*U.S. Department of Health and Human Services*
*National Institutes of Health*
*National Institute of Dental and Craniofacial Research*
*Division of Intramural Research*

# ANNUAL REPORT SUMMARY

# 2002

Department of Health and Human Services
National Institutes of Health

National Institute of Dental
and Craniofacial Research

Division of Intramural Research

# Annual Report
# Summary

2002

For Administrative Use Only

Fiscal Year 2002 Annual Report

National Institute of Dental and Craniofacial Research
Division of Intramural Research

# Overview

Henning Birkedal-Hansen, Scientific Director

**National Institute of Dental and Craniofacial Research**
**Division of Intramural Research**
**Overview**

Fiscal Year 2002 proved to be a highly productive year for the Division of Intramural Research (DIR) on many fronts. Significant advances in research were reported in leading biomedical journals and at international meetings and conferences. The highlights of our scientific findings are summarized in this document. The creativity of the individual scientists and the scientific leadership in the Branches, Sections and Units resulted in important new insights and advances at both the conceptual and technological levels. This Annual Report details the scientific advances made by the six Branches and four single-standing units which provide the organizational infrastructure for our 28 independent investigators. DIR scientists published some 300 articles in this fiscal year. A significant number of these (≈50) were published in high-profile biomedical research journals *(Science, Cell, Nature, Nature Neuroscience, Nature Genetics, Nature Cell Biology, Nature Biotechnology, NEJM, Development, J. Exp. Med., PNAS, J. Biol. Chem., EMBO J. etc)* suggesting that our findings reach a broad range of scientists in many fields both inside and outside of the dental, oral and craniofacial research community.

The Pain and Neurosensory Mechanisms Branch (PNMB) was reviewed by the Board of Scientific Counselors, which found many strengths in the program and recommended continued strong support of the Branch's efforts. Prior to the review, Dr. Raymond A. Dionne was appointed to the position of Branch Chief after having served in an acting capacity.

The Division conducted an analysis of its clinical research infrastructure by an outside expert panel. The panel came to the conclusion that conducting and building a vibrant clinical research program in accordance with all applicable regulations is a full time job. Since Dr. Dionne assumed the position of Chief of the PNMB, it was decided to open a national search for a Clinical Director. The search is currently in progress and will hopefully be concluded in the near future.

On the administrative front a search was conducted for a Principal Administrative Officer which resulted in appointment of Ms. Mary (Dede) Daniels. In addition, the Division recruited an additional Administrative Officer, Mr. Paul Kiehl in order to upgrade the service to the Branches and the investigators.

Renovation and infrastructure improvements proceeded with full force in the basement of Building 30 for creation of a new state-of-the-art animal facility. In addition, the renovation of the H. Trendley Dean Conference Room was completed and an extra floor was added on top of the conference room that will provide space for additional activities.

Plans to recover additional space either at Navy or in the Twinbrook complex have been abandoned because of the high cost of renovation/rental. Instead the division will focus

its efforts and resources on bringing research space in Building 10 and 30 up to reasonable and contemporary standards.

Dr. Scott Diehl, Chief of the Molecular Epidemiology and Genetics Section/Unit left the Institute to pursue other research interests. Dr. Diehl's departure created a void in the area of human craniofacial genetics, which we will fill through a national search for a senior investigator who can build such a program and take full advantage of the NIDCR programs and the NIH environment. During the past year, Dr. Stephen Leppla transferred from NIDCR to the National Institute of Allergy and Infectious Diseases (NIAID). His expertise and experience in anthrax research have become vitally important to NIAID's efforts against bioterrorism. Dr. Jerry Keith, Dr. M.A. Ruda, Dr. Frank Robey and Dr. Richard Gracely also left the Institute to pursue other career opportunities. As a result of these departures the number of Principal Investigators fell to 28 (25 Senior and 3 Tenure Track Investigators). The Division is focusing on rebuilding by recruiting for tenure track positions in pulpal stem cell biology, structural biology, host-microbial interactions and mucosal immunology.

DIR scientists also were highly active in organizing scientific meetings and workshops, presenting invited talks at international scientific meetings and giving invited presentations at universities across the country. In addition, our scientists continue to develop an extensive net of national and international collaborative projects with colleagues in the United States and abroad. These activities are detailed annually in a separate document titled "Interactions with the Scientific Community."

Henning Birkedal-Hansen
Scientific Director

# Craniofacial Developmental Biology and Regeneration Branch

Kenneth Yamada
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# CRANIOFACIAL DEVELOPMENTAL BIOLOGY AND REGENERATION BRANCH 2002

The Craniofacial Developmental Biology and Regeneration Branch (CDBRB) focuses on four general objectives: understanding the mechanisms of normal and defective craniofacial development at genetic, molecular, and cellular levels; characterizing related processes in tissue repair and cancer; discovering novel genes, biologicals, and biomimetics relevant to diagnosis, repair, and therapy; and developing biologically based methods to replace or regenerate tissues that are defective or damaged. We place particular emphasis on research characterizing the interactions between cells and extracellular molecules. Project areas include identification of mechanisms of morphogenesis, characterization of the gene regulation, organization, and function of extracellular matrix molecules and their receptors, and analysis of signaling pathways from the cell surface to the nucleus. These ongoing basic research innovations will provide the basis for novel translational and patient-oriented applications. This past year, our researchers continued to generate a variety of exciting research advances and to receive international recognition. We also continued to place high priority on training younger scientists to become independent leaders in academia and industry. In addition, we provided extensive service and citizenship activities on behalf of NIDCR, NIH, and our research fields.

Researchers in the CDBRB have made substantial progress and exciting scientific breakthroughs during the past year as reflected in the 68 publications in our annual report bibliography. Several selected research advances are highlighted below. More comprehensive summaries of the major new findings in our Branch can be found in the Project reports of each Section.

CDBRB initiated genome projects to catalogue genes expressed in oral and craniofacial tissues and to discover novel genes important for tooth, oral, and craniofacial development. Previously, CDBRB researchers discovered the gene for ameloblastin, a developmentally regulated, tooth-specific extracellular matrix protein, which maps close to the locus of the congenital disorder amelogenesis imperfecta. Analyses of ameloblastin-null mice by the Molecular Biology Section reveal that ameloblastin is a key cell adhesion molecule essential for amelogenesis and regulation of the polarity and differentiation of dental epithelium. They also analyzed expression patterns of cDNA clones from mouse tooth germ and craniofacial cDNA libraries using cDNA microarrays. Several new genes have been identified as being expressed predominantly in specific developing tissues such as tooth. These genes have been further characterized by expression in tissue culture and in mice. In studies of salivary gland development, gene expression profiling of multiple embryonic stages using microarray and laser micro-dissection/SAGE approaches by the Cell Biology and Developmental Mechanisms Sections has identified a variety of additional genes that may be important in salivary development. CDBRB members also serve as Project Officers of a major contract with Washington University, St. Louis, in association with researchers at Johns Hopkins

University and Necker-Enfant Malades Hospital, to discover human craniofacial genes that are active in specific tissues during early development. All clones, cDNA libraries, and antibodies will continue to be made freely available to dental, craniofacial, and other investigators to promote research in the area.

Our knowledge about gene regulation and the extracellular matrix is being used to examine pathology in animal models and in human diseases. For example, the Molecular Biology Section has created gene knockout mice to study the biological roles of perlecan. Perlecan-null mice showed severe chondrodysplasia. Subsequently, mutations in perlecan genes were identified in two human disorders, the severe neonatal lethal dyssegmental dysplasia (Silverman-Handmaker type) and the relatively mild Schwartz-Jampel syndrome with chondrodystrophic myotonia. They also found that perlecan plays an important role in neuromuscular junction function by retaining acetylcholinesterase at this site. In addition, transcription factors that regulate cartilage collagen genes have been identified and characterized.

Laminin and laminin peptides have been implicated by CDBRB members in differentiation, tumor growth, and metastasis. Active sites of laminin chains have been identified in a variety of biological processes using synthetic peptide and recombinant protein approaches by the Molecular and Cell Biology Sections. Many of these molecules have potent cell type-specific effects on cell adhesion, angiogenesis, salivary gland differentiation, and tumor metastasis. For example, one peptide can cause melanoma cells to metastasize to the liver and breast cancer cells to bone, yet it has no effect on angiogenesis; in contrast, another peptide stimulates angiogenesis and increases lung tumor cell colonization five-fold. The receptors for both have been identified: the former is a cell surface heparan/chondroitin sulfate-containing molecule, and the latter is an integrin. These studies may lead to the development of new therapeutic reagents.

The Cell Biology Section has identified potent extracellular regulators of cell migration and tumor metastasis. Ongoing studies on thymosin beta-4 have established roles in endothelial, keratinocyte, and corneal epithelial cell migration. It decreases inflammation and accelerates wound repair in the rat cornea and in dermal wounds in aged and diabetic animals, suggesting its potential for promoting human wound healing. Unexpectedly, thymosin beta-4 was also found to promote hair growth in rats and aged mice. It acts by increasing stem cell migration and protease production.

Successful regeneration of salivary gland function is likely to require re-establishing the complex branched organization of acini to provide sufficient epithelial surface area for fluid production. The Cell Biology and Developmental Mechanisms Sections have consequently focused on determining the mechanisms and regulation of early salivary branching morphogenesis. Both FGF receptors and the PI 3-kinase pathway were found to be important in early gland development. Expression of the metallothionein gene was found to be induced at least six-fold by laminin. When metallothionein expression in salivary gland tumor cells is increased by transfection, the acinar structures formed in vitro are larger, and the tumors formed in vivo are smaller and more differentiated. Furthermore, as cells become malignant, many lose their expression of this protein. This

gene is likely to have a role in salivary differentiation and may function as a tumor suppressor. Other studies are characterizing cell adhesions to matrix-derivatized biomaterials. These approaches are building the knowledge base necessary to develop creative therapeutic approaches for the repair or replacement of salivary glands and other tissues. For example, CDBRB is collaborating with the Gene Therapy and Therapeutics Branch to develop a first-generation artificial salivary gland.

Integrins are major mediators of cell interactions with the extracellular matrix. The Developmental Mechanisms Section has dissected mechanisms of integrin-mediated responses by identifying components of integrin adhesive complexes and exploring how signal transduction is initiated. A novel component of integrin complexes was identified as kinectin; integrin clustering transiently redistributes over half of total cellular kinectin to adhesion complexes. Initial signaling could be triggered by simple dimer formation of focal adhesion kinase, but MAP kinase activation requires formation of larger complexes.

