in

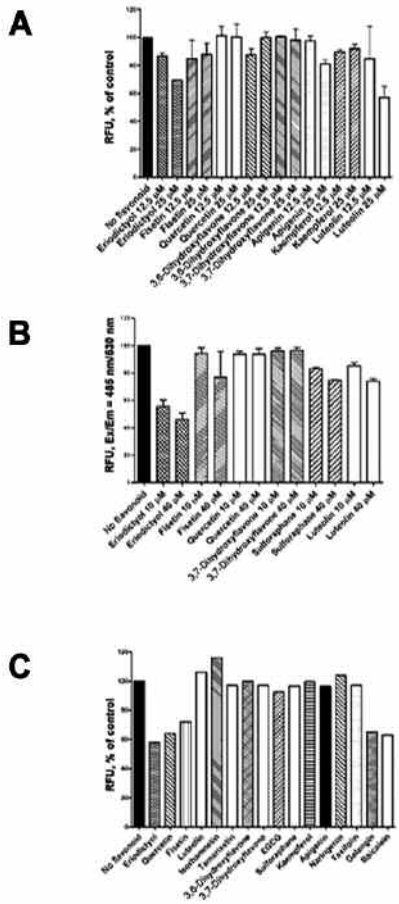

Fig. 1. ROS production in human RPE cells after exposure to A2E (a component of lipofuscin) and light (A), all-*trans* retinal and light (B), and hematoporphyrin IX and light (C) is decreased by flavonoids and other compounds. EGCG = (–)-epigallocatechin gallate; RFU = relative fluorescence units.

RPE cells exposed to a variety of bioflavonoids and other compounds that we have identified with this screening assay. We are validating and expanding these results. We hope to identify additional compounds and combinations of compounds that have greater potency and efficacy than the ones we have identified so far.

In other projects, we are studying new approaches to block the degeneration of photoreceptor cells in rods and cones. We have novel models that we are testing to understand the biochemical mechanisms that lead to neuronal degeneration in macular degeneration, and we will be testing some of these ideas in upcoming clinical trials.

This research is the result of a partnership formed between the Scripps Mericos Eye Institute and Scripps Research to bring together the promise of biomedical research and the practice of medicine.

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Computational Analysis of Molecular Specificity in 2-Component Signaling

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In both prokaryotes and eukaryotes, a large number of pathways with proteins with identical structural folds are used to interpret and propagate vastly different signals specific for unique targets. A central question in understanding signal transduction is how a signaling protein distinguishes its true partner from the much larger number of similar partners present in the cell. In a collaboration between Scripps Research and the University of California, San Diego, we have developed a sequence-based method, independent of structural considerations, for identifying specificity-determining interactions between proteins for which genomic data indicate a large number of examples of functionally coupled pairs. This method was applied to the phosphotransfer domains of bacterial 2-component signaling proteins. Using this method, we identified a network of residue-residue interactions and generated a 3-dimensional structure consistent with the exemplary

cocrystal structure obtained for the Spo0B-Spo0F complex and mutation studies of this pair. We also identified an interaction network that links long-distance interactions with pair specificity of 2-component signaling proteins.

The method provides a simple scoring procedure that can be used to identify potential cross-phosphorylation between functional pairs and to assign orphan 2-component signaling proteins with no known mate to their signaling partners. Although, currently, we are applying the method to 2-component signal transduction systems for which structural and mutational data allow proof of principle, the method may generate interaction structures for less-characterized protein pairs if sufficient functional pairs exist in genomic data.

Molecular Dynamics of Response Regulators

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Recognition specificity by sensor kinases in 2-component signal transduction depends on the composition of amino acid residues in the surface of the response regulator with which the sensor interacts. Part of this surface of response regulators consists of several dynamic loops generated by the folding of strands and helices to form the core of the regulators. Using nuclear magnetic resonance chemical-shift perturbation experiments with specific mutants of the Spo0F response regulator, we found that the conformation of the $\beta4$-$\alpha4$ loop and the $\alpha4$ helix dictates kinase specificity. The presentation of the loop, and therefore kinase recognition, can be altered by perturbations of core residues that propagate to the surface. These results further support our earlier hypothesis that molecular recognition processes are significantly influenced by intraprotein communication networks in the core of the response regulator. These networks are critical in providing the precise surface to the appropriate sensor kinase in signal transduction, for which hundreds of structurally similar proteins have evolved from gene duplication to carry out as many different signaling functions within the cell.

Regulatory Connections Between Cell Division and Cell Wall Synthesis

J.A. Hoch, T. Fukushima, H. Szurmant

Rapid growth and cell division in bacteria require precise coordination of several complex processes, such as DNA synthesis, septum formation, and cell wall synthesis and restructuring. The mechanism by which this coordination is accomplished remains unknown. Recent results have shown that the YycFG 2-component signal transduction system is an essential component of this regulation. The transcription factor YycF regulates the balance of synthesis of autolysins, which are required for cell wall restructuring during cell division, and autolysin inhibitors, which prevent cell wall degradation in nondividing conditions. This balance is determined by the phosphorylation level of YycF, which is in turn controlled by the activity of the sensor kinase YycG. The key to understanding this coordination is determining what controls the activity of YycG.

Our immunofluorescence studies on the cellular location of the YycG sensor kinase revealed that this kinase is located at the division septum in dividing cells. Furthermore, transcription studies have shown that YycG is activated to phosphorylate YycF only when YycG is present at the division septum. Thus, coordination of septation and cell wall restructuring required for cell division is accomplished by signals acquired by YycG at the division septum.

Sensor Kinases and Control of Sporulation in *Bacillus subtilis*

J.A. Hoch, T.J. Muff, A.E. Dago II

In sporulating bacteria, a redundancy of sensor kinases capable of initiating sporulation is the rule. It is thought that multiple sensor kinases allow the bacterial cell to respond to a wide variety of signals in different environments and physiologic conditions. However, in the laboratory, attempts to discover the activating signals of multiple kinases have been unsuccessful. We have generated a new series of bacterial strains to overcome this obstacle. Using a recently developed marker-

less deletion strategy, we produced strains that place sporulation under the control of single kinases. These strains allow experimentation in controlled conditions to identify which kinases are being activated and to focus on the activating signals.

Regulation of Virulence Gene Expression in *Bacillus anthracis*

M. Perego, J.A. Hoch, F. Scaramozzino, C. Bongiorni, A. Wilson

Bacillus anthracis is the spore-forming gram-positive soil bacterium that is the etiologic agent of anthrax. The 2 major virulence determinants of *B anthracis* are the multicomponent toxin and the capsule, which are expressed from genes on 2 virulence plasmids: pXO1 and pXO2, respectively. Expression of toxin and capsule genes is strictly dependent on the product of another pXO1-encoded gene, *atxA*. Activity of AtxA, the transcription factor encoded by *atxA*, is regulated by phosphorylation-dephosphorylation of 2 histidine residues through an undefined mechanism that we are investigating. Using newly adapted transposon mutagenesis vectors for *B anthracis*, we initiated studies to identify additional factors that affect the expression of virulence genes. Our results have revealed genes for the heme–cytochrome *c* pathway involved in the expression of the toxin gene. Additionally, genes involved in the carbon dioxide/bicarbonate–dependent induction of toxin transcription have been identified. The mechanisms by which the products of these genes affect the expression of *B anthracis* virulence genes are now being investigated.

Negative Regulation of Development in Bacilli

M. Perego, C. Bongiorni

Initiation of spore formation in gram-positive bacilli is regulated by the phosphorelay signal transduction system. Multiple positive and negative signals are integrated by the phosphorelay through the opposing activities of histidine protein kinases and aspartyl phosphate phosphatases. The phosphatases belong to 2 families: Rap and Spo0E. Rap proteins act as negative regulators of the initiation of sporulation by dephosphorylating the Spo0F response regulator intermediate of the phosphorelay; the Spo0E proteins act as negative regulators of the phosphorelay by dephosphorylating the Spo0A response regulator and transcription factor for the initiation of sporulation.

A structure-function analysis of the Spo0E protein was carried out by analyzing a series of alanine substitution mutant proteins in the conserved signature motif centered on the sequence serine–glutamine–glutamic acid–leucine–aspartic acid. By means of in vivo analysis, biochemical assays, and fluorescence resonance energy transfer spectroscopy, we found that the aspartate residue at position 43 is critical in Spo0E catalytic activity. We have proposed that this residue may function in a manner similar to the one described for the catalytic mechanisms of nucleotidase members of the haloacid dehalogenase family. These proteins use an aspartyl nucleophile as their common catalytic strategy, and the active site of haloacid dehalogenase proteins has the same geometry and identity as the conserved amino acids in the active site of response regulators such as Spo0A.

Signal Transduction in *Enterococcus faecalis*

M. Perego

Enterococci are commensal bacteria within the intestinal tract in mammals but also can cause disease in compromised hosts. The acquisition of resistance to multiple antibiotics by enterococci makes infections caused by these microorganisms clinically challenging. The ability of the bacteria to adapt and respond to different environmental stimuli, including the host environment, led my group to investigate the role of 2-component signal transduction in the physiology and pathogenesis of *Enterococcus faecalis*.

We identified 17 2-component systems consisting of a sensory histidine kinase and a cognate response regulator. Among them, the *fsr* system was involved in the formation of biofilms through regulation of expression of the gene that encodes the zinc metalloprotease gelatinase. The ability of *E faecalis* to form biofilms increases the ability of the microorganism to colonize humans and persist at sites of infection.

Biofilms are bacterial communities that grow as surface-attached aggregates encased in an exopolymer

matrix. A deletion of the gene encoding gelatinase results in a strain strongly affected in biofilm growth, thus pointing to this enzyme as a target for antibacterial intervention. Gelatinase is synthesized as a precursor 509 amino acid polypeptide that is subject to 2 processing events that cleave the N-terminal 192 amino acids to produce the secreted active enzyme. Our studies have indicated that production of fully active gelatinase also requires a processing event at the C-terminal end. This processing is autocatalytic and results in the loss of the terminal 14 amino acids. The processing of the C-terminal tail of gelatinase may have a regulatory role in cell division and cell lysis through regulation of autolysin activity.