Although such tissue culture approaches have provided substantial conceptual advances, cells generally interact with a three-dimensional extracellular matrix rather than flat substrates in vitro. Methods for analyzing cell functions and signaling in three-dimensional matrices were developed by the Developmental Mechanisms Section. The newly discovered "3D-matrix adhesions" are unusually long, thin structures formed by human and mouse cells growing in 3D matrices. These adhesions have a molecular composition distinct from previously described adhesions to matrix molecules and substrates. Cells interacting with 3D matrices show substantially enhanced rates of attachment, as well as accelerated morphological changes, migration, and even cell proliferation rates, when compared to interactions with a variety of 2D matrices or collagen gels. Signal transduction also differs in 3D matrices compared to 2D surfaces, with a marked decrease in specific phosphorylation of focal adhesion kinase and a slight increase in MAP kinase activation. These findings provide insights into mechanisms of cell-matrix interactions that should eventually benefit cell-based tissue engineering.

Besides publishing extensively in leading journals, our Branch distributes its research materials widely by licensing, donating to repositories, and providing numerous gifts to research colleagues. Products generated by CDBRB members that were licensed by companies included Matrigel, invasion substrates, and monoclonal antibodies against integrins. CDBRB has donated hundreds of cDNA clones to the ATCC and completed over 60 formal Material Transfer Agreements with extramural researchers over the past year to provide our reagents as gifts. Members of the Branch have also received support from outside organizations. NASA provided funds to study salivary gland cell differentiation in microgravity, and non-NIH salary support for postdoctoral members of CDBRB has come from various sources.

CDBRB members continue to be invited as featured speakers at a variety of international meetings and symposia. Examples from this past fiscal year included Hynda Kleinman as a featured speaker at the Biochemical Society of Korea's Annual Meeting and at the International Symposium on Immunology and Aging, Kenneth Yamada as a symposium speaker at the International Association for Dental Research, and Yoshihiko Yamada as

an invited speaker at the Basement Membranes Gordon Research Conference. Our members continue to serve on the editorial boards of a number of leading journals. Examples include J. Cell Biology (K. Yamada, Editor, and H. Kleinman, board member); Cancer Research (H. Kleinman, Associate Editor); J. Cellular Physiology (K. Yamada, Editor); Matrix Biology (Y. Yamada and K. Yamada, Associate Editors); J. National Cancer Institute (H. Kleinman, Associate Editor); J. Biological Chemistry (Y. Yamada, board member), J. Cell Science (K. Yamada, board member), and a variety of other editorial boards. Members also served on various review panels, on the Board of the Metastasis Research Society (H. Kleinman), and on the Council of the International Society for Matrix Biology (H. Kleinman and K. Yamada). CDBRB members provide extensive service on more than two-dozen NIH and NIDCR committees, including the NIH Senior Biomedical Research Service Policy Board, NIH Central Tenure Committee, and NIH Committee on Scientific Conduct and Ethics.

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# Craniofacial and Skeletal Diseases Branch

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## CRANIOFACIAL AND SKELETAL DISEASES BRANCH
## 2002

The Branch has continued to concentrate our efforts on gaining an in depth understanding of the skeleton, defined as bone, cartilage, teeth, and their associated soft tissues. The rationale for this emphasis is based not only on the critical role that skeletal tissues play in oral health, but also on the increasing incidence of skeletal disorders, and the lack of efficacious therapy for most of these disorders. The goal is to apply cutting edge basic, translational and clinical research in order to impact current medical practice. To accomplish this goal, studies focus on the cells, genes and macromolecules of skeletal tissues in order to elucidate the fundamental mechanisms that govern development, growth and maintenance of the normal skeleton, and to determine the pathogenic mechanisms in genetic and acquired skeletal disorders. These studies are performed by three Principal Investigators who are highly collaborative, not only within the Branch, NIDCR and NIH, but also worldwide. Each group is focused on specific aspects of skeletal metabolism that has positioned the Branch to address major physiological questions, to devise new hypotheses, and to test them through in vitro and in vivo analyses, and through clinical observation.

The Skeletal Biology Section, directed by Dr. Pamela Gehron Robey, has focused on stem cells that form hard tissues, in particular, bone marrow stromal stem cells (BMSSCs) and dental pulp stem cells (DPSCs). Human post-natal bone marrow stromal stem cells (BMSSCs) have potent osteogenic capacity, but progressively lose their "stemness" during *ex vivo* expansion. The expression of human telomerase reverse transcriptase (hTERT) was forced in BMSSCs by viral transduction, and it was found that this extended their lifespan and enhanced their osteogenic potential. The enhanced bone forming ability of BMSSCs-T was correlated with a higher and sustained expression of the early pre-osteogenic stem cell marker, STRO-1. To further characterize DPSCs, the self-renewal capability, multi-lineage differentiation capacity was further examined. Stromal-like cells were re-established in culture from primary DPSCs transplants and re-transplanted into immunocompromised mice to generate a dentin-pulp-like tissue, demonstrating their self-renewal capability. Although bone and dentin are extremely similar in composition, the organization of their matrix, as dictated by their respective stem cells is quite different. To identify possible differences between progenitors of osteoblasts and odontoblasts at the level of transcription, we compared the gene expression profiles of BMSSCs and DPSCs. Using commercially available arrays, a few differentially expressed genes, including insulin-like growth factor binding protein 7, and collagen type I alpha 2, were more highly expressed in BMSSCs while collagen type XVIII alpha 1, insulin-like growth factor 2, discordin domain tyrosine kinase 2, NAD(P)H menadione oxidoreductase, homolog 2 of Drosophila large disk and cyclin-dependent kinase 6 were highly expressed in DPSCs. However, it was noted that more representative arrays may be needed to further distinguish differences between the two populations.

The matrix proteins of bones and teeth play key roles in the structure and functions of these tissues. The objective of the Molecular Biology of Bones and Teeth unit, lead by

Dr. Marian F. Young, was to study their function and regulation using a combination of in vitro and in vivo analyses. To determine the function of matrix proteins in vivo, transgenic mice that are deficient in one or more Small Leucine Rich Proteoglycans (SLRPs) were generated. SLRPs are an expanding family of proteoglycans found in the extracellular matrix that contains tandem repeats of a motif rich in leucine. Previously, it was found that the biglycan (Bgn) deficient mouse fails to achieve peak bone mass and develops osteoporosis. Recent data showed that *bgn* deficient mice have diminished numbers of bone marrow stromal stem cells (BMSSC), the bone cell precursors, and that this deficiency increased with age. The cells also had a reduced response to TGF-beta, reduced collagen synthesis and relatively more apoptosis than cells from normal littermates. In addition, calvarial cells isolated from BGN deficient mice had reduced expression of late markers of osteogenic differentiation such as bone sialoprotein and osteocalcin and diminished ability to accumulate calcium judged by alizarin red staining. Because of their high degree of homology and the issue of compensation of one SLRP for another, mice deficient in Bgn and the sister molecule, decorin (Dcn) were generated. The double *bgn/dcn* KO mice had more severe osteopenia than the single *bgn* KO indicating redundancy in SLRP function in bone tissue. To further determine whether compensation could occur between different classes of SLRPs, we generated mice deficient in both *Bgn* (class I) and fibromodulin (*fm*) a class II SLRP highly expressed in mineralizing tissue. These doubly deficient mice had an impaired gait, ectopic calcification of tendons and premature osteoarthritis. TEM analysis showed that like the decorin and biglycan KO, they had severely disturbed collagen fibril structures. In summary, these studies present important new models of multiple skeletal diseases and provide the opportunity to characterize the network of signals that control the integrity of mineralized tissue through SLRP activity.

The fundamental question of how cells of bones and teeth assemble and mineralize their respective matrices in such a coordinated and superbly biofunctional way is still largely unanswered. The Matrix Biochemistry Unit, headed by Dr. Larry W. Fisher, has been performing a variety of experiments and collaborations to help determine the structure-function relationship of several of the more interesting non-collagenous proteins. One of the most abundant proteins in the mineralized matrices of bones and dentin is a protein Dr. Fisher discovered several years ago, bone sialoprotein (BSP). It is a member of a family of proteins that he recently named the SIBLING (Small Integrin-Binding LIgand, N-linked Glycoprotein) family. BSP, osteopontin (OPN), dentin sialo-phosphoprotein (DSPP), dentin matrix protein-1 and (DMP-1) enamelin are clustered within a 375,000 basepair region of human Chromosome 4 and likely represent a series of gene duplications. As the family name implies, BSP is a phosphorylated, sulfated glycoprotein that binds to integrins, particularly the vitronectin receptor, through its RGD tripeptide. Although BSP in adults is generally limited to the skeleton, work with our colleagues has shown that this molecule is likely a very good marker for the appearance of many osteotrophic cancers. This year it was determined that serum levels of both BSP and/or another member of the SIBLING family, OPN, are elevated in lung, breast, prostate and colon cancers. It was found that the complex between the SIBLING and a protein (Factor H) found in all sera must be disrupted in order to properly measure the levels of BSP and OPN in patient sera. It was also shown that DMP1 can also bind to complement Factor H and protect some tumor cells from being killed by the complement pathway of our

immune system. Furthermore, there was evidence that the mechanism for the protective properties of these proteins involves the complement protein, Factor I. This may be one mechanism that allows trophoblasts of the developing placenta to evade the complement system of the maternal blood supply and may also be similarly used by tumor cells during metastasis.

The Skeletal Clinical Studies program, currently under the direction of Dr. Pamela Gehron Robey, has continued to focus on two aspects: the role of BMSSCs in skeletal disease, and the use of BMSSCs for tissue regeneration. Patient accrual is continuing in four clinical protocols (97-DK-0055, 98-D-0145, 98-D-0146, 00-D-0183) for the study and treatment of fibrous dysplasia of bone (FD) and the McCune-Albright Syndrome (MAS). FD/MAS is known to arise from a post-zygotic mutation in the GNAS1 gene (R201C and R201H) which regulates adenylyl cyclase and ultimately results in excess cAMP production, which profoundly affects the ability of BMSSCs to differentiate and form bone. FD is found in a broad range of severity, ranging from monostotic (single bone) to polyostotic (many bones) and often in association with the MAS, which in addition to FD has multiple endocrinopathies and skin hyperpigmentation. To date, over 100 patients have been enrolled and studied extensively through clinical chemistries and various imaging modalities, and the clinical data is currently being closely analyzed. Of note, it was found that a significant percentage of the patients (28%) suffered from undiagnosed growth hormone (GH) excess, which further exacerbated their craniofacial lesions. These patients were treated with standard therapies (cabergoline and long-acting octreotide either singly or in combination) to block GH secretion from the pituitary, but GH was not totally suppressed as in other types of GH disorders. Currently, patients are being treated with a growth hormone receptor antagonist (Pegvisomant) to try and prevent further expansion of their craniofacial fibrous dysplasia. Current studies are focused on examining the progression of the disease as assessed by many different parameters (mutation load within lesions, bone scan intensity, fracture history). In screening the patients for enrollment, two who were diagnosed elsewhere were found not to have fibrous dysplasia, but rather other disorders (gnathodiaphyseal dysplasia and skeletal angiomatosis). These two cases point out the need for astute physical examination coupled with novel histological analysis and mutation analysis in order to distinguish between benign fibro-osseous lesions. The group is also progressing towards another IND application to the FDA for use of ex vivo expanded bone marrow stromal stem cells to be used in bone regeneration in the context of fibrous dysplastic lesions.

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# Gene Therapy and Therapeutics Branch

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## GENE THERAPY AND THERAPEUTICS BRANCH
## 2002

The Gene Therapy and Therapeutics Branch (GTTB) provides a model of translational research with a bench to clinic continuum. The GTTB has both a primary tissue-specific focus, asking questions related to salivary gland biology, pathology and management, as well as a primary applications focus, gene transfer technology. Saliva is the primary protective agent for the mouth and upper GI tract and thus is of primary importance to health maintenance. Perturbations of salivary secretory mechanisms can consequently lead to serious oral health problems. The GTTB is committed to the notion that significant advances in clinical care will come from our understanding of biological mechanisms and an inter-disciplinary approach to problem solving. This reporting period has seen substantial scientific progress.

The production of salivary fluid is due to neurotransmitter stimulation of transepithelial secretion of $Cl^-$ by acinar cells. A $Na^+$-$K^+$-$2Cl^-$ cotransporter, NKCC1, located in the acinar basolateral membrane drives much of this $Cl^-$ flux. This transporter is the rate-limiting step in salivary secretion. The Membrane Biology Section (MBS) continues to concentrate on understanding the structure, function and regulation of this important transport protein. The NKCC1 molecule consists of large cytosolic N and C termini (35 kDa and 50 kDa, respectively) on either side of central hydrophobic domain (50 kDa) containing 12 membrane-spanning segments. The latter contains the regions primarily responsible for ion transport and substrate affinity, while the cytosolic N and C termini are primarily involved in transport regulation.

Over the past year the MBS has made significant progress in its projects involving NKCC1. For example, staff members have identified and characterized functional regions of NKCC1 using primarily cysteine scanning mutagenesis. One of the resulting mutants (A483C) has proven to be particularly interesting since it renders the NKCC1 protein sensitive to inhibition by several sulfhydryl reagents that have no effect on the wild type transporter. Inhibition of NKCC1 at this site is independent of the presence of sodium and potassium but requires chloride, indicating that this effect is sensitive to the conformation of the protein. Mutations of surrounding amino acids indicate that the sensitive region corresponds to one face of an alpha helix suggesting that this is the secondary structure of membrane spanning segment 6. A second example involves identifying sites responsible for NKCC1 molecular dimerization. To determine regions of the protein responsible for dimer formation MBS staff expressed a series of truncation mutants of NKCC1 in HEK293 cells. They found that mutants containing 6 or more membrane spanning segments of NKCC1 tend to form dimers, however, the most significant contributor to dimerization interestingly appears to be the cytosolic C terminus of the protein.

Previously, the MBS developed a novel system for determining the transmembrane topology of integral membrane proteins like NKCC1 using intact cells, by employing a fusion protein consisting of the green fluorescent protein followed by a portion of the putative membrane spanning region of the protein of interest, then a glycosylation tag. Recent studies with this system to explore the membrane integration of the critically

important water channel AQP1 in intact cells indicate that AQP1 integrates into the membrane cotranslationally. Other studies use this system to study the topology of the putative calcium channel Trp1 (see below), which appears involved secretory signaling in salivary and other exocrine glands. MBS findings indicate that the pore region of the Trp1 is much more deeply embedded in the membrane than previously suspected and suggest that the pore is actually a part of a complexly folded hydrophobic pocket.

Neurotransmitter stimulation of fluid secretion from salivary glands is mediated via a biphasic elevation in cytosolic [$Ca^{2+}$], an initial transient increase due to internal release and a latter sustained increase due to $Ca^{2+}$ influx. GTTB's Secretory Physiology Section (SPS) is focused on understanding such $Ca^{2+}$ influx using salivary gland cells as models. This process appears to be mediated via store-operated $Ca^{2+}$ entry (SOCE), a pathway ubiquitously present in all non-excitable cells. The molecular mechanism for this influx has not yet been determined in any cell type. Recently, the transient receptor potential (TRPC) gene family of ion channel proteins has been proposed as molecular components of the store-operated Ca-2+ influx channel (SOCC). The SPS has made major progress towards characterizing SOCE and identifying the role of Trp1 in the SOCE mechanism in salivary gland cells. For example, in this reporting period the SPS showed that calmodulin and TrpC1 are involved in the $Ca^{2+}$-dependent inactivation of SOCC. Staff members identified two calmodulin-binding domains in TrpC1, aa 715-749 and aa 758-793. Calmodulin mediates $Ca^{2+}$-dependent feedback inhibition of SOCE via binding to the second domain, aa758-793, in the C-terminus of TrpC1. These findings reveal an integral role for TrpC1 in the regulation of SOCE and provide a mechanism for the $[Ca^{2+}]_i$-dependent feedback inhibition of SOCC.

Using a mutagenesis approach, the SPS recently also demonstrated that TrpC1 directly contributes to SOCC activity and that acidic amino acid residues in the TrpC1 putative pore domain (between the 5th and 6th TM regions) are involved in SOCC function. Their results demonstrate for the first time that a TrpC protein is a pore-forming subunit of SOCC and represent a major advancement in understanding of the mechanism of SOCE in salivary gland cells. Furthermore, in this reporting period the SPS has also shown that TrpC1 monomers interact via the first ankyrin-repeat domain in their N-terminus, leading to a proposal that multimerization of TrpC1 monomers, via these ankyrin repeats, is required for SOCC function. Additionally, the SPS recently identified the binding site for caveolin-1 in the TrpC1 N-terminus and shown that caveolin-1 is required for the plasma membrane localization of TrpC1 and TrpC3. Further, yeast-two hybrid analyses by SPS staff demonstrated that TrpC1 interacts with the SNARE proteins VAMP2 and SNAP, and show that VAMP2 is involved in the trafficking of TrpC3 and caveolin to the plasma membrane.

The AAV Biology Unit (AAVBU) has continued to make considerable progress in their focus on understanding the interactions of adeno-associated virus (AAV) with its host cell. The AAVBU staff previously cloned two novel AAV serotypes (AAV4 and AAV5) and showed in vitro and in vivo that these vectors may be useful to mediate gene transfer to cells and tissues poorly transduced by the commonly utilized serotype 2 AAV vectors. For example, a recombinant serotype 5 vector is ~50 fold more efficient in mediating gene transfer to cultured human airway epithelia than an AAV2 vector. Likewise,

serotype 4 AAV vectors appear more useful in targeting salivary glands than AAV2 vectors. These new viral isolates are being studied both as natural mutations of other serotypes for understanding the biology of this genus of virus and because of their unique cell tropism, as novel vectors for gene transfer. While heparin sulfate proteoglycans are important in AAV2 binding and transduction, AAV4 and AAV5 interact with the cell surface by a distinct mechanism. AAVBU studies indicate these latter serotypes bind to the cell surface via another charged carbohydrate, sialic acid. Treatment of cells with neuraminidase, competition with soluble sialic acid conjugates or resialyation experiments with specific sialyltransferases all indicate that serotypes 4 and 5 both require 2-3 sialic acid linkages for efficient binding and transduction. However, and in keeping with their different cell tropism, AAV4 requires an O-linked 2-3 sialic acid while AAV5 requires a 2-3 N-linked sialic acid.

The Gene Transfer Section (GTS) studies clinically relevant applications of gene transfer technology to salivary glands. During this reporting period the GTS addressed fundamental and practical questions necessary to move its gene transfer approaches into the clinic. For example, the GTS completed a detailed study of the toxicology and biodistribution of an adenoviral vector administered to a single rat submandibular gland. In general, there were no adverse systemic consequences to animals over the course of this study (15 days). Also, GTS staff members began work on the design of an optimal transgene cassette for salivary glands, using a variety of different promoters and other elements, in order to enhance therapeutic protein expression after gene transfer. Furthermore, in order to determine which viral vector facilitates optimal salivary expression the GTS is testing several adenoassociated virus (AAV) serotypes (2, 4, 5), conventional E1/E3 deficient adenoviral vectors, and hybrid (adenoviral with retroviral elements) vectors in rodent models using erythropoeitin (epo) as a therapeutically relevant reporter gene. Epo is readily secreted from transduced salivary glands into the bloodstream and its concentration and function are easily measured (the latter by changes in hematocrit) *in vivo*. Mechanistic studies on hybrid adeno-retroviral (AdLTR) vectors, previously reported by the GTS, have been considerably extended, by showing that AdLTR vectors infect and transduce epithelial cells like a conventional adenoviral vector, but integrate into genomic DNA much more frequently. Additionally, the GTS began to employ the AAV serotype 2 vector encoding human IL-10 that it reported last year in a pre-clinical Sjogren's syndrome (SS) model, NOD mouse submandibular glands. This maneuver prevented age and gender-related autoimmune-associated salivary flow reductions and diminished glandular lymphocytic infiltrates in this SS model. The GTS also made significant progress in finding a possible autologous cell source for the artificial salivary gland device from an unexpected source, hematopoetic progenitor cells, which also may be useful in treating SS patients (see below).