Transmembrane Helix Complexes in 2-Component Signaling

H. Szurmant, C.L. Brooks III, J.A. Hoch

Bacteria adjust to environmental conditions by using 2-component signal transduction systems. Because the proteins in the systems regulate important functions in bacteria and are not present in mammalian genomes, they have been recognized as a promising antibacterial drug target. Some of these systems are essential for cell viability. An example is the YycFG system that is conserved in gram-positive bacteria, such as the important pathogens *Staphylococcus aureus* and *Streptococcus pneumoniae*.

The YycFG system plays a role in regulating cell division and cell wall homeostatic processes. The input sensed by this system remains unknown. Our goal is to identify these signals and the mechanism by which they lead to activation. In a transposon mutagenesis approach, we identified 2 proteins, YycH and YycI, with individual transmembrane helices involved in regulating the activity of the transmembrane YycG sensor histidine kinase. The role of these proteins has now been further analyzed by using structural, computational, and mutagenic approaches.

Structures of soluble parts of both proteins revealed a novel fold that precluded insights into the molecular mechanism of activity. On the basis of the structures, we did further mutagenesis studies. Site-directed mutagenesis on conserved residues within YycH and YycI surprisingly had little effect on the activity of the proteins. Deletion of entire individual domains similarly had no effect, and truncation studies ultimately revealed that the N-terminal transmembrane helices of YycH and YycI were sufficient to modulate the activity of the YycG kinase.

Because modulation is achieved at the transmembrane level, we concluded that the YycGHI complex is an attractive model for studies of protein helix interaction within a membrane environment. Because transmembrane complexes are not readily amenable to experimental structural resolution, we used replica exchange molecular dynamics calculations combined with an implicit membrane model in collaboration with C.L. Brooks, Department of Molecular Biology, to gain insights into the structure of the transmembrane helix complex. A model derived from these simulations was the basis for mutagenesis studies of the transmembrane helix regions. The results of the mutagenesis studies proved remarkably consistent with the computational model.

We are using localization, crystallization, and protein-interaction approaches to gain further insights into the signals detected by the YycGHI complex and the mechanisms of activation of the signal transduction system.

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Cytochrome P450: Regulation, Structure, and Function

E.F. Johnson, N. Bumpus, M.-H. Hsu, R.L. Reynald, S. Sansen, Ü. Savas

Enzymes in the cytochrome P450 superfamily primarily serve 2 purposes in human physiology. Some P450s catalyze specific biotransformations in autocrine, paracrine, and endocrine signal transduction pathways; others play defensive roles by converting toxic compounds to less toxic and more soluble products that are more easily excreted than are the parent compounds. Each xenobiotic-metabolizing P450 generally oxidizes structurally diverse substrates, leading to a wide-ranging protective capacity for elimination of toxic chemicals. Often these enzymes are induced in response to exposure to chemicals or altered physiologic states. We wish to understand how the structural diversity and transcriptional regulation of P450s that metabolize xenobiotics contribute to a person's ability to avoid the adverse effects of environmental chemicals or alter the clearance and bioavailability of therapeutic drugs.

Although extensive information on the conditional expression of P450 genes in various experimental animal species is available, in humans the transcriptional responses of P450 genes to environmental stimuli and to physiologic changes are poorly understood. To address this problem, we use human cell lines, primary cultures of human cells, and transgenic mice to study mechanisms that regulate human family 4 P450 genes. These genes encode enzymes that are involved in both signal transduction and the metabolism of endogenous lipids and xenobiotics. Studies with cell lines are providing new information about endocrine and autocrine signal transduction pathways that govern the conditional expression of these genes in response to nutritional, hormonal, and xenobiotic signals.

Research is in progress to test whether more complex physiologic conditions such as pregnancy or caloric restriction alter the expression of the human enzymes in transgenic mice. We found that peroxisome proliferator–activated receptor α (PPARα), a nuclear receptor, has a role in the elevated expression of the human gene for CYP4A11 in response to fasting and administration of hypolipidemic drugs. Additionally, expression of the human transgene is significantly diminished in animals that lack the gene for PPARα, indicating a critical role for this receptor in the maintenance of normal levels of CYP4A11 expression (Fig. 1). Several studies indicate that a relatively common genetic variant of CYP4A11 with diminished functional activity is a risk factor for hypertension. On the basis of our results, we anticipate that PPARα agonists could counter this deficiency by increasing the expression of CYP4A11, and conversely, pathologic conditions that diminish PPARα activity could further diminish the protective effects of this enzyme.

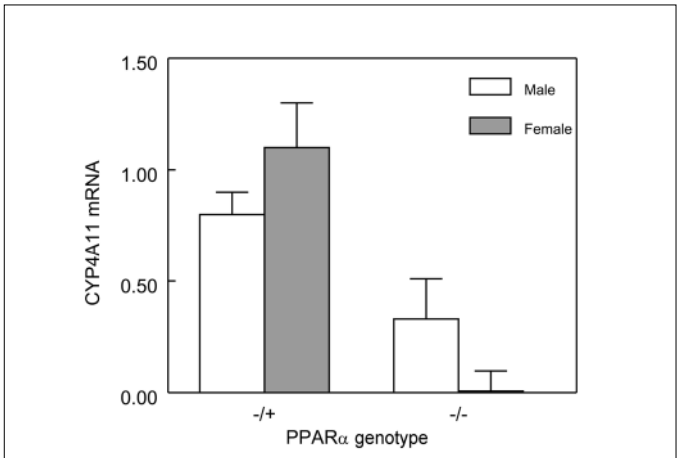

Fig. 1. Expression of human CYP4A11 mRNA in livers from male or female transgenic mice that are either heterologous (–/+) or homozygous (–/–) carriers of a disabled gene for PPARα. In the absence of PPARα, expression of the transgene is dramatically diminished in female mice and less extensively in male mice.

In collaboration with C.D. Stout, Department of Molecular Biology, we are defining the atomic structures of human P450 enzymes to understand the structural basis for the broad yet unique catalytic selectivity of each enzyme. This information can be used to better understand oxidation of drugs and toxins and the potential for metabolic drug-drug interactions. Toxicity and poor metabolic properties are significant barriers to the development of new drugs.

Mammalian P450s are tethered to the endoplasmic reticulum by a transmembrane segment at the amino terminus and by additional interactions of the catalytic domain with the cytoplasmic side of the membrane. Although membrane proteins are difficult to crystallize, we developed methods to express, purify, and crystallize genetically modified mammalian P450s that retain a native catalytic domain. Using this approach, we have determined the atomic structures of several of the most important human drug-metabolizing P450s: 1A2, 2A6, 2C8, 2C9, and 3A4.

To better understand key determinants of substrate binding to these enzymes, we are characterizing the

structures of the enzymes with bound substrate molecules. Substrates bind in an internal cavity, where they are positioned near the heme prosthetic group, which reduces molecular oxygen to generate the oxidant that combines with the substrate to form the product. For P450 2C8, pharmacophore models suggest that therapeutic drugs that are oxidized by this enzyme often have anionic groups roughly 12 Å from the site of oxidation. To better understand how these observations are related to the binding of substrates to the enzyme, we determined structures for P450 2C8 with 3 anionic substrates—9-*cis*-retinoic acid and the drugs troglitazone (Rezulin) and montelukast (Singulair)—and with the neutral drug felodipine (Plendil) bound to the enzyme. The results indicate that the pharmacophore models oversimplify the role of the anionic moiety, because 2 distinct modes of binding are evident that position the anionic group in 2 different parts of the active-site cavity. Moreover, our studies also indicate that 2 of the relatively small retinoic acid molecules bind to the enzyme at the same time. One molecule is positioned for oxidation; the second binds in a more distal part of the active-site cavity, where it secures the first retinoic acid molecule for oxidation. Multiple substrate occupancy generally leads to complex kinetic properties. Overall, these findings provide a clearer picture of the substrate interactions with P450 2C8 that determine the contribution of the enzyme to drug metabolism.

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Matrix Metalloproteinases in Peripheral Blood in Alzheimer's Disease

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Alzheimer's disease is the most common age-related neurodegenerative disorder. Patients with the disease experience progressive deterioration of cognitive function, particularly functions related to memory. Both genetic and environmental factors seem to play a role in the onset of the disease. One pathologic hallmark of Alzheimer's disease is the formation and extracellular aggregation of amyloid β-peptide (Aβ) in senile plaques and cerebral blood vessels. Aβ seems to play a key role in the pathogenesis of Alzheimer's disease; all mutations that underlie familial cases of the disease are implicated in the processing and accumulation of this peptide. The mechanism of Aβ generation is rather well known, but its catabolism and degradation are not well understood.

Matrix metalloproteinases (MMPs) can degrade components of the extracellular matrix in a variety of physiologic and pathophysiologic conditions such as stroke, intracerebral hemorrhage, and multiple sclerosis. Growing evidence indicates that MMPs play an important role in the pathogenesis of Alzheimer's disease and may be involved in the processing pathway of Aβ. Whether their functions in the pathogenesis of Alzheimer's disease are protective or destructive is not known.

In a pilot study, we compared the activity of MMP-2, MMP-3, MMP-9, and MMP-10 in plasma from patients with Alzheimer's disease with the activity of the enzymes in plasma from healthy volunteers (controls). On the basis of prior evidence of alterations in the genes that encode MMPs in patients with Alzheimer's disease, we hypothesized that the 2 groups would have significant differences in the activity of circulating MMPs.

From patients treated at the Central Institute of Mental Health, Mannheim, Germany, we recruited 8 women and 6 men 56 to 78 years old (median, 67.4 years) who had Alzheimer's disease. Disease duration in these patients varied from 0.5 to 6 years (median, 3 years). The sex- and age-matched control group consisted of 8 women and 6 men 52 to 74 years old (median, 66.5 years) who had no history of ischemic stroke, recent head trauma, or major cardiac, renal,

hepatic, or malignant disease. The study was approved by the ethics committee of the Medical School, University of Heidelberg, and all patients or their relatives gave informed consent.