SS is an autoimmune disease, characterized as a widespread "epitheliitis", which results in dryness of the lining surfaces of the body, producing, most notably, dry mouth and dry eyes. GTTB's SS Clinic conducts clinical investigations and clinical trials, and also collaborates with laboratory investigators to elucidate pathogenic mechanisms operative in this disease. Recent studies in the SS Clinic have demonstrated that elevated IgG levels were predictive of positive labial salivary gland biopsies, a hallmark sign of SS. Also, staff members are examining the effect of different immunomodulatory treatments

on the course of SS. For example, the SS Clinic is now conducting a placebo-controlled, randomized, clinical trial (RCT) of a new biologic agent, etanercept, which is an inhibitor of tumor necrosis factor alpha. The latter is found in increased amounts in salivary and lacrimal gland tissues of SS patients. Recently, another RCT, which studied dehydroepiandrosterone, was completed. This study was based on the hypothesis that sex hormones exert immunomodulatory effects on SS. The results of this RCT showed, however, that the agent had no efficacy in treating the disease. Patients with SS have a much higher risk (~40X) of developing malignant lymphoma than the general population, and SS Clinic staff have hypothesized that increased inflammation in salivary tissue, manifesting as higher lymphocytic focus scores, as well as increased serological reactivity and higher immunoglobulin levels, may be risk factors for monoclonal expansion of B cells in SS patients. This hypothesis is being examined utilizing a gene rearrangement approach in studies with B cells obtained from the minor salivary glands of SS patients and is ongoing. To potentially facilitate a novel treatment for SS patients, SS Clinic staff and GTS staff have asked whether bone marrow progenitor cells, received by transplant patients, are capable of differentiation into either/both buccal mucosal or salivary epithelial cells. Results thus far with buccal mucosal cells strongly support the existence of such differentiation. The SS Clinic is also examining whether subsets of SS patients are identifiable by antibodies specific for distinct autoantigens. For example, patients with either primary SS and patients with limited scleroderma were found to differ markedly in their pattern of serum anticentromere antibody recognition. In addition, in 15% of SS patients these anticentomere antibodies exclusively recognized CENP-C, and were uniformly associated with antibodies to Ro and La.

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# Oral Infection and Immunity Branch

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## ORAL INFECTION AND IMMUNITY BRANCH
## 2002

The Oral Infection and Immunity Branch (OIIB) plans, fosters and carries out research relating to the causes, diagnosis, treatment and prevention of infectious and inflammatory diseases. Efforts to understand the functional and molecular organization of infectious organisms, and research into the cellular, biochemical and molecular components of inflammatory, immune and sensory responses provide the basis for dissecting the interactions between pathogens, noxious agents and the host defense system. These multifaceted approaches define fundamental mechanisms of host defense, how these pathways become dysregulated in disease, and how to intervene for the benefit of the host.

The past year has witnessed significant changes in the staff and environment of the Oral Infection and Immunity Branch. After nearly four years of renovations involving serial transfer of our research staff to swing space, all senior investigators have finally been relocated to their newly renovated research facilities. Although disruptive to our research progress, we are grateful for the opportunity to upgrade our working environment, which not only has enriched our research capabilities, but also morale as some of our research space was in shambles. During the past year, two of our senior investigators have left the Branch. Dr. Jerry Keith resigned from the NIDCR after 12 years to join the Frauenhofer Institute in Newark, Delaware. In support of the NIH Bioterrorism effort, Dr. Steve Leppla was recruited to NIAID to head up their anthrax program. Unfortunately, he will continue to occupy newly renovated space in OIIB until the new Bioterrorism building is constructed in 2005. With the departure of these two senior scientists, exciting new opportunities for recruitment and new directions have emerged and two national searches for tenure-track investigators in mucosal immunity and in host-pathogen interactions are currently in progress.

Mentoring young investigators continues to be a major emphasis of the Branch that includes our joint training programs with Children's National Medical Center for Infectious Disease Fellows and with the Department of Periodontology at the University of Maryland for Periodontal Disease Fellows. In addition, Dr. Sharon Gordon successfully completed and defended her Ph.D. thesis, a joint effort between John Hopkins University and OIIB. The entire Branch participated in a 5 hr scientific integrity workshop on authorship, publication practices and peer review led by Dr. Francis Macrina, VCU. Senior investigators mentoring their fellows and interns continue to generate diverse and exciting advances in our research portfolio, providing opportunities for translational and clinical directions. During the past year, Senior Investigators in the Branch have received recognition for their innovative research and their commitment to sharing their discoveries, transgenic animals and novel reagents with the extramural community. Recognition of the accomplishments of OIIB Senior Investigators was evident by their numerous invitations to organize, chair and speak at national and international meetings, opportunities to participate on editorial boards, and invitations to edit books and write authoritative chapters. Within the Branch, OIIB Employee of the Quarter Awards also recognized special achievements.

In another productive year, the Branch has made numerous advances and remarkable progress in many of its multiple unique and diverse research programs, as reflected in the publications in our annual bibliography. Among the scientific advances, including those featured in prestigious journals such as *Nature*, *Cell*, *Immunity*, *Proceedings of the National Academy of Sciences*, *Journal of Clinical Investigation* and *Journal of Biological Chemistry* are those highlighted in the 2002 Annual Report.

Remarkable progress continues in understanding the molecular mechanisms that enable mammals to detect and discriminate between chemosensory signals with identification of an amino acid taste receptor by members of the OIIB Taste and Smell Unit. This G protein coupled receptor, comprised of a heterodimer, T1R1+3, responds to a range of amino acids in mice, whereas in humans, the receptor combination specifically recognizes glutamate. Detailed comparison of the expression of the T1R and T2R families suggest that attractive (sweet) and repellant (bitter) responses are encoded by activation of nonoverlapping populations of cells in the periphery, which may be essential for stimuli that evoke such opposite behavioral responses. Transgenic animals are being developed to specifically investigate the functional significance of these individual receptors in taste.

Receptor engagement also underlies the host response to infectious pathogens as well as pathogen-pathogen interactions. In this regard, studies continue on defining the structure and function of bacterial surface receptors that mediate microbial adhesion, colonization and initiation of host defense. As the characterization of the *S. gordonii* 38 receptor polysaccharide gene cluster and flanking regions has been completed, evidence documents that receptor polysaccharide production and coaggregations with other oral bacteria depend on a cluster of 14 genes. The findings indicate that surface expression of receptors depends on Wzy-dependent polymerization of a heptasaccharide repeating unit, providing insight into potential disruption of coaggregation events. Another consequence of the interaction amongst oral bacteria is the exchange of soluble communication signals. One of these diffusible signals is autoinducer 2(AI-2), which has been identified in *S. gordonii,* and is synthesized by the gene product of *luxS*. Inactivation of *luxS* blocks synthesis of AI-2 and the mutants form biofilms with enhanced cellular mass, consistent with failure to accurately assess local cell densities with unregulated accumulation.

In addition to adherence to one another, oral bacteria also adhere directly to the tooth surface. Sucrose is the precursor for glycan synthesis that facilitates attachment of *S. mutans*, and its fermentation to lactic acid initiates dental caries by promoting demineralization of tooth enamel. The belief that microorganisms are unable to metabolize the five isomers of sucrose, suggests the potential of these sweet non-cariogenic compounds as substitutes for dietary sucrose. However, innovative studies conducted in the Branch during the past year have revealed rapid dissimilation of these isomers by several bacterial species including Fusobacteria, Klebsiella, Bacillus and Clostridia. Unique transport proteins and novel phospho-glycosylhydrolases participate in the metabolism of the isomers, and the relevant genes have been cloned, sequenced, and proteins expressed for characterization. The absence of these genes in *S. mutans* explains the failure of the oral bacterium to ferment the isomers. In view of the potential for inter-species transfer of genetic information, studies with sucrose isomers suggest caution in the widespread use of palatinose and leucrose as sucrose substitutes.

Importantly, the determination of the solution-state conformations of the phosphorylated derivatives of sucrose and its isomers may permit the rational design of sucro-based inhibitors for selective targeting of pathogenic microbes.

Microbes such as *B. anthracis* are also dependent on interactions with receptors on their targeted host cells. *B. anthracis* produces a tripartite anthrax toxin, the components of which function coordinately to bind cell receptors, enter cytoplasmic vesicles, and escape to the cytosol to alter cAMP and disrupt MAP kinase pathways. The unique ability of the protective antigen (PA) to bind to cell surface receptors, recently identified as tumor endothelial marker 8 (TEM8), provides the nidus for defining the linked intracellular events culminating not only in infected cell death, but also the death of the host. Additional studies have focused on further identification of virulence genes based on the availability of the nearly complete DNA sequence of the *B. anthracis* genome. These important studies and the development of PA as a vaccine candidate will be transferred to the NIAID.

In leukocytes, disruption of receptor signaling events may also provide a mechanism to control potentially negative consequences. Studies continue to dissect out the intracellular events in a mast cell model pursuant to engagement of Fcepsilon receptor I, which culminate in release of histamine and other inflammatory mediators. Members of the Receptor and Signal Transduction Section continue to identify critical regulatory intermediates in the signaling pathway including the protein tyrosine kinase Syk, Cbl, E3 ubiquitin ligase, and Gab2, a scaffolding molecule which interacts with multiple molecules in the signaling cascade to downregulate aggregated receptor complexes. Another important new breakthrough in the past year is represented by the first isolation and characterization of a lineage committed bone marrow mast cell precursor.

Aberrant host responses to noxious, infectious or self-antigens can result in substantial pathology and/or the development of autoimmune diseases. Prevention/inhibition of autoimmune diseases and allotransplant rejection as well as the evolution of cancer pathogenesis and immune deficiency rely in large part on a population of endogenous suppressor T lymphocytes. These CD4+CD25+ T cells are functionally anergic and have suppressor activity, but the mechanisms underlying their suppression remain hotly contested. Current studies in the Cellular Immunology Section have provided novel evidence that CD4+CD25+ suppressor T cells express surface bound active TGF-beta, which, through its upregulated signaling receptor, TBRII, is critical in maintenance of their anergic status and in driving suppression of normal T cells. Phosphorylation of Smad2/3, key mediators for TGF-beta signaling, is an intracellular consequence of this interaction that can be reversed by engaging the glucocorticoid induced TNF receptor. These data link multiple divergent observations into a coherent picture of CD4+CD25+ T cell mediated suppression.

Failed suppression of autoreactive T cells results in the development of autoimmune type 1 diabetes. The ability to distinguish type 1 from non-autoimmune type 2 diabetes is important for therapeutic intervention trials. Members of the Oral Medicine Section have previously identified and isolated one of the major autoantigens, IA-2, in type 1 diabetes

and over 70% of patients have autoantibodies to IA-2. IA-2 is a member of the transmembrane protein tyrosine phosphatase (PTP) family and is located in secretory granules of neuroendocrine cells. However, because of two amino acid substitutions in the catalytic domain of the molecule, IA-2 is enzymatically inactive and its function is not known. During the last year, the function of IA-2 was assessed by targeted disruption of the gene and IA-2-/- animals exhibited statistically significant elevations of glucose and depressed insulin release. From these studies, it appears that IA-2 is involved in glucose-stimulated insulin secretion and represents an intervention target.

## ORAL INFECTION AND IMMUNITY BRANCH

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# Oral and Pharyngeal Cancer Branch

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## ORAL AND PHARYNGEAL CANCER BRANCH

## 2002

Cancer of the head and neck area is the sixth most common neoplastic disease in the developed world, representing a very serious public health problem based on annual morbidity and mortality rates. The molecular and etiological factors involved in the development of head and neck tumors, including oral cancers, are still largely unknown. Members of our Branch work on complementary basic, translational, and clinical aspects of cancer research, in an effort to understand the molecular basis for malignant transformation as well as tumor invasion and metastasis, and to use this knowledge for the development of molecular markers of disease progression and novel therapeutic approaches for oral malignancies.

During the current reporting period, we have continued to expand the breadth and depth of our program by making a concerted effort in areas of direct relevance to head and neck cancer research. This includes the continuing efforts of Dr. Myung Hee Park, Dr. Thomas Bugge, Dr. Adrian Senderowicz, and Dr. J. Silvio Gutkind's teams, whose research programs address the most basic molecular mechanisms involved in normal and aberrant cell proliferation, tissue remodeling, squamous carcinogenesis and tumor metastasis, as well as the development and evaluation of novel mechanism-based therapeutic approaches for head and neck cancer patients. These directions are expected to provide a better understanding of the still unknown molecular alterations that lead to oral malignancies, thus broadening the horizon on developing potential oral tumor markers and treatments modalities.

Our Branch has been highly productive during this year, and has made substantial contributions to the field, providing new concepts and shedding new light on questions of fundamental importance for cancer biology. We have also developed a large number of novel reagents, such as new genes, expression vectors, cell lines, and bioactive peptides of value to biomedical research, and provided them to hundreds of investigators in the U.S. and abroad. Another tradition of our Branch has been our high priority on the training of postdoctoral investigators to become independent leaders in the field; we have tried to continue and strengthen this commitment.

During the current reporting period, significant progress has been made in a number of research efforts at the OPCB. A variety of arbitrarily selected research advances are highlighted below. The progress report for each project provides a more comprehensive description of the major findings in our Branch.

The near completion of the human genome project and the recent development of novel, highly sensitive high-throughput techniques have now afforded the unique opportunity to perform a comprehensive molecular characterization of normal, precancerous, and malignant cells. As part of these efforts, the Head and Neck Cancer Genome Anatomy Project (HN-CGAP) was established as a joint program between NCI and NIDCR aimed to identify the nature of those genes expressed during HNSCC development. Recently, we have used the bioinformatic tools in the CGAP website to analyze the database from

the HN-CGAP libraries derived from laser-capture microdissected (LCM) normal and HNSCC tissues contributed by our ongoing effort. Differential expression of genes between normal and tumor tissues were further confirmed using an oral cancer specific cDNA microarray. A very high number of genes were discovered from these libraries, and highly expressed known genes uniquely expressed in normal or HNSCC libraries were also identified, whose contribution to HNSCC progression can now be investigated. We have also made a concerted effort to study protein expression profiles during HNSCC tumor development. In collaboration with the NCI, we have demonstrated the feasibility of utilizing antibody arrays for the study of protein expression in LCM-procured material, an effort that helped to identify a number of unexpected alterations in the otherwise phenotypically normal tissue adjacent to neoplastic lesion. We have also characterized gene expression profiles in NHGK (normal human gingival keratinocytes) and several HNSCC's, using cDNA microarrays, which revealed groups of genes upregulated or down regulated in HNSCC. Genes upregulated in HNSCC include oncogenes and growth regulatory factors. Genes commonly downregulated in HNSCC include many keratins, differentiation markers, growth arrest or tumor suppressor gene, cell surface and extracellular matrix proteins and various protease inhibitor proteins. These efforts, together with other multi-institutional genomic and proteomic initiatives are expected to contribute to the complete understanding of the molecular pathogenesis of HNSCCs, thus helping to identify new markers for the early detection of preneoplastic lesions and novel targets for pharmacological intervention in this devastating disease.

Proteolytic modification of the extracellular matrix is essential for physiologic tissue remodeling, as well as for the progression of a number of chronic, degenerative diseases including cancer invasion, and metastasis. We have addressed the biochemistry, biology, and pathology of selected cell surface-associated serine proteases, in particular, their relation to the development, regeneration, and malignant transformation of keratinized epithelium. Studies included the role of the plasminogen activation (PA) system in cancer development. For example, we found that plasminogen (Plg) promotes the orthotopic progression of fibrosarcoma in mice, and that host Plg and tumor cell-produced Plg activator cooperate to promote fibrosarcoma progression independently of host Plg activator, Plg activator inhibitor, and urokinase Plg activator receptor (uPAR). Furthermore, we have linked tumor cell Plg activation to the promotion of tumor angiogenesis. On the other hand, Plg-deficiency impairs both squamous cell carcinoma progression and skin wound healing. In collaboration with the MMPU, we have found that Plg activation facilitates keratinocyte-mediated collagen breakdown via the direct activation of matrix metalloproteinase-13 and possibly other fibrillar collagenases. The novel pathway underscores the likely existence of multiple, cell type and context-specific pathways for collagen dissolution, which may be relevant to physiological remodeling of the skin and oral cavity, as well as to HNSCC progression. Building on our expertise in LCM and cDNA array analysis, we set out to identify novel serine proteases associated with skin wound healing and squamous cell carcinogenesis. This analysis led to the identification of the type II transmembrane serine protease, matriptase, as a protease that is expressed by reepithelializing wound keratinocytes and in HNSCC. By introducing a null mutation into the mouse matriptase gene, we have found that this novel membrane serine protease has pleiotropic functions in epidermal development. We are currently unraveling the specific functions of matriptase in epidermal development, and are

determining the contribution of matriptase to skin wound healing and squamous carcinogenesis.

Receptors exhibiting an intrinsic tyrosine kinase (RTKs) activity or that are coupled to heterotrimeric G proteins (GPCRs) can effectively stimulate growth promoting pathways in a large variety of cell types and, if persistently activated, these receptors can also behave as dominant-acting oncoproteins. In this regard, the ability to stimulate GTPases of the Rho family appears to be a central component of the mitogenic and transforming pathway utilized by these cell surface receptors. Work in our laboratory led to the identification of a novel family of guanine-nucleotide exchange factors (GEFs) that provides a direct link between heterotrimeric G proteins of the $G_{12/13}$ family to Rho stimulation. Recently, we observed that tyrosine kinases such as FAK, can phosphorylated this family of RhoGEFs, thus providing evidence of the existence of a novel biochemical route by which tyrosine kinases may regulate the activity of Rho. Using a yeast two-hybrid approach to screen for molecules interacting with the PDZ-domain of one of these RhoGEFs, PDZ-RhoGEF, we identified PlexinB2 as a candidate interacting molecule. Plexins represent a novel family of transmembrane receptors that transduce attractive and repulsive signals mediated by the axon-guiding molecules semaphorins, which have been also implicated in cell migration and metastasis. The association of Plexins with PDZ-RhoGEF thus provides a direct molecular mechanism by which semaphorins acting on Plexin B can control Rho, thereby regulating cell migration and growth. However, which genes are in turn regulated by Rho GTPases, and how they contribute to cell growth is not known. To address this issue, we investigated the global gene expression pattern induced by Ras and Rho GTPases by cDNA microarray analysis. By this approach, we identified key genes that were up- or down- regulated with a pattern both unique and common to Ras and Rho GTPases. These results will provide important information to begin unraveling the complexity of the molecular mechanisms underlying the transforming potential and cell cycle effects induced by these small GTPases.

We have expanded our drug evaluation effort at the NIDCR, whose goal is to develop novel therapies aimed at improving the quality of life and life expectancy of oral cancer patients. We have focused on novel molecule affecting the cell cycle and angiogenesis, and exploited caharteristics of cancer cells to develop novel cytotoxic agents. Two drugs, UCN-01, a novel protein kinase C (PKC) inhibitor, and perifosine, a novel alkylphospholipid with antitumor properties, were found to exhibit potent antiproliferative effects in HNSCC cells. For example, UCN-01 caused a $G_1$/S arrest and apoptosis in all HNSCC lines tested, including radiation-resistant cells, and displayed a very significant antitumor response in *in vivo* xenograft studies using a HNSCC model. Moreover, the first Phase I trial of UCN-01 in patients with refractory neoplasms was performed in collaboration with NCI with interesting results. Thus, HNSCC patients may be a good population for testing UCN-01 in the Phase II clinical setting. Basic studies on UCN-01 and perifosine may facilitate their biochemical assessment in tissues from patients during the course of future clinical trials. Parallel efforts have focused on flavopiridol, a novel cdk inhibitor with potent antiproliferative activity in *in vitro* and *in vivo* models of HNSCC. The basic mechanisms by which flavopiridol induce cell cycle arrest and apoptosis were investigated. Furthermore, the first Phase I trial of bolus flavopiridol in cancer patients was recently completed in collaboration with the NCI, and

based on the encouraging results of this trial, we opened a Phase II protocol in patients with recurrent/metastatic HNSCC in collaboration with NCI and NIDCD. We have also taken advantage of our expertise on deoxyhypusine hydroxylase function to explore whether its inhibitors may display antiproliferative activities. Indeed, we have recently found that an inhibitor, ciclopirox, displays potent antiangiogenic and antitumor activity. Similarly, as the acquisition of cell surface urokinase plasminogen activator activity is a hallmark of human malignancy, we exploited this tumor-related activity to design novel anticancer approaches by engineering an anthrax toxin that is specifically activated by cell surface uPA. In collaboration with the BTTS, OIIB, we observed that the engineered toxin displayed limited toxicity to normal tissue but potent tumoricidal activity that was strictly dependent on tumor cell surface PA, showing that a simple change of protease activation specificity converts anthrax toxin from a highly lethal to a potent tumoricidal agent.