Venous blood samples were drawn from each subject at time of admission into the study. Activity levels of MMP-2, MMP-3, MMP-9, and MMP-10 were assessed by using zymography. We found no significant differences between the 2 groups in the activity of MMP-2 or MMP-10. In contrast, compared with the control group, the patients with Alzheimer's disease had significant increases in the activity of MMP-3 and significant decreases in the activity of MMP-9.

Figure 1 shows scatterplot matrices for pairwise activity levels of plasma MMP-2, MMP-3, MMP-9, and MMP-10 for both groups. Among the patients with Alzheimer's disease, correlations of absolute magnitude of 0.30 or greater were observed for MMP-2 vs MMP-9 (r = 0.43), MMP-2 vs MMP-10 (r = 0.33), MMP-2 vs MMP-10 (r = –0.58), and MMP-9 vs MMP-10 (r = –0.38). In contrast, all pairwise correlations among MMP-2, MMP-3, MMP-9, and MMP-10 in the controls were of absolute magnitude 0.26 or less. Among the patients with Alzheimer's disease, activity levels of MMP-2, MMP-3, MMP-9, and MMP-10 did not appear strongly related to duration of disease; all correlations were of absolute magnitude 0.30 or less.

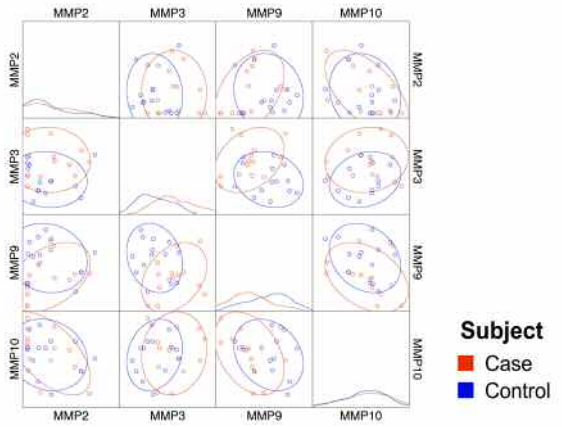

Fig. 1. Scatterplot matrices of the plasma activity levels of MMP-2, MMP-3, MMP-9, and MMP-10 from 14 patients with Alzheimer's disease (cases) and 14 matched controls. Pairwise activity levels are shown in the off-diagonal cells, along with 75% confidence ellipses separately for the patients with Alzheimer's disease and the controls. Separate density displays (frequency polygons) for the 2 groups are given in the diagonal cells. Compared with the controls, patients with Alzheimer's disease have significant increases in MMP-3 activity and significant decreases in MMP-9 activity.

How should these findings be interpreted? At autopsy, MMP-9 has been detected in regions near senile plaques in the brains of patients who had Alzheimer's disease, and this enzyme can degrade Aβ in vitro, suggesting a protective role for MMP-9 in Alzheimer's disease. We speculate that the lack of MMP-9 to cleave the Aβ could be one reason for amyloid aggregation in the brains of patients with Alzheimer's disease. In contrast, the augmented activity of MMP-3 might reflect growth factors and inflammatory cytokines that regulate MMP expression. For example, previous studies have shown elevated expression of inflammatory cytokines in the brains of patients with Alzheimer's disease, and this elevated expression could be responsible for the release of MMP-3. Also, treatment with Aβ can increase the production of MMP-3 in cultured astrocytes, and this effect may also occur in vivo. In summary, our findings suggest a potential role of MMPs in the pathogenesis of Alzheimer's disease.

Molecular Genetics of Hemostasis and Thrombosis

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THE MOUSE MODIFIER OF HEMOSTASIS LOCUS

Inactivation of *Gp6*, the gene for mouse platelet glycoprotein VI (GPVI), causes the anticipated defects in platelet response to collagen in vitro. However, $Gp6^{-/-}$ littermates have a dichotomous phenotype in vivo. Some $Gp6^{-/-}$ mice have normal bleeding times and normal rates of in vivo thrombus formation, whereas other $Gp6^{-/-}$ littermates have extremely prolonged bleeding times and do not form consolidated thrombi. The elimination of *Gp6* creates a "sensitized" murine model

in which the penetrance of the bleeding phenotype depends on genetic background, and modifier genes can be characterized.

We identified a dominant locus, *Modifier of hemostasis (Mh)*, in linkage disequilibrium with the bleeding phenotype, mapping an 8-Mb locus of chromosome 4. One of the candidate genes in *Mh* is *Klf4*, the gene for Kruppel-like factor 4, an important transcription factor widely expressed by key cellular elements of the vasculature, including endothelial cells, mural cells, and fibroblasts. A strain-related difference in *Klf4* expression correlates precisely with the in vivo bleeding/thrombosis phenotype. Expression of GPVI varies 5-fold in humans and is also implicated in abnormalities of human thrombosis. Further characterization of *Mh* and the composite candidate genes (e.g., *Klf4*) responsible for the bleeding/thrombotic phenotype will make an important contribution to understanding the in vivo regulation of hemostasis.

TRANSCRIPTIONAL AND EPIGENETIC REGULATION OF THE INTEGRIN COLLAGEN RECEPTOR LOCUS *ITGA1-PELO-ITGA2*

The human genes for integrin subunits α_1 (*ITGA1*) and β_2 (*ITGA2*) are adjacent to each other on chromosome 5 (5q11.2). *ITGA1* lies only 32 kb upstream from *ITGA2*, which probably arose by ancestral duplication of *ITGA1*. Selective suppression of *ITGA1* within the megakaryocyte lineage is due to locally restricted modifications in *ITGA1* transcription initiated by megakaryocyte differentiation from precursors. Suppression is highly localized and specific; the physically proximal *ITGA2* and even the gene *PELO*, which is embedded within intron 1 of *ITGA1*, are not affected. Methylation of cytosines within the dinucleotide sequence CpG is a common mechanism of transcriptional suppression in vertebrates, which can be involved in the establishment and maintenance of cell type–specific gene expression, and methylation patterns of tissue-specific genes differ unequivocally from tissue to tissue.

We found that CpG methylation plays a role in coordinated suppression of *ITGA1* during human megakaryocyte differentiation. We also found that the onset of CpG methylation of the *ITGA1* promoter is rapid, occurring in a coordinated fashion with the active CpG demethylation of other megakaryocyte-specific genes, such as *Gp6*.

ATYPICAL CONGENITAL AMEGAKARYOCYTIC THROMBOCYTOPENIA WITH IMPAIRED EXPRESSION OF PLATELET GPVI

Severe thrombocytopenia and megakaryocytopenia are hallmarks of congenital amegakaryocytic thrombocytopenia (CAMT). Mutations of *MPL*, the gene for Mpl, the thrombopoietin receptor, are the known cause of CAMT, and the lack of response to thrombopoietin is a diagnostic criterion. We have characterized the first case of CAMT not caused by a *MPL* mutation.

In this patient, platelets are devoid of Mpl protein, and thrombopoietin-induced phosphorylation of Janus tyrosine kinase 2 does not occur. The patient has a unique and severe reduction in platelet GPVI but normal levels of all other platelet-specific glycoproteins. Further sequencing eliminated other potential causes for the lack of Mpl, such as mutations in the downstream signal transduction intermediates Janus tyrosine kinase 2 or the transcription factor AML1. These manifestations could be caused by a single genetic defect that affects expression of both the kinase and the transcription factor. Alternatively, a defect in thrombopoietin signaling might be directly related to the decreased expression of GPVI. We favor this second explanation, because we recently reported that thrombopoietin can directly initiate active CpG demethylation during human megakaryocyte differentiation in vitro, resulting in the transcription of *Gp6*. Thus, this case of atypical CAMT is the first in vivo confirmation of this epigenetic mechanism that initiates megakaryocyte (platelet) GPVI expression.

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Adult Stem Cells in Articular Cartilage

S. Grogan, M. Lotz

Recent findings suggest that mesenchymal stem cells may be present in articular cartilage. We analyzed the spatial organization of these progenitor cells and their involvement in tissue homeostasis and in the development of osteoarthritis. Mesenchymal stem cells throughout normal and osteoarthritic human articular cartilage were examined by using immunohis-

tochemistry and flow cytometry with antibodies to 3 markers for mesenchymal stem cells: Stro-1, Notch-1, and vascular cell adhesion molecule-1. To document chondrogenic activities, we stained for Sox9, a transcriptional regulator of chondrocyte-specific genes. DNA microarrays were used to detect differences in progenitor markers in the cartilage zones and between normal and osteoarthritic tissues.

The general staining patterns for the 3 markers in normal and osteoarthritic cartilage were similar. In normal cartilage, more than 68% of cells located within the superficial zone were positive for the stem cell markers; the percentage decreased to approximately 32% in the middle and deep zones. In nonfibrillated cartilage, compared with staining in normal tissue, staining for the markers was reduced in the superficial zone and increased in the middle zone in osteoarthritic tissue; in fibrillated cartilage, staining in the deep zone was greater in normal tissue than in osteoarthritic tissue. Most cells in clusters of proliferating cells characteristic of osteoarthritic cartilage were positive for the stem cell markers. Sox9 staining was nuclear only in normal cartilage; cluster cells showed both nuclear and cytoplasmic staining. Results of flow cytometry and DNA arrays supported zone-specific and osteoarthritis-related differences in the expression of stem cell markers.

These findings indicate that a surprisingly high number of cells express markers for progenitor cells throughout cartilage, with a greater proportion localized in the superficial zone and the upper part of the middle zone. The increased presence of progenitor markers in osteoarthritis cell clusters implicates the involvement of these cells in the abnormal cell activation and differentiation characteristic of osteoarthritis.

Rho Kinase and Mechanotransduction in Chondrocytes

D. Haudenschild, M. Lotz, D.D. D'Lima

Mechanical forces regulate chondrocyte proliferation, survival, differentiation, gene expression, and biosynthetic responses. The type and duration of mechanical stimulation determine the outcome of the cellular responses, which in their extreme manifestations can range from cell proliferation to cell death and from matrix formation to matrix destruction. We showed earlier that dynamic compression of agarose-embedded chondrocytes induced remodeling of the actin cytoskeletal that depended on the activation of Rho kinase. A key regulator of cartilage formation is the transcription factor Sox9, which directly controls the expression of the major proteoglycans and collagens that make up the cartilage extracellular matrix, including type II collagen and aggrecan. The activity of Sox9 is controlled through various posttranslational modifications, including phosphorylation, which can affect the cellular localization and DNA-binding affinity of the factor.