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# Pain and Neurosensory Mechanisms Branch

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## PAIN AND NEUROSENSORY MECHANISMS BRANCH
## 2002

The mission of the Branch is to increase understanding of the mechanisms underlying pain and inflammation, improve the diagnosis and treatment of chronic orofacial pain, and to disseminate this information to the biomedical community with emphasis on the needs of the dental profession. Orofacial pain has been a major problem since dentistry's primitive origins in the pre-anesthetic era. Pain as a barrier to oral health care has forced dentists into the forefront of pain research, as evidenced by the early establishment of the NIDCR pain research program in the 1950's to the international leadership of pain research to this day. Unfortunately, the vexing problem of chronic orofacial pain remains a therapeutic enigma that can progress from transient symptoms in most patients to chronic suffering with significant potential for iatrogenic injury with aggressive treatment. The PNMB is now making the transition from a broad program of basic and clinical research to a more focused translational program targeting the molecular-genetic mechanisms that contribute to the development of pain chronicity in the orofacial region and interventions to prevent or reverse these mechanisms.

### Rationale for a Translational Pain Research Program

Multiple sources indicate that the prevalence of pain and inadequate analgesia continues to be a large public health problem. A national pain survey indicates that approximately 34 million adults suffer from some form of chronic pain. The frequent undertreatment of pain associated with cancer in both adults and children is described in the *Management of Cancer Pain, Clinical Practice Guideline* (Jacox et al. 1994). Improvements in pain and symptom control modalities and their application to palliative care needed in the late stages of cancer and other chronic diseases have not kept pace with medical advances prolonging life (Foley and Gelband, IOM 2001). As a consequence, an estimated 75% of patients with advanced cancer experience moderate to severe pain, with severe unrelieved pain reported by 40% of the population at the end of life. Many studies suggest that pain is under-treated in children following surgery with an estimated 36-80% of pediatric patients being undermedicated on the first day after surgery, depending on the criteria used. A recent study of symptoms and suffering at the end of life in children found that 89% suffered significantly from at least one symptom in their last month of life, most commonly pain, fatigue, or dyspnea despite aggressive treatment.

Rational drug development has failed to produce a single breakthrough in efficacy, despite many successes in the laboratory, supportive of the need for alternative approaches to analgesic research to optimize opportunities for translating scientific advances into improved pain relief through the development of more effective and safer treatments.

### Organizational Strategy for the PNMB Translational Research Program

The PNMB remains the only NIH intramural research program focusing on the neurobiology of pain and analgesia. As such, we are uniquely poised to capitalize on findings and mechanisms emerging from the intramural neuroscience community (>200 investigators and fellows), the resources of the Clinical Center, our network of

collaborations both on campus and nationally, and the technological advances inherent in the fields of genomics and proteomics.

**Hypothesis Generating Basic Studies**

Studies of the mechanisms of the transduction of nociceptive afferent stimuli at the primary afferent ending and nociceptive processing in the dorsal spinal cord comprise the basic research targets of the Neuronal Gene Expression Unit (NGEU). A primary research target is the molecular and physiological properties of the heat/capsaicin activated vanilloid receptor (VR1), one of the main channels responding to and integrating thermal and chemical noxious stimuli. These studies have suggested that acidic pH encountered in tissue inflammation modulates the conductance and affinity properties of VR1 rendering the receptor more sensitive to nociceptive stimuli and neuroactive substances. Two populations of VR1 in the cell have been identified: one at the plasma membrane, and a high concentration of VR1 in the endoplasmic reticulum. The potential functional role of endoplasmic reticulum VR1 to increase cystolic $Ca^{++}$ concentration is consistent with increased peripheral release and depolarization processes in the nerve ending, suggestive of a mechanism to modulate C-fiber activity in chronic inflammatory processes.

The cellular consequences of VR1 over-activation were explored with the ultrapotent agonist resiniferatoxin (RTX) to demonstrate calcium cytotoxicity suggestive of a cell deletion approach for the treatment of chronic intractable pain. Studies of RTX administered by the intrathecal route demonstrated a long-lasting suppression of pain signaling by thermal nociception and inflammatory hyperalgesia, but not for high threshold mechanosensitive nociceptors. Intraganglionic administration into the trigeminal ganglion resulted in a rapid loss of a capsaicin-induced behavioral measure (eye wipe response) that was sustained up to 5 months. The effect was unilateral and no abnormal behavior or appearance was noted, supportive of a selective effect on VR1-mediated nociception and warranting preclinical and clinical evaluation of this therapeutic strategy.

Preliminary evaluation of vanilloid agonists as preemptive analgesics was performed in the oral surgery model of acute pain by injecting capsaicin at three sites around an anesthesized third molar prior to its extraction. The highest dose of capsaicin resulted in less postoperative pain than saline injections at 3-4 hours after surgery and at 24 and 48 hours indicating that the observations in the rat studies are transferable to the human. Preclinical studies with RTX administered peripherally into one hind paw resulted in: (1) single dosing producing long duration pain inhibition; (2) a peripheral site of action and minimization of central nervous system involvement; (3) selectivity due to inactivation of only VR1 expressing nerve terminals. RTX treated animals were also insensitive to carrageenan-induced inflammatory hyperalgesia.

This series of experiments ranging from characterization of the vanilloid receptor *in vitro*, evaluation of agonists *in vivo* to demonstrate proof of concept, and clinical evidence of therapeutic potential show promise for development of a novel therapeutic strategy that may be useful for preemptive analgesia and the management of intractable pain. Similar

strategies are being employed in the dorsal horn of the spinal cord to identify novel genes that are expressed by persistent nociceptive input that may provide insights into synaptic plasticity and molecular alterations in persistent pain states. Identification of the cystatin C gene following peripheral inflammation in animals led to demonstration that cystatin C, a secreted cysteine protease inhibitor, was increased in CSF during labor in humans. While the functional significance of this phenomenon is not readily apparent, this study demonstrates the potential for an integrated genomics-proteomics approach to advance clinical pain research.

**Translating from "Bench to Bedside"**

The ability to assess the therapeutic potential of a novel therapeutic strategy in humans is limited by the sensitivity of the human pain model to detect changes in biologically-relevant endpoints. Demonstration of shifts in the stimulus-response to experimental pain stimuli has been widely used to achieve sensitivity but may be irrelevant to the study of pain from tissue injury or nerve damage. The oral surgery model has proven useful for over 25 years for predicting the clinical utility for opioids and non-opioid drugs but is now largely used in multicenter studies to achieve rapid evaluation of investigational drugs for pharmaceutical studies. The PNMB has extended the oral surgery model to evaluate analgesic mechanisms, neuroendocrine responses to pain and intraoperative stress, and the relationship between local release of inflammatory mediators and pain reports of pain and analgesia. This approach serves as a paradigm for translation clinical trials to provide proof of concept for novel therapeutic strategies.

Arguably the only pharmacologic improvement for pain in the last decade has been the introduction of the selective COX-2 inhibitors as effective analgesics with a greater safety profile than dual COX-1/COX-2 inhibitors. Preclinical evaluation of selectivity was done using *in vitro* systems or *ex vivo* studies with blood cells taken from humans chronically administered the selective COX-2 inhibitor or a traditional NSAID. An early report soon after release of celecoxib suggested that the in vivo selectivity in humans was dose related and less than predicted by the preclinical trials. Use of microdialysis probes with measurement of perfusate for markers of COX-1 and COX-2 over the first few hours after oral surgery permitted direct assessment of the *in vivo* selectivity of a COX-2 inhibitor in humans. While administration of ibuprofen suppressed products of both COX-1 and COX-2 at all time points in comparison to placebo, celecoxib only suppressed products of COX at time points associated with COX-2 expression. No effect was seen on thromboxane $B_2$ or prostaglandin $E_2$ over the first 60 minutes post-surgery. Parallel studies conducted in collaboration with the NGEU demonstrated expression of COX-2 at 60 and 120 minutes post-surgery with continued expression of COX-1 throughout the postoperative period, consistent with its role as a constitutive enzyme. These data not only support the selectivity of a COX-2 inhibitor in a human model of pain from tissue injury but also illustrate the sensitivity and versatility of the oral surgery model as a powerful tool for translational pain research.

**Developing Novel Therapeutics for Chronic Pain**

The approach to evaluating mechanistic hypotheses and novel therapeutics for chronic pain has traditionally involved administration of a maximally tolerated dose of a drug

with activity predictive of an effect on a mechanism of interest, e.g. dextromethorphan for neuropathic pain. The failure of many pharmaceutical programs based on activity in rat neuropathic models to demonstrate activity in human trials has prompted reconsideration of the approach taken by PNMB investigators in the search for novel therapeutics for chronic pain. A small subset of subjects with idiopathic trigeminal neuralgia took part in a pilot study examining the effects of topiramate, a drug that blocks AMPA/kainate currents and sodium channels, with a reduction in all patients in comparison to placebo. The series of clinical trials evaluating NMDA receptor blockers suggests that they have a poor therapeutic ratio in the treatment of neuropathic pain and that other interventions directed at glutamate receptors should be investigated. Subsequent studies in the oral surgery model with an investigational mixed blocker of AMPA receptors and parallel studies at another institution with the same drug are efficacious for reducing signs of evoked hyperalgesia, in distinction to spontaneous pain. These findings are supportive of the need to evaluate drugs for neuropathic pain across a wide variety of clinical conditions, use signs of hyperalgesia as the primary outcome, and continue to evaluate drugs that are more selective for the GluR5 kainate receptor.

The wide inter-individual response to nerve injury and analgesic drugs invites speculation on a genetic component to neuropathic pain. Collaborative research by a scientist on a sabbatical visit to PNMB resulted in identification of a trait for autotomy (a putative model for phantom limb pain) on a portion of mouse chromosome 15. This is leading to a clinical genetics in a large cohort of patients with painful lumbar disc herniation and nerve root compression. The recently approved clinical protocol will collect DNA from up to 500 patients and examine candidate genes in the Laboratory of Neurogenetics, NIAAA where a fellow in the clinical trials unit has been learning genotyping procedures. If successful, this study may provide a basis for larger genetic association studies in chronic pain that might increase understanding of the inter-individual variation in the development of chronic pain following nerve injury.

**Future Directions for Translational Pain Research**

The training, experience, and research interests of the three PNMB senior investigators are complimentary and provide an experienced nucleus for collaborative efforts, as well as semi-autonomous research within their own research units. We will continue to address the problems of chronic orofacial pain with several approaches based on the expertise of current staff and through eventual recruitment of collaborators, trainees and staff with complimentary expertise needed to provide national scientific leadership in the field of chronic orofacial pain. A chronic orofacial pain clinic has been scheduled to coincide with the Pain and Palliative Care Service outpatient clinic to evaluate patients for ongoing and planned studies of temporomandibular disorders. Continued progress in the development of investigational treatments for intractable pain (RTX, the substance P-Pseudomonas exotoxin fusion protein, and unforeseen novel strategies) will form the basis for small pilot studies in carefully selected patients to test the safety and potential effectiveness as alternatives to unrelieved pain or the risk of iatrogenic injury through surgical interventions. A significant part of the problem of inadequate treatment for chronic orofacial pain and the persistence of non-validated treatment modalities is the continued reliance on training in pain diagnosis and management by the dental school

faculty with little or no advanced training in pain treatment or research. The current clinical research fellowship developed under the leadership of the Scientific Director and the Office of Education now has six fellows, with three of the current fellows engaged in pain research. The standardized didactic track for the trainees, requirements for developing their own clinical protocols, and the clinical and pain research environment is conducive to most of these fellows being placed into dental schools to foster a tradition of pain management and pain research that supercedes current practices. This long-term approach needs to be supplemented by continuing efforts at re-educating dental practitioners relying on inadequate training and research expertise to differentiate between popularized but unproven treatments for pain and validated, safe treatments. PNMB staff will continue to use traditional approaches to continuing education (talks, review articles, chapters, texts, etc) but are in the process of re-designing the Branch website to provide therapeutic updates, the latest results of our research that is relevant to treatment of orofacial pain, and as a possible link to web-based clinical trials at collaborative study sites. In addition, several chapters in Dr. Max's web-based text are devoted to diagnosis and treatment of chronic orofacial pain.

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# Functional Genomics Unit

Ashok Kulkarni

## FUNCTIONAL GENOMICS UNIT
## 2002

The Functional Genomics Unit (FGU) focuses on research breakthroughs to dissect molecular pathways pertinent to the disorders affecting craniofacial and dental systems as a result of genetic abnormalities and environmental factors. The Unit's major research focus is centered on functional genomics. Since its inception in 1996, the key research work of the Unit has concentrated on the following studies:

In the first set of studies, we continue to define molecular roles of cyclin dependent kiase-5 (Cdk5) in neuronal phosphorylation, neuronal migration and NMDA receptor biology to gain insight into its involvement in brain development, function and neurodegenerative disorders. We generated Cdk5 null mice which exhibit perinatal mortality associated with gross lesions in the brains and spinal cords. These mice lacked normal stratification of the neurons along with cerebellar defoliation, ectopic facial nerve nucleus, accumulation of neurofilaments in the neuronal cell bodies and ballooned motor neurons. Further studies confirmed a typical inverted cortex in these mice with a special "cell autonomous" role of Cdk5 in neuronal migration. The reconstituted Cdk5 expression in the neurons corrected this phenotype indicating its critical role is only associated with neuronal cell types. We have also generated Cdk5 "conditional" knockouts that exhibit abnormal gait, postures and reflexes mimicking ALS-like condition indicating a possible peripheral neuropathy. Our ongoing studies indicate synergistic actions of Cdk5, reelin and mDAB in brain development and particularly in positioning of facial branchiomotor and inferior olive neurons, involvement of Cdk5 in NMDA receptor biology and neuronal apoptosis.

In the second project, we continue to work on Fabry disorder because of its unique nature as a painful and fatal metabolic disorder and the challenges it presents in developing much needed therapeutic approaches. Following the cloning of the murine gene of alpha-galactosidase A, the gene involved in Fabry disease, we generated null mice, which exhibit lipid inclusions in the target organs typically seen in Fabry patients. Subsequent studies revealed that the aging of these mice accentuates and bone marrow transplantation ameliorates the phenotpye of these mice indicating a potential for BMT as a therapy for some of the Fabry patients. Additionally, we identified greater prevalence of dental and oral defects in Fabry patients and mutant mice. Our recent studies indicate lipid inclusions in salivary glands of the Fabry mice. We are currently analyzing salivary gland function in these mice.

In the third project, we have made rapid advances in characterizing the role of amelogenins in enamel formation. Amelogenins are mainly expressed by ameloblasts and secreted to form a major component of the enamel matrix. These proteins play an important role in enamel formation, defects in which are implicated in amelogenesis imperfecta. Amelogenin null mice generated in our laboratory displayed characteristic discoloration and attrition of teeth associated with defective enamel formation. Presence of amelogenins in cementum, as shown by immunostaining using anti-amelogenin antibodies, suggests a potential role for these proteins in cementum. The objective of the

present study was to analyze the defects in the cementum in the amelogenin null mice in order to delineate the function of amelogenin during cementogenesis. Teeth of amelogenin null mice and their littermate controls at different ages were analyzed using light microscopy, electron microscopy and micro-radiography. EM analysis of the null teeth revealed cobbled roughness on their root surface whereas wild-type mice have a relatively smooth surface. Light microscopic analysis of tooth sections of the null mice displayed significant increase in the number of cementicles (~6 fold increase in the null mice). A number of lacunaes were noticed in the cementum of the null mice indicating resorption of cementum and dentin. Periodental-ligament fibers were frequently seen deeply embedded into these lacunaes. These findings suggest that amelogenin plays an important role not only in enamel formation but may also participate in cementogenesis.

These mice display unique phenotype with enamel hypoplasia, cuspal attrition, and enamel undermineralization similar to that seen in amelogenesis imperfecta. We continue to delineate in vivo role of dentin sialophosphoprotein gene (dspp) in dentinogenesis. We have cloned and characterized the structure, regulation and functions associated with the dspp gene products. We have developed a transgenic animal model with a reporter gene (ß-galactosidase) under the control of 5.7 kb 5' flanking dspp and the analysis of these mice validates this promoter for tooth specific expression of the candidate genes. We have now generated dspp null mice that display a novel phenotype with numerous dentin defects.

We continue to study role of transforming growth factor-ß (TGF-ß) in inflammation and tooth mineralization. Our ongoing studies indicate important nature of the autocrine role of TGF-ß isoforms. The targeted perturbations in TGF-ß expression in teeth resulted in gross abnormalities in tooth mineralization, abnormal ECM formation, and in some cases tooth discoloration and loss. We have also recently identified expression of numerous members of crystallin family and down regulation of their expression by TGF-ß1 in teeth. Our ongoing studies are focused on delineating role of TGF-ß signaling pathway and the downstream target genes involved in tooth development and disease.

Besides publishing extensively, our Unit distributes its research materials by licensing, donating to repositories, and providing numerous gifts to research colleagues. FGU has filed numerous invention reports and completed many Material Transfer Agreements with extramural researchers to provide mouse models and other reagents. FGU members continue to be invited as featured speakers at national and international meetings and they are also actively engaged in collaborations with members of the extramural research community. FGU members are also active in mentoring summer interns from high schools, universities and dental colleges.

**FUNCTIONAL GENOMICS UNIT**
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# Immunopathology Section

Larry Wahl

## IMMUNOPATHOLOGY SECTION
## 2002

Research in the Immunopathology Section focuses on the biological mediators and signal transduction pathways involved in the modulation of human monocyte functions that may contribute to the immunopathology associated with various disease states. Connective tissue destruction is associated with many diseases in which the monocyte/macrophage is a prominent cell. Since matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) are believed to play a major role in the destruction and remodeling of connective tissue, a major emphasis has been placed on how these enzymes and inhibitors are regulated. Research is also conducted on the role of MMPs and TIMPs in the regulation of cellular functions that are not directly related to connective tissue metabolism.

As monocytes migrate into an inflammatory site they are exposed to multiple cytokines that influence the MMPs produced by these cells. Our previous work has demonstrated that either TNF-alpha or GM-CSF enhanced MMP-9 production by monocytes whereas both cytokines are required for the induction of MMP-1. Our ongoing research in this area has focused on interferon (IFN)-gamma. IFN-gamma alone does not induce monocyte MMP production, however in the presence of GM-CSF it stimulates the production of TNF-alpha that in turn results in the synthesis of MMP-1. In contrast to MMP-1, IFN-gamma inhibited TNF-alpha-induced MMP-9 through a caspase-dependent pathway involving caspase 8 and downstream caspases as demonstrated by the restoration of MMP-9 with specific caspase inhibitors. Western analysis revealed that only when IFN gamma was added with TNF-alpha was the p26 kDa fragment of caspase 8 detected indicating the initiation of the second cleavage process that leads to the active p18 and p10 fragments of caspase 8. Activation of the caspase pathway by IFN-gamma did not result in increased apoptosis as shown by annexin V staining, demonstrating that caspases regulate MMP-9 through an apoptosis-independent pathway. Thus IFN-gamma in the presence of GM-CSF and/or TNF-alpha differentially regulates monocyte MMPs through induction of TNF-alpha and a novel mechanism involving caspases.

Migration of monocytes from the vasculature into sites of inflammation where they differentiate into macrophages may result in changes in the expression of inflammatory mediators, such as MMPs and TIMPs. To examine this possibility monocytes and monocyte-derived macrophages after 14 days of culture were compared for their production of specific MMPs and TIMPs in response to stimulation by TNF-alpha and GM-CSF. Maturation of monocytes into macrophages resulted in decreased MMP-1 expression whereas MMP-9 was increased. Moreover, whereas monocytes produce mainly TIMP-1 and little or no TIMP-2 the opposite is true for macrophages. Our previous studies have shown that MMP-1 production by cytokine-stimulated monocytes is PGE2-dependent whereas MMP-9 is PGE2-independent. Analysis of cyclooxygenase-2 (COX-2) revealed that, unlike monocytes, cytokines failed to induce this enzyme in macrophages. However, COX-2 and MMP-1 were induced in macrophages when PGE2 was added with the cytokines. Our initial data show that the inability of cytokines to stimulate COX-2 in macrophages is related to decreased cytosolic phospholipase-A2 activity resulting in a decrease in the release of arachidonic acid that can be utilized by

COX-1 to generate the initial PGE2 required for the induction of COX-2. These findings indicate that the PGE2 supplied by monocytes and other cells at an inflammatory site in conjunction with cytokine stimulation enables macrophages to produce MMP-1 through a PGE2-dependent pathway.