Rho kinase has profound effects on the chondrocyte actin cytoskeleton, which is instrumental in determining the chondrocyte phenotype. Pharmacologic inhibition of Rho kinase alters chondrogenic differentiation via unknown mechanisms. How Rho kinase is linked to altered chondrocyte gene expression remains unknown, and a direct interaction between Rho kinase and Sox9 has not been reported. We found that Sox9 contains a consensus phosphorylation site for Rho kinase. Rho kinase directly phosphorylates Sox9 at serine 181 in vitro, and the overexpression of Rho kinase or the activation of the RhoA pathway in chondrosarcoma cells increases phosphorylation of the serine. Serine 181 is contained within a nuclear localization signal, and its phosphorylation can enhance Sox9 nuclear import and DNA binding. Rho kinase also causes a dose-dependent increase in transcription of a Sox9-luciferase reporter construct. Taken together, these results indicate a new pathway that directly links Rho kinase to increased production of cartilage matrix via phosphorylation of Sox9.

High Mobility Group Box Protein 2 in Joint Aging and Osteoarthritis

N. Taniguchi, B. Carames, M. Lotz

Osteoarthritis is the most common joint disease and typically begins with an aging-related disruption of the articular cartilage surface. Mechanisms leading to the surface degeneration have not been determined. We showed that high mobility group box protein 2 (HMGB2) is uniquely expressed in the superficial zone of human and murine cartilage. Both species

experience an aging-related loss of HMGB2 expression. Mice genetically deficient in HMGB2 have an earlier onset and more severe osteoarthritis than normal mice do. These changes in HMGB2-deficient mice are associated with a profound reduction in cartilage cellularity, because of increased cell death. Chondrocytes from HMGB2-deficient mice are also more susceptible to induction of apoptosis in vitro.

We also showed that HMGB2 interacts with the Wnt signaling protein β-catenin. Thus, HMGB2 is the first transcriptional regulator specifically expressed in the superficial zone of cartilage and supports chondrocyte survival. Aging is associated with a loss of HMGB2 expression, and this loss is a novel mechanism and risk factor for the development of osteoarthritis.

Extracellular Sulfatases in Cartilage Biology and Pathophysiology

S. Otsuki, M. Lotz

Osteoarthritis is characterized by destruction of articular cartilage, subchondral bone remodeling, and joint inflammation. Chondrocyte death and loss of glycosaminoglycan have been linked with the degradation of articular cartilage. Abnormal activation and differentiation of chondrocytes are central pathogenetic processes, in part related to an altered signaling environment that affects multiple growth factor pathways. Several major growth factor pathways in cartilage, including Wnt, bone morphogenetic protein, and fibroblast growth factor 2, are regulated by the specific sulfation pattern of heparan sulfate. Sulfation of glycosaminoglycan is controlled by sulfotransferases and sulfatases and plays a critical role in cartilage homeostasis. A novel class of extracellular glucosamine-6-sulfatases has recently been identified; mammals have 2 isoforms, Sulf-1 and Sulf-2, that affect heparan sulfate 6-O sulfation.

We found that osteoarthritic cartilage is characterized by overexpression of Sulf-1 and Sulf-2. This observation suggests that the increased sulfatase expression in osteoarthritis and resulting changes in heparan sulfation lead to abnormal activation of several central growth factor pathways and to joint destruction.

Role of Noncoding RNA in Arthritis Pathogenesis

S. Miyaki, R. Higashiyama, H. Asahara

Rheumatoid arthritis is characterized by chronic synovitis and cartilage and bone destruction. The cytokines IL-1 and TNF are central mediators of the pathogenesis of rheumatoid arthritis. Inhibition of these cytokines results in an improvement in clinical signs and symptoms and prevents progressive tissue destruction. However, a subset of patients with rheumatoid arthritis does not respond to inhibitors of TNF-α and IL-1β. Further research to elucidate the pathogenesis of rheumatoid arthritis might open the door to innovative therapies.

The recently discovered microRNAs consist of approximately 22 nucleotide noncoding forms of RNA that are important for a diverse range of biological functions. New concepts are emerging that some specific microRNAs might be associated with human diseases such as cancer or viral infection; however, microRNAs in rheumatoid arthritis have not been analyzed. We were the first to identify several microRNAs that are specifically upregulated in the synovial tissues in rheumatoid arthritis but not in osteoarthritis. Among these microRNAs, microRNA-146, which was recently reported as a regulator of innate immune responses, markedly increased in response to stimulation with TNF-α and IL-1β in fibroblasts from patients with rheumatoid arthritis. We will characterize other microRNAs that are also specifically expressed in synovial tissues in rheumatoid arthritis and examine their function to obtain a comprehensive view of the microRNA network in the pathogenesis of this type of arthritis.

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Regulation of Cerebral Angiogenesis by the Extracellular Matrix

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Proteins of the extracellular matrix play an important role in regulating the growth of blood vessels, both during development and in adulthood. To investigate the potential role of these proteins during developmental angiogenesis in the CNS, we characterized expression of different extracellular matrix proteins and β_1 integrins during development. We found that cerebral endothelial cells make a switch in expression, from fibronectin and the $\alpha_4\beta_1/\alpha_5\beta_1$ integrins during angiogenesis to laminin and the $\alpha_1\beta_1/\alpha_6\beta_1$ integrins in adults. Further studies revealed that fibronectin promotes survival and proliferation of brain endothelial cells and that this effect is mediated via the $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins.

Because of the suggested angiogenic role for $\alpha_5\beta_1$ during CNS development, we examined whether this induction was recapitulated during angiogenesis in the CNS in adults. Mice were subjected to hypoxic conditions (10% oxygen) for 0, 4, 7, and 14 days, and expression of fibronectin and specific integrins was determined. During this time, a strong angiogenic response occurred throughout the CNS. Using a marker specific for angiogenic cerebral endothelial cells, we discovered that angiogenic capillaries had strong induction of fibronectin and the $\alpha_5\beta_1$ integrin. This response was maximal at the earliest time studied (4 days), but declined thereafter, in concordance with the reduced number of angiogenic endothelial cells. Combined with the defined angiogenic role in other systems, these findings are consistent with the notion that the interaction between fibronectin and the $\alpha_5\beta_1$ integrin may be instrumental in driving angiogenesis in the CNS in adults.

In another project, we have defined the expression profile of matrix metalloproteinases (MMPs) in different glial cell types. Because increased MMP activity contributes to the pathogenesis of many neurologic conditions, determining which glial cell expresses which MMP is important. In all previous studies of astrocytes, the mixed glial culture system, which contains variable amounts of microglia, was used. The normal expres-

sion of high levels of MMPs by microglia complicated the analysis. To overcome this problem, we devised a novel strategy to prepare astrocyte cultures devoid of microglia by differentiating neural stem cells into astrocytes. Because microglia are derived from the hematopoietic, not neural, lineage, the resulting cultures are highly pure astrocytes that contain no microglia, as confirmed by a total lack of cells positive for microglial markers such as Mac-1. Using this system, we have shown that astrocytes and microglia express distinct sets of MMP genes. Specifically, we found that microglia and not astrocytes are the major source of MMP-9 after stimulation by lipopolysaccharide or TNF-α.

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Transcriptional Modulation of HIV Type 1 Expression and Latency

K.V. Morris, P. Hawkins, A.-M. Turner

Transcriptional gene silencing mediated by small noncoding RNA operates in human cells via the antisense strand of small interfering RNAs and interactions with DNA methyltransferase 3a (DNMT3a) and the protein Argonaute 1 (Ago 1). The use of noncoding RNAs to regulate gene expression is a shift in the current understanding of gene regulation. Our goal is to understand the mechanism of RNA-mediated regulation of gene expression in human cells and to use our findings to devise therapies for HIV type 1 (HIV-1) infection and cancer.

Building on our recent research and an emerging model of small noncoding RNA regulation of gene transcription, we found that we can establish long-term stable silencing of gene expression by targeting the promoter for ubiquitin ligase (UbC) with small noncoding, small hairpin RNAs (shRNAs; Fig. 1A). Interestingly,

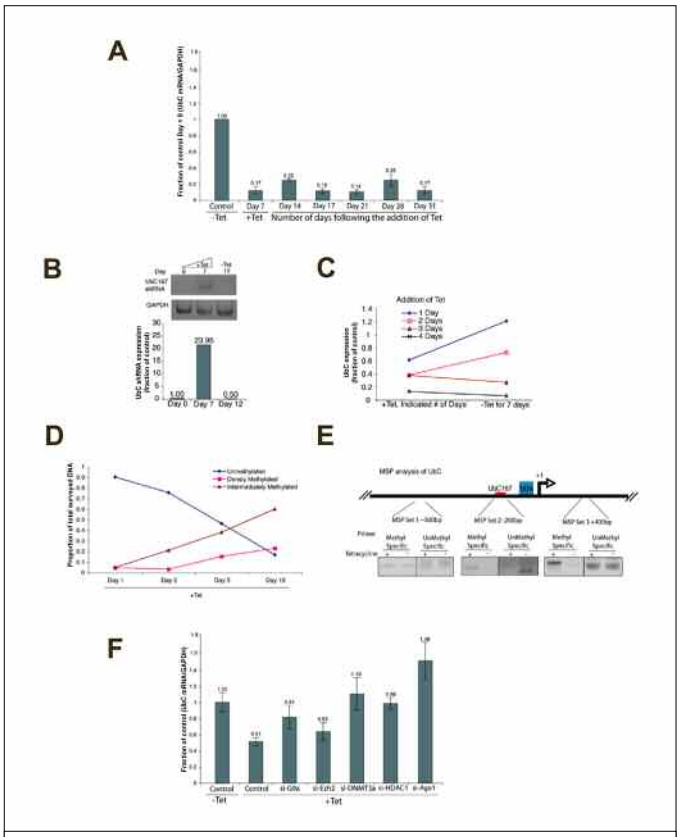

Fig. 1. Establishment of long-term gene silencing. See text for explanations of parts A–F.

this event requires that the promoter be targeted for about 4 days (Figs. 1B and C). After about 4 days, a notable increase occurs in DNA methylation at the targeted gene promoter (Figs. 1D and 1E). We also discovered that the initiation of transcriptional gene silencing requires Ago-1, DNMT3a, and histone deacetylase 1 (HDAC-1; Fig. 1F) and that maintenance of long-term expression requires DNMT1 and DNMT3a. Combined with our previous results, these data strongly indicate that small noncoding RNAs can modulate transcriptional gene silencing in humans.

Similar long-term silencing occurred when HIV-1 was targeted with a mobilization-competent vector expressing a small antisense RNA (362as) targeted to the long terminal repeat (LTR)/promoter of HIV-1 (Fig. 2A). HIV-1 expression in cultures treated with 362as was reduced for approximately 4 weeks (Fig. 2B). The suppressive activity of 362as appeared to require Ago-1, DNMT3a, and HDAC-1 (Fig. 2C). Taken together, these data suggest that gene promoters targeted by small noncoding

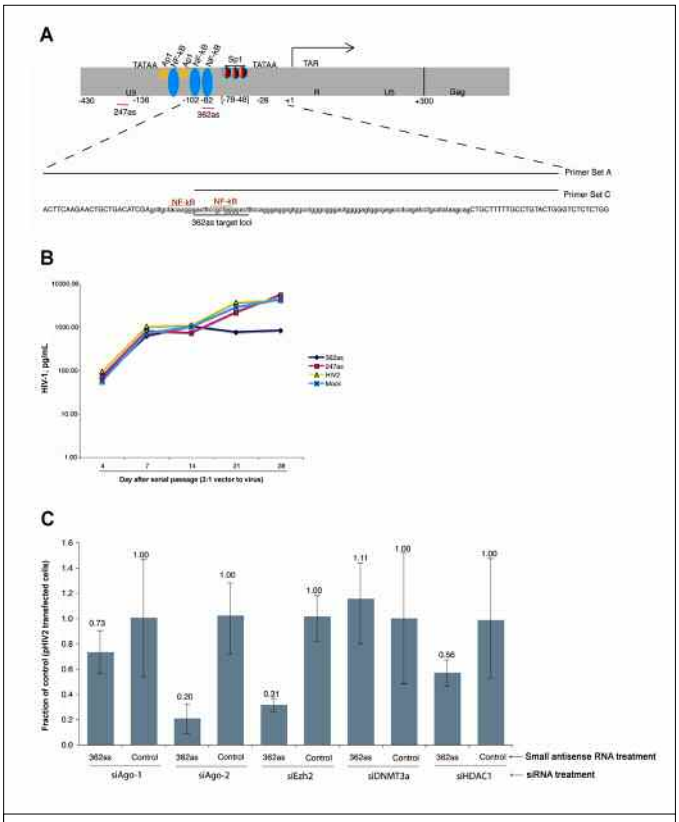

Fig. 2. Long-term silencing of HIV-1 by treatment with the small antisense RNA 362as. See text for explanations of parts A–C.

RNAs can modulate long-term stable gene expression and that small noncoding RNAs targeted to the HIV-1 LTR/promoter can be delivered to cultures infected with HIV-1 and spread along with the virus via the action of a mobilization-competent vector (Fig. 3).

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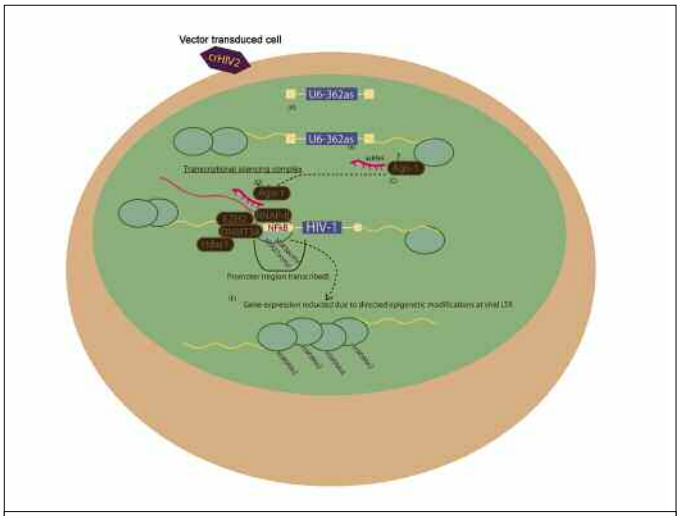

Fig. 3. Model for transcriptional gene silencing of HIV-1 gene expression mediated by a mobilization-competent vector. A, The mobilization-competent vector integrates into the genome of the target cell where (B) the small antisense RNA is expressed, which then (C) interacts with Ago-1 and localizes to the elongating transcript (D), which contains the target loci for the small antisense RNA. The Ago-1 small antisense RNA might also localize at the targeted site with DNMT3a, HDAC-1, and possibly Ezh2. The result of the localizing of this complex at the targeted promoter is histone methylation and transcriptional gene silencing of HIV-1 gene expression.

Cytoprotective Blood Coagulation Proteases and Their Receptors

L.O. Mosnier, S.K. Coit, M.G. Matias

Cytoprotective blood coagulation proteases (CYTOCOPs) are a novel class of proteases that in addition to their established role in blood coagulation have direct cytoprotective effects on cells. The cytoprotective effects of CYTOCOPs hold novel therapeutic promise for treatment of complex diseases such as sepsis, stroke, and other inflammation-induced diseases.

Activated protein C (APC) is the archetype CYTOCOP and is currently the only drug approved by the Food and Drug Administration to reduce mortality in severe sepsis. Other CYTOCOPs include factor VIIa, factor Xa, and meizothrombin. Our understanding of the molecular mechanisms of CYTOCOPs is limited and is mostly derived from recent studies on APC. Beneficial in vivo effects of APC do not require its anticoagulant activity. Instead they are mediated by the cytoprotective effects of APC, which require interaction of APC with cellular receptors, including endothelial cell protein C receptor and protease-activated receptor 1. Similar to

APC, factor VIIa has direct cellular antiapoptotic and anti-inflammatory effects that are independent of the role of factor VIIa in coagulation. The receptor profile for factor VIIa on cells is strikingly similar to that of APC, suggesting that shared molecular events are responsible for these direct effects on cells. We are using a molecular engineering approach to define structural regions on the CYTOCOPs for interaction with cytoprotective receptors and the relative contribution of these regions to coagulation effects vs direct effects on cells.

Sensitivity of the CYTOCOP receptors to inflammatory mediators limits the bioavailability of these receptors during inflammatory disease and thus the efficacy of cellular CYTOCOP activities on cells when such activities are needed most. We are using a molecular engineering approach to restore the bioavailability of CYTOCOP receptors through biochemical means to improve the therapeutic efficacy of the cryoprotective effects of CYTOCOPs during disease. Combined, use of these 2 molecular engineering approaches will provide detailed structure-function information on the receptor and protease domains required for cytoprotective effects of CYTOCOPs on cells and the molecular mechanisms involved.

In another project, we are investigating the anti-inflammatory role of activated thrombin activatable fibrinolysis inhibitor (TAFIa). TAFIa is an unstable carboxypeptidase with specificity for C-terminal lysine and arginine residues. In addition to inhibition of fibrinolysis, TAFIa has anti-inflammatory activities via the inactivation of the peptides bradykinin and the C3a and C5a components of complement. The des-Arg forms of these peptides have diminished bioactivities and are intermediates on pathways for metabolism of these peptide mediators. Our ultimate goal is to create a stable TAFIa enzyme that is anti-inflammatory but not antifibrinolytic. Currently, we are identifying surface-exposed loops and residues involved in substrate recognition and enzymatic activity.

Autoimmunity Induced by Xenobiotics

K.M. Pollard, D.M. Cauvi, C. Toomey

We focus on mechanisms involved in the initiation of autoimmunity and how the interplay between genetics and environment influences the expression of systemic autoimmune diseases.

DECAY-ACCELERATING FACTOR AND T-CELL ACTIVATION

Recently, we showed that reduction in decay-accelerating factor 1 (DAF1 or CD55) is an important step in the activation of $CD4^+$ T cells in murine systemic autoimmunity. We found that mice prone to systemic lupus erythematosus have endogenously low levels of DAF1 and that induction of systemic autoimmunity in B10.S mice by treatment with mercuric chloride reduces expression of DAF1 on activated ($CD44^{high}$) $CD4^+$ T cells. In mice deficient in the T-cell costimulatory molecule CD28, activated T cells did not accumulate, and expression of DAF1 was not modulated after exposure to mercury, suggesting that interaction between T cells and antigen-presenting cells is necessary to reduce DAF1 expression.

To examine this possibility further, we stimulated splenocytes of B10.S mice in vitro either with antibodies to CD3 and CD28 to mimic cell-surface events of interaction between T cells and antigen-presenting cells or with phorbol 12-myristate 13-acetate and ionomycin, which increases intracellular levels of free calcium and activates protein kinase C independently of cell-surface interactions. Compared with untreated control cells, cells treated with antibodies to CD3 and CD28 had significantly reduced expression of DAF1, as detected by flow cytometry, whereas cells treated with 12-myristate 13-acetate and ionomycin had no change in DAF1 expression. These results were confirmed by using real-time polymerase chain reaction analysis, which indicated that treatment with antibodies to CD3 and CD28 also reduced DAF1 mRNA. Thus, regulation of DAF1 requires cell-surface interactions involving CD3 and/or CD28.

Other investigators have shown that DAF is localized into lipid rafts and that the outcome of cross-linking events depends on whether the lipid-raft clusters cross-link with CD55 or with CD28. These observations suggest that the presence of DAF on the cell surface influences T-cell activation by competing with other costimulatory molecules, such as CD28. Thus, reduced levels of DAF would be expected to favor more efficient T-cell activation via antibodies to CD28. Conversely, modulation of T-cell function by antibodies to CD55 probably is influenced by the presence of CD28. In future experiments, we will focus on the relationship of T-cell activation to the cell-surface density of CD28 and DAF (CD55).

REGULATION OF DAF EXPRESSION

Differences in the expression of DAF1 between autoimmune-prone and healthy mice suggested possible genetic differences. To examine this possibility, we compared DAF1 expression in NZB and DBA/2 mice congenic for a fragment on the distal end of chromosome 1 that includes the gene for DAF1. Significantly, transfer of this genetic locus between strains did not show that expression of DAF1 is controlled within the locus, suggesting that genetic polymorphisms do not explain the differences in DAF1 expression. This idea was supported by the results of DNA sequencing of the DAF1 open reading frame and the 2.5-kb 5′ end of the ATG start site.

We have shown that transcription of the murine gene for DAF1 relies on 2 Sp1 sites and a CREB site within the DAF1 promoter, and the results of chromatin immunoprecipitation confirmed the presence of Sp1 bound to the appropriate region of the promoter. Using real-time polymerase chain reaction to determine the levels of the transcription factors Sp1, Sp3, CREB, and CREM, we found no differences in splenocytes from NZB and DBA/2 mice. Future studies will focus on transcription factor levels and phosphorylation within T-cell subsets, especially $DAF1^{low}$, $CD44^{high}$, $CD4^{+}$ T cells.

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Autoantibodies to Tumor-Associated Antigens as Reporters From the Immune System

E.M. Tan

Studies in my laboratory and in other laboratories indicate that some patients with cancer mount immune responses that are manifested as antibodies to certain cellular proteins. These autoantibodies can be detected in the circulation of cancer patients. The target antigens are cellular proteins that are abnormal or aberrantly expressed. My colleagues and I have been characterizing and isolating these autoantibodies and using them to identify the cancer-associated cellular proteins that provoke autoantibody responses. These cancer-associated cellular proteins have been called tumor-associated antigens (TAAs).

Hepatocellular carcinoma is a form of liver cancer that offers unusual advantages for our studies. Patients with some liver ailments, such as chronic hepatitis (due to hepatitis B or C viral infections) and liver cirrhosis, are highly disposed to development of hepatocellular carcinoma. Patients with such ailments are followed up frequently to detect malignant transformation as early as possible. We were the first to discover that novel autoantibodies to TAAs can be detected in the sera of patients with hepatitis or cirrhosis many weeks or months before clinical detection of a malignant tumor. It appears that the immune systems of the patients were telegraphing the occurrence of tumor cells by producing autoantibodies to the TAAs involved in malignant transformation. We therefore called autoantibodies to TAAs reporters from the immune system, and we have pursued this line of research with the goal of using autoantibodies to TAAs as immunodiagnostic markers for detection of cancer.

Several TAAs have been identified in different types of cancer. A consistent finding has been that any autoantibody to any TAA so far identified has a frequency that rarely exceeds 20%–25%, whatever the type of cancer. The reason for this low percentage has not been elucidated. In earlier studies, we had observed that cancer patients usually have more than a single kind of autoantibody. Patients with the same type of cancer might each have multiple autoantibodies, but no common patterns of antibody specificities exist.

With these background observations and the objective of increasing the sensitivity of using spontaneously occurring autoantibodies as an immunodiagnostic system for detecting cancer, we are designing TAA arrays consisting of several proven TAAs. So far, we have designed arrays containing 8–10 different antigens and have tested hundreds of serum samples from patients with different types of cancer. The sensitivity for cancer detection with the arrays has been 90% for some types of cancer, a level acceptable for diagnostic use. We are continuing to collaborate with former postdoctoral colleagues who are in faculty positions in other institutions to further improve the TAA arrays. Some of the advantages of cancer immunodiagnosis with TAA arrays are that the technique is noninvasive, should be less costly than current techniques, and, perhaps more importantly, offers the possibility of earlier diagnosis.

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Scripps Genomic Medicine

E.J. Topol, N.J. Schork, K.A. Frazer, S.S. Murray, C.M. Nievergelt, C. Bloss, O. Harismendy, M. Jirout, N. Malo, N. Rahim, E. Smith, A. Torkamani, K. Ohlsen, G. Zhang, O. Libiger

Scripps Genomic Medicine (SGM), a new research collaboration between Scripps Health and Scripps Research, was initiated in early 2007. The goal of the collaboration is to pursue and advance translational genomics research, that is, research to identify and characterize the genomic basis of disease in a way that the results can be used to facilitate translation of relevant findings into clinically useful insights and technologies. The motivation for SGM was rooted in the belief that the combined clinical resources at Scripps Health (which treats more than 1 million patients in the greater San Diego area) and the state-of-the-field basic research at Scripps Research could set precedents in applied biomedical research. Activities to be pursued within SGM include using genetic information to (1) develop genomic panels of disease risk assessment for prevention and (2) identify and characterize drug targets and drug designs. We will also be developing panels of genetic markers useful for charting disease progression and pursuing studies that will advance individualized medicine, prevention, and pharmacogenomics.

The program was founded by Eric Topol, professor, Department of Molecular and Experimental Medicine, who is a world-renowned cardiologist and cardiovascular geneticist. Dr. Topol is also chief academic officer at Scripps Health and director of the Scripps Translational Sciences Institute, an umbrella entity that encompasses SGM as well as joint educational and training initiatives between Scripps Health and Scripps Research. Joining Dr. Topol are Kelly Frazer, professor, Department of Molecular and Experimental Medicine, and current director of genomic biology at SGM, who formerly led a state-of-the-art functional genomics team at Perlegen Sciences, Mountain View, California, and the International HapMap Project; Sarah S. Murray, assistant professor, Department of Molecular and Experimental Medicine, and current director of genetics at SGM, a human geneticist who developed the most widely used high-throughput genotyping platform in the world while at Illumina, San Diego, California; and Nicholas Schork, professor, Department of Molecular and Experimental Medicine, and current director of research at SGM, a highly revered quantitative geneticist who has guided the analysis aspects of some of the largest applied genetics programs in the world.

As expected with such a far-reaching program, the research projects undertaken by the primary faculty associated with SGM in the past year have been diverse, as described in the following.

Dr. Topol's recent research has built on his extensive experience in clinical research and genetics. In the past year, he has overseen a number of extremely large clinical trials investigating novel drugs used to treat cardiovascular disease. He has also investigated the usefulness of biomarkers assayed during the course of treatment to monitor the efficacy of the drugs used. In addition, he has investigated the association of genetic and proteomic markers with cardiovascular diseases and has written extensively about the usefulness of genomic technologies in clinical and biomedical research.

Dr. Schork is interested in the quantitative and statistical aspects of genetics and genomics investigations. In the past year, he has developed novel statistical methods for assessing the relationship between genetic background diversity and phenotypic expression, analyzing microarray data, and assessing the association between genetic variations and phenotypic expression. He has also considered the impact on protein structure of amino acid substitutions associated with disease.

Dr. Frazer focuses on functional genomics and the development of high-density maps of polymorphic sites in genomes. In the past year, she has concentrated on evaluating the effect of genetic variation on molecular physiologic end points, such as transcription, and has developed and implemented schemes for identifying imprinted genes on a genome-wide scale. In addition, she has led 2 of the largest polymorphism discovery initiatives in the world: one on laboratory mice and one on humans. On the basis of the insights obtained from her research, she has also written on the relative merits of genomic strategies in biomedical research.

Dr. Murray focuses on all aspects of human genetics, including the design, implementation, and analysis of high-throughput genotyping and related assays. In the past year, she has considered the design and power of genome-wide association studies and the construction of large-scale maps of polymorphic sites in the human genome. She has also written extensively

on the usefulness of genetic maps and genotyping assays in biomedical research.

Dr. Nievergelt has developed panels of genetic markers that can be used to distinguish individuals with different geographic origins, has collaborated with Dr. Schork to develop novel analytic methods for testing the significance of differences in genetic background among participants enrolled in studies of a phenotype of interest, and has applied population association analysis methods in studies to identify genetic variations that influence disease phenotypes.

Since the inception of SGM in early 2007, the number of personnel has increased from 4 to 32; many of the staff have been hired to facilitate collection of samples from patients through the Scripps Health system. SGM will continue to grow and foster collaborations among investigators at Scripps Research and among investigators at its partner institution, Scripps Health.

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Evolution of Resistance of HIV Type 1 to Protease Inhibitors, Gene Delivery to Disrupt HIV Type 1, and Regulation of Hematopoietic Development

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Our research interests include the structural and biochemical evolution of the resistance of HIV type 1 (HIV-1) proteases, gene delivery strategies to disrupt cellular entry of HIV-1, and normal and abnormal regulation of myeloid development by the transcription factors PU.1 and cyclin D–interacting Myb-like protein (DMP1).

HIV-1 PROTEASE RESISTANCE

In patients infected with HIV-1, treatment with inhibitors of HIV reverse transcriptase, integrase, and protease suppresses replication of the virus. However, in some patients, HIV-1 variants evolve that escape the approved drug treatments by developing a broad-based resistance to the protease inhibitors. A molecular understanding of the resistance to protease inhibitors is needed so that strategies can be developed to design protease inhibitors that target drug-resistant viruses and are less likely to induce inhibitor-resistant viruses.

In collaboration with J.H. Elder and C.D. Stout, Department of Molecular Biology, and A. Brik and C.-H. Wong, Department of Chemistry, we showed that evolution of HIV-1 protease from a form susceptible to inhibitors to a form that is broadly resistant resulted in changes in viral fitness and protease structure. Structural changes in the resistant proteases included alterations in the active site, flap, and basal regions that promote structural alterations. To better understand how selected inhibitors disrupt the function of proteases, we used a novel protease inhibitor, AB2. We found that interactions between AB2 and the backbone of resistant proteases are necessary to maintain inhibitor function in proteases that are broadly resistant to inhibitors (Fig. 1).

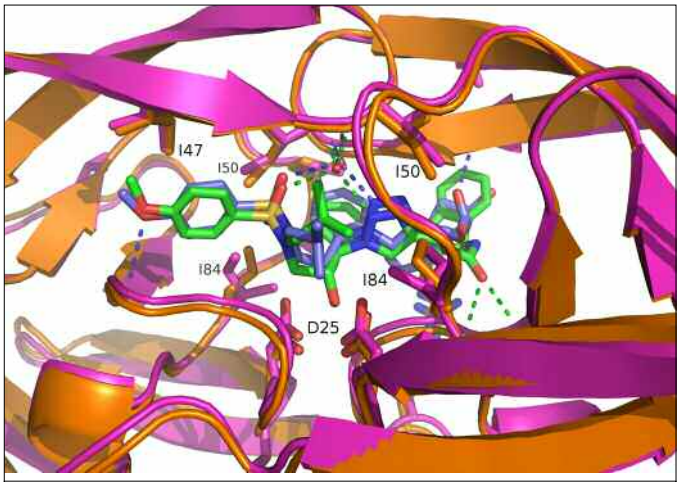

Fig. 1. Superposition of the protease inhibitor AB2 (green) bound to wild-type HIV-1 protease (purple ribbon) and to HIV-1 6X protease (orange ribbon), which is resistant to protease inhibitors, shows similar binding of AB2 to the 2 proteases. Hydrogen bonds between the inhibitor and the proteases are represented by green dashed lines for the wild-type protease and blue dashed lines for the 6X protease. The "flap water" is shown as a red sphere beneath the flaps forming 4 hydrogen bonds bridging the I50 protease residues and AB2.

The structural changes that occur during the development of resistance provide insight into the biochemical basis for the loss of activity of protease inhibitors.

Moreover, alterations in protease, and in its substrate gag/pol, are necessary to restore viral fitness. We are continuing investigations on the relationship between structure and function in wild-type proteases, mutant proteases broadly resistant to inhibitors. and gag/pol

to better understand the mechanisms of viral adaptation required to overcome the effect of protease inhibitors.

HIV-1 VECTOR DELIVERY OF CCR5-INTRABODY GENES TO HUMAN HEMATOPOIETIC CELLS

CXCR4 and CCR5 are the main chemokine receptors for HIV-1 entry into cells, and blocking these receptors limits entry of the virus. Naturally occurring polymorphisms of the gene for CCR5 indicate that disruption of the gene provides protection from viruses that use CCR5 to gain entry. Because polymorphisms are present in healthy persons, the use of genetic intervention strategies that prevent or limit expression of CCR5 may provide protection from initial infection and limit the spread of HIV-1.

We have reported that intracellular expression of a CCR5-specific single-chain antibody (intrabody) efficiently disrupted expression of CCR5 on the T-cell surface and protected cells from HIV-1 infection. Moreover, we have shown that human stem cells expressing the CCR5-intrabody develop into T cells and that the decreased expression of CCR5 protects cells against HIV-1 challenge and imparts a survival advantage in the presence of HIV-1 infection. Thus, it seems that gene delivery can provide gene programs that will protect and allow expansion of protected cells during HIV-1-infection.

Currently, we are disrupting the function of viruses that use either the CXCR4 or the CCR5 receptor for entry, the so-called R5X4 viruses. We are using combination vectors that genetically target chemokine receptors and viral and cellular pathways critical for viral entry and replication.

MYELOID DIFFERENTIATION

PU.1, a member of the Ets family of transcription factors, is expressed in hematopoietic cells and is necessary for directing myeloid development and for regulating genes required for monocyte/macrophage and neutrophil function. PU.1 has 3 major domains: the transactivation, PEST, and Ets/DNA-binding domains. PU.1 interacts with other transcription factors, and domains of PU.1 have been implicated in its function.

Myeloid development is controlled by temporal gene expression of PU.1 and interactions among specific transcription factors. We are addressing which PU.1 domains regulate myeloid lineage–specific commitment, differentiation, and function. Inappropriate PU.1 expression in developing erythroblasts contributes to erythroleukemia. Recently, we found that PU.1 interacts with p53, thereby disrupting p53 transcriptional control of the cell cycle. In turn, this event supports uncontrolled cellular growth, and subsequent events support erythroleukemia. Studies are under way to map the PU.1 domains necessary for p53 interaction that disrupts p53 function.

Cancer often originates from inactivation and/or deregulation of the control of gene expression. The transcription factor DMP1 positively regulates expression of human $p14^{ARF}$ (ARF) and CD13/aminopeptidase N, thus playing a role in cell-cycle control, differentiation, and function of hematopoietic and nonhematopoietic cells. The tumor suppressor ARF is critical for positive regulation of p53, which in turns controls cellular proliferation and modulates apoptosis. We have identified 2 novel and developmentally expressed human DMP1 splice variants: β and γ. We found that the β variant functions as a dominant-negative regulator of the originally reported DMP1 protein and thus regulates ARF. Recently, we discovered a p53 feedback loop that regulates the activity of DMP1 in normal cells, thus dampening its ability to regulate ARF and p53.

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Molecular Genetics of Cancer

P.K. Vogt, D. Bai, N. Dannemann, A. Denley, A. Galkin, M. Gymnopoulos, J. Hart, P. Hillmann, B. Hofmann, H. Jiang, P. Pavlickova, J. Shi, M. Sun, L. Ueno, L. Zhao

Our main area of research is molecular mechanisms of carcinogenesis. We study viral and cellular oncoproteins and tumor suppressors,

defining their functions in oncogenesis and identifying molecular targets for therapeutic intervention. In high-throughput screens, we look for small molecules that can interact with these targets and inhibit or reverse oncogenic cellular transformation. Although most of our work originated in studies of retroviruses, our current focus is on cancer cells; viruses are used as tools but are not the main topic of our research.

PHOSPHATIDYLINOSITOL-3′-KINASE IN CANCER

Oncogenic transformation of cells requires changes in gene activities, regulated at the level of transcription, translation, or posttranslational modification. These changes result in a gain of function for specific growth-promoting genes and a loss of function for growth-attenuating genes.

The activities and functions of cells are controlled by a system of interacting signaling chains that regulate movement, differentiation, metabolism, growth, and replication. In cancer, growth-promoting signals have a gain of function. One of these signaling chains originates in phosphatidylinositol-3′-kinase. The catalytic subunit of this lipid kinase, p110α, is mutated in a high percentage of several common cancers, including cancers of the breast, prostate, and colon. Most of these cancer-specific, somatic mutations map to 1 of 3 "hot spots" in the coding sequence of p110α; each mutation consists of a single nucleotide exchange resulting in a single amino acid substitution in the protein. The high frequency of the 3 hot-spot mutations strongly suggests that they are selected for and that they endow cells with a growth advantage. We have shown that these hot-spot mutations induce a gain of enzymatic function, cause a constitutive downstream signal, and make the protein oncogenic in cell culture and in animals.

The reasons for targeting mutant p110α in cancer are compelling: The mutations are cancer specific and do not occur in normal tissues. They are "driver" mutations, contributing to the oncogenic phenotype of the cell, and gain of function is much easier to correct with small molecules than is loss of function. We are using cell-based assays to screen targeted combinatorial chemical libraries for mutant-specific inhibitors of p110α.

TARGETING PROTEIN-PROTEIN INTERACTIONS WITH SMALL MOLECULES

Protein-protein interactions are fundamental to the regulation of cellular activities. Numerous oncoproteins depend on such interactions for function. Examples are the dimerization of the transcription regulators Myc and Max and LEF and β-catenin and the binding of Ras to the catalytic subunit p110α of phosphatidylinositol-3′-kinase. In collaboration with D.L. Boger and K.D. Janda, Department of Chemistry, we are screening targeted chemical libraries for inhibitors and for stabilizers of protein-protein interactions. Recently, we have concentrated on the Myc-Max and Max-Max dimers.

Increased levels and enhanced function of Myc are common in cancer. These changes are due to gene amplification, elevated transcription, or activated translation. In many cancers, a correlation exists between the gain of function in Myc, tumor grade, and poor prognosis, suggesting that Myc plays an important role in the causation and progression of cancer.

Myc is a transcription factor that functions only as a dimer with another protein, Max. The structure of the Myc-Max dimerization interface is known; single amino acid substitutions at critical sites can break or stabilize dimerization. We have isolated several small molecules that interfere with the dimerization of Myc and Max. As a consequence, these molecules also prevent Myc DNA binding, Myc-dependent transcriptional activation, and Myc-induced oncogenic transformation.

The Myc-Max dimer belongs to a complex network that includes activators as well as repressors of transcription. All of the activators and repressors function as dimers with the Max protein, making Max the common denominator of the network. Max is also the only component of the network that can form homodimers, albeit weak and transcriptionally inactive homodimers. Small molecules that specifically stabilize the Max homodimer would trap this essential partner and make it unavailable for heterodimerization and for transcriptional regulatory activities. Such compounds would downregulate the entire network.

We have searched for small molecules that could bind specifically to Max and stabilize the Max homodimer while leaving Myc-Max dimerization unaffected. The search was performed in silico, using the helix-loop-helix leucine zipper dimerization domain of Max and the National Cancer Institute diversity set of compounds. We used the software docking program Autodock, developed here at Scripps Research, and the computations were performed on the supercomputer at the University of California, San Diego. The list of candidates identified in silico was then screened by using fluorescence resonance energy transfer followed by cell-based assays for inhibition of Myc. The final compound that passed all these tests inhibits Myc-dependent cell growth, Myc-mediated transcriptional activation, and Myc-induced oncogenic transformation.

The candidate inhibitors and stabilizers are currently being further optimized. We are also proceeding with screens that focus on the phosphatidylinositol-3′-kinase pathway. These screens include the Ras-p110α interaction and the binding of the signaling protein Rheb (Ras homolog enriched in brain) to its cellular target FKBP38.

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Maintenance of Genome Stability in Mammalian Cells

X. Wu, C. Nievera, H. Wang, K.H. Koh, L. Truong

Genome instability is a hallmark of the malignant phenotype and a driving force for tumorigenesis. Cell-cycle checkpoints have evolved to monitor the integrity of the eukaryotic genome, activate DNA repair pathways, and ensure the completion of DNA repair before progression of the cell cycle.

DNA double-strand breaks (DSBs) are the major inducer of chromosomal rearrangements, leading to genome instability. However, DSBs occur routinely as a result of endogenous cellular processes, including oxidative metabolism, mechanical stress, and replication fork collapse, and as a result of exogenous events, such as exposure to ionizing radiation. Homologous recombination and nonhomologous end joining are 2 major pathways involved in the repair of DSBs in mammalian cells.

In one area of our research, we focus on the Mre11-Rad50-Nbs1 complex (MRN), which plays an essential role in the repair of DSBs. Recently, we analyzed the interaction of the Mre11 complex with the breast cancer suppressor Brca1 and its association protein CtIP. We found that the supercomplex formation of Brca1-CtIP-MRN is cell-cycle dependent, requiring the activity of cyclin-dependent protein kinases. The interaction is also stimulated by DNA damage. Interestingly, several investigators have shown that CtIP is a functional homolog of yeast repair proteins Ctp1 and Sae2, suggesting that the supercomplex of Brca1-CtIP-MRN may be involved in DNA repair. Indeed, our findings indicated that CtIP, like Brca1 and MRN, plays an important role in mediating repair of DSBs via homologous recombination. We further discovered that this supercomplex is involved in end resection to generate the single-stranded DNAs required to initiate repair via homologous recombination. We argue that the formation of the supercomplex Brca1-CtIP-MRN that depends on cyclin-dependent protein kinases and is stimulated by DNA damage contributes to the activation of homologous recombination–directed DSB repair used in the S and G_2 phases of the cell cycle.

A second focus of our research is understanding how DNA replication is controlled so that DNA is replicated once and only once per cell cycle. Rereplication of the genome, or even a segment of it, could lead to genome instability. We found that the S-phase checkpoint mediated by the ataxia telangiectasia–mutated and Rad3-related (ATR) pathway acts as a surveillance mechanism to prevent rereplication, so that disruption of licensing control by overexpression of the licensing factor Cdt1 does not induce significant rereplication in mammalian cells when the ATR checkpoint is intact. Single-stranded DNA accumulated by uncontrolled DNA unwinding mediated by mini-chromosome maintenance due to Cdt1 overexpression is the initial signal to activate the checkpoint. We further found that rereplication leads to the generation of DSBs, which activate the ATM-mediated repair pathways. We propose that repair of rereplication-associated DSBs is essential for cell viability and the maintenance of genome stability.

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NADH Dehydrogenases

T. Yagi, A. Matsuno-Yagi, B.B. Seo, E. Nakamaru-Ogiso, M. Marella, J. Barber-Singh, J. Torres-Bacete, P.K. Sinha, N. Castro-Guerrero

STRUCTURE AND FUNCTION OF NADH DEHYDROGENASES

The proton-translocating NADH-quinone oxidoreductase (complex I; Fig. 1) in the mitochondrial respiratory chain catalyzes the first step of electron transport by oxidation of NADH, providing 2 electrons for reduction of quinone coupled to the translocation of 4 protons across the membranes to produce an electrochemical gradient. Complex I has an L-shaped structure and is composed of 2 segments: a peripheral arm and a membrane arm. The membrane arm of *Escherichia coli* complex I contains 7 hydrophobic subunits. Of these subunits, NuoM, a homolog of the mitochondrial ND4 subunit, is proposed to be involved in proton translocation and quinone binding. Therefore, we conducted site-directed mutation of the conserved amino acid residues of NuoM and investigated the properties of the mutants. Mutation of highly conserved glutamic acid at position 144 and lysine at position 234 led to almost total elimination of energy-transducing complex I activities and to increased production of superoxide radicals. The NADH dehydrogenase activities of the 2 mutants were almost normal. Because these 2 residues most likely are located in the transmembrane segments of NuoM, the results strongly suggest that the residues participate in proton translocation. Although it is hypothesized that histidine interacts with a quinone head group, mutations at 4 histidines moderately inhibited complex I activities and had almost no effect on the K_m values for quinone or the IC_{50} values of capsaicin-40, a competitive inhibitor for the quinone-binding site. The data suggest that NuoM has no catalytic quinone-binding site in contrast to the previous prediction.

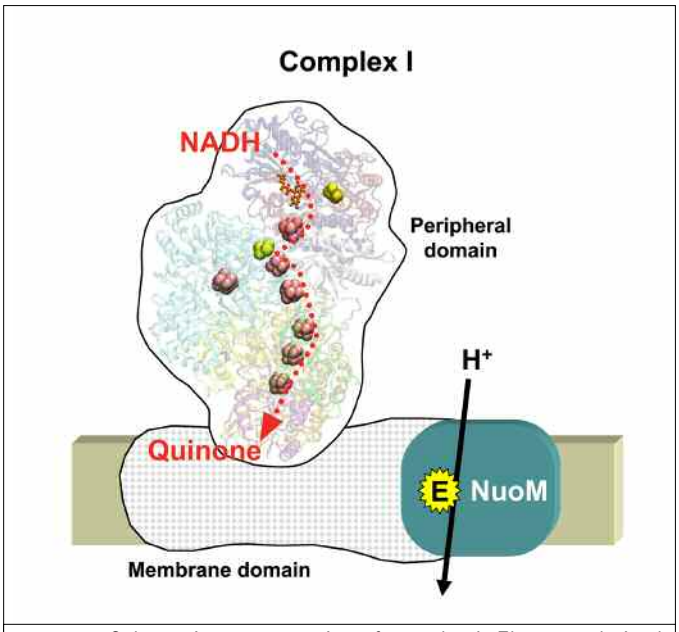

Fig. 1. Schematic representation of complex I. Electrons derived from NADH oxidation pass several iron-sulfur clusters in the peripheral domain and are accepted by quinone (red dotted line). Coupled to the electron transfer, complex I pumps protons from the cytoplasmic phase to the periplasmic phase and constructs the electrochemical gradient across the membranes. Glutamic acid at position 144 of subunit NuoM is directly involved in proton translocation of complex I. NuoM is located near the distant end of the membrane domain. Therefore, the mechanism of the energy transduction of complex I may involve a long-range conformational change induced by electron transfer.

MOLECULAR REMEDY OF COMPLEX I DEFECTS

Mitochondrial dysfunction, most notably defects in complex I, is closely related to the etiology of sporadic Parkinson's disease; many patients with the disease have decreased complex I activity. Furthermore, complex I inhibitors can induce parkinsonian signs in animals. We showed that expression of alternative NADH dehydrogenase 1 (Ndi1) can rescue mammalian cells from complex I dysfunction. Using Ndi1, we investigated the key events in cell death in a rat dopaminergic cell line, PC12 (Fig. 2). Inhibition of complex I caused activation of a specific kinase pathway and release of mitochondrial proapoptotic factors. The traditional caspase pathway does not seem to be involved, because caspase 3 activation was not observed.

Our data suggest that overproduction of reactive oxygen species (ROS) by complex I inhibition is responsible for triggering the kinase activation, for the release of the proapoptotic factors, and then for cell death. Nearly perfect prevention of apoptotic cell death by Ndi1 agrees with our earlier observation that Ndi1 diminishes rotenone-induced ROS generation from complex I. It is evident that Ndi1 keeps the redox potential high even in the presence of rotenone. Under these conditions, ROS formation by complex I is minimal.

Chronic rotenone exposure in rats seems to reproduce pathophysiologic conditions of Parkinson's disease more closely than do acute mouse models as manifested by neuronal cell death in the substantia nigra and Lewy body–like aggregations. Using the rotenone rat model, we investigated the protective effects of Ndi1. A single, unilateral injection of recombinant adeno-associated virus carrying the *NDI1* gene into the substantia nigra

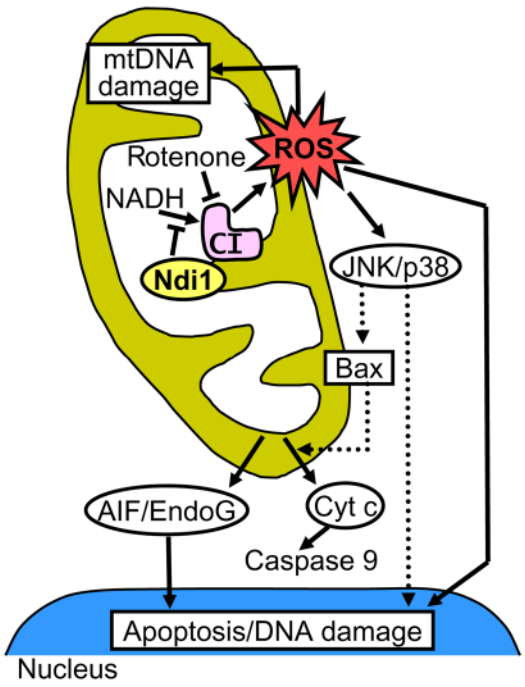

Fig. 2. Speculative mechanism of PC12 cell death caused by complex I inhibition. Inhibition of complex I promotes the production of ROS within hours. ROS can cross the lipid bilayer and target directly both mitochondrial and nuclear DNA. The presence of ROS in the cytoplasm induces the phosphorylation of kinases such as p38 kinase and JNK. Activation of the kinase pathway seems to be linked to the mitochondrial apoptosis machinery through the Bax phosphorylation, resulting in the release of the proapoptotic factors such as AIF and EndoG. From the mitochondria, cytochrome *c* (Cyt c) is released earlier than is AIF/EndoG and participates in caspase 9 activation. Ndi1 suppressed ROS overproduction due to complex I inhibition because rotenone cannot increase the NADH to NAD^+ ratios in the matrix of the Ndi1 mitochondria. The alternative NADH dehydrogenase keeps the NADH to NAD^+ ratio in the matrix high enough to provide the redox environment that leads to suppression of ROS production.

resulted in the expression of Ndi1 in the entire substantia nigra of that side. Clearly, the introduction of Ndi1 into the substantia nigra induced resistance to the deleterious effects of rotenone exposure as indicated by the levels of tyrosine hydroxylase and dopamine. The presence of Ndi1 also prevented cell death and oxidative damage to DNA in dopamineregic neurons in rotenone-treated rats. Unilateral protection also led to unilateral rotation of the rotenone-exposed rats in the behavioral test. These findings indicate the powerful neuroprotective effect of Ndi1 in a rotenone rat model of Parkinson's disease.

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