The ratio of low-density lipoprotein (LDL) to high-density lipoprotein (HDL) is well established as a contributor to the pathology associated with human atherosclerosis and possibly other diseases where the monocyte is a prominent cell. Oxidation of LDL (ox-LDL) results in its binding to and uptake by monocytes/macrophages, a process that is involved in the formation of foam cells. We examined these lipoproteins for their ability to regulate monocyte MMP-1 and -9 that are thought to be important in the rupture of vulnerable atherosclerotic plaques. While ox-LDL alone did not induce monocyte MMPs, when added to TNF alpha and GM-CSF stimulated monocytes it caused a significant enhancement of MMP-1 and a slight increase in MMP-9. Moreover, the enhancement of MMP-1 production by ox-LDL occurred through, in part, a PGE2-dependent pathway as indomethacin suppressed and PGE2 restored MMP-1 production. In contrast to ox-LDL, HDL suppressed the induction of monocyte MMPs by cytokines. Thus the ratio of LDL to HDL is critical in determining the production of MMPs by monocytes at an inflammatory site.

Angiotensin II (Ang II)-mediated hypertension increases the risk for acute coronary syndrome a consequence of atherosclerotic plaque rupture, which may be caused by MMPs. Since Ang II may impact on monocyte/macrophage functions in the atherosclerotic plaque as well as other chronic inflammatory lesions we examined the potential role of Ang II in the regulation of monocyte MMPs. Monocytes have all the components of the renin-angiotensin system to produce Ang II. This was demonstrated by the release of Ang II from TNF-alpha and GM-CSF stimulated monocytes. Addition of exogenous Ang II to activated monocytes caused a significant increase in MMP-1 and MMP-9 production. Labeling studies revealed that monocytes have receptors for Ang II. Ang II mediates many of its effects through the AT1 receptor, however the AT1 receptor antagonist, [Sar1, Ile8]-Ang II enhanced the production of MMPs by activated monocytes. In contrast the AT2 receptor antagonist, PD123319, suppressed MMPs. Thus Ang II induces an increase in MMP production through the AT2 receptor and when the AT1 receptor is blocked there is an enhancement of MMP production due to an increase in the amount of Ang II that binds to the AT2 receptor. Addition of Ang II or the AT2 receptor agonist to cytokine stimulated monocytes resulted in a significant increase in PGE2. This is, in part, the mechanism through which Ang II increases MMP production since indomethacin or aspirin blocked the enhancement of MMPs by Ang II. These findings demonstrate that, in addition to atherosclerosis, Ang II may be an important mediator of monocyte MMPs in chronic inflammatory lesions. Moreover, aspirin and/or angiotensin converting enzyme inhibitors, such as captopril, may be beneficial in treatment of these lesions.

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# Matrix Metalloproteinase Unit

Henning Birkedal-Hansen

## MATRIX METALLOPROTEINASE UNIT
## 2002

The Matrix Metalloproteinase (MMP) Unit investigates the role of MMPs and their inhibitors in physiologic and pathologic processes. Recently, we have focused particular attention on membrane-bound metalloproteinases and their dual role in degrading the extracellular matrix and activating other members of the MMP family.

**Mechanisms of Bone Formation in Response to Targeting the MT1-MMP Gene**
Previous studies have causally linked the phenotypic defects including growth retardation, skeletal deformities, craniofacial defects, skin fibrosis, dwarfing and arthritis-like joint abnormalities of the MT1-MMP deficient mouse to inadequate degradation and remodeling of selected type I and type II collagen matrices. Studies conducted over the past year have yielded further insights into the bone phenotype of the MT1-MMP deficient mouse. It appears that all intramembraneous bone formation takes place on a scaffold of cartilage either in the chondrocranium, Meckel's cartilage (mandible, condyle, inner ear,) or clavicle. This cartilage primordium never calcifies but is dissolved by an only recently recognized process that is totally dependent on MT1-MMP. The observation that these mice fail to dissolve the cartilagineous precursor of Ranvier's node, which is responsible for the longitudinal growth of cortical bone, provides an explanation for why the animals are severely growth inhibited. While growth plates are seemingly normal at least for the first 40 days, the failure to dissolve Ranvier's node cartilage prevents timely longitudinal growth of the long bones. The mice also show severe eruption defects of the molar teeth.

In collaboration with Dr. Wouter Beertsen in Amsterdam we have observed that cells of the periodontium phagocytize large quantities of collagen fibrils, but apparently are not able to metabolize it further in the absence of MT1-MMP. This failure to remodel type I collagen of the Sharpey's fibers leads to failure to erupt and secondarily to stunted root development.

**An MT1-MMP-Indepdent Pathway for Collagen Degradation**
Previous work has demonstrated the pivotal role of matrix metalloproteinase MT1-MMP [MMP-14] in MMP activation and fibrillar collagen turnover in fibroblasts. Here we demonstrate an alternative cell surface-associated pathway for MMP activation and collagen dissolution that utilizes the plasminogen activation (PA) cascade and is independent of MMP-14. MT1-MMP deficiency did not affect collagen dissolution. Keratinocytes deficient in either uPA or tPA also retained the ability to breakdown collagen. Interestingly, however, collagen dissolution was completely eliminated in keratinocytes with combined deficiency in both uPA and tPA. Combined PA deficiency also completely blocked the activation of MMP-13 by keratinocytes. In wild type keratinocytes the activation MMP-13 could be prevented by exogenously added PAI-1 and aprotinin, but not by TIMPs, suggesting that plasmin activates MMP-13 directly. We propose that the PA cascade promotes keratinocyte-mediated collagen breakdown via the direct activation of MMP-13 and possibly other fibrillar collagenases.

### Targeting of Two other Membrane-Associated Matrix Metalloproteinases

MMP-19 and MT4-MMP (MMP-17) most likely are linked to the plasma membrane through GPI-anchors and thus distinct from all other members of the MMP family. To further explore the function of these enzymes, we targeted each of these genes for disruption in mice; MMP-19, in collaboration with Dr. Carlos Lopez-Otin, Oviedo, Spain. The mice that are viable and do not display macroscopic pathological features are being subjected to ongoing phenotypic analysis.

### Targeting of a Tooth Specific Matrix Metalloproteinase

Previous studies have shown that enamelysin (MMP-20) is expressed only by odontoblasts and ameloblasts, and only during certain stages of tooth development. In collaboration with Dr. John Bartlett at Forsyth Research Institute, Boston we have uncovered a series of abnormalities in enamel formation and in the adhesion of enamel to dentin.

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# Molecular Structural Biology Unit

Dennis Torchia

## MOLECULAR STRUCTURAL BIOLOGY UNIT
## 2002

The principal research goal of the Molecular Structural Biology Unit (MSBU) is to elucidate the structure and dynamics of proteins and associated molecules at the molecular level in order to provide a basis for understanding function. The main research tool used in this work is high resolution, multidimensional, nuclear magnetic resonance (NMR) spectroscopy. Three projects are currently active: (1) HIV-1 protease, as either the free enzyme or bound to a high affinity protease inhibitor; (2) Ribosomal proteins S4 and L11, either free or bound to a target RNA molecule; (3) VIAF, a protein that regulates apoptosis. Progress that has been made during the past year, relating the structure and dynamics of these molecules with their functions is discussed below.

**HIV-1 protease**

The active HIV-1 protease is a homodimer made up of monomers containing 99 amino acid residues. All drugs directed against the protease have targeted the substrate-binding site of the dimer. Although at least six drugs have been developed that binding tightly to the active site, one unfortunate consequence of targeting a single drug binding site on the protease has been the emergence of viral strains which carry multi-drug resistant mutations on their protease constructs. For this reason, there is considerable interest in identifying other protease sites that are suitable drug targets. Although the protease monomer is completely inactive, it is folded and therefore contains numerous potential alternative drug-binding sites, making it an attractive anti-HIV target. In order to identify drug-binding sites of the folded monomer, we have been made a series HIV protease constructs with the goal of obtaining a stable folded monomer in solution which would be suitable for studies of drug interactions using NMR spectroscopy. We have shown that N- and C-terminal deletion mutations, as well as point mutations R87K, D29N and T26A all shift the monomer-dimer equilibrium in favor of the folded monomer. However, all these constructs are prone to aggregation. In order to alleviate this undesirable property we have designed proteins in which the N- and C-terminal regions are linked by intra-monomer disulfide bonds. In particular, cysteine residues were introduced at positions 2 and at either 97 or 98 of the protease amino acid sequence. A procedure for the efficient preparation an intra-chain linked monomer was developed and we have shown that the Q2C/L97C monomer construct exhibits a fold similar to that observed for the monomer subunit in the active dimer. It is anticipated that monomeric proteases of this kind will aid in the discovery of novel inhibitors, aimed at binding to the monomer at the dimerization interface. This extends the target area beyond that of current inhibitors, all of which bind across the active-site formed by both monomers in the active dimer. We anticipate that these inhibitors may address problems related to multidrug-resistance invariably observed with current HIV-1 protease drugs.

Although hundreds of crystal structures of active HIV-protease bound to potent inhibitors are available, structures of are not available of the protein bound to substrate because of rapid catalysis. The binding of the fully active HIV-1 protease to peptide substrate IV was studied in solution using NMR spectroscopy. Unfortunately we were not able to follow the binding of the substrate to the active protease, because many of the protease amide signals were missing from the HSQC spectrum of the protease recorded after

adding the substrate. This result was ascribed to severe broadening of the protease signals due to chemical exchange of the protease with various products of the catalytic reaction. In contrast, an inactive protease structural homologue, $PR_{D25N}$, binds to the substrate in the slow exchange limit, and yields high quality HSQC spectra over a wide range of substrate concentrations. Following sequential assignment of free and substrate bound $PR_{D25N}$, substrate titration HSQC experiments and $^{15}N$-edited NOESY experiments were recorded at 20 degC, pH 5.8. Analysis of these spectra yielded an equilibrium dissociation constant, $K_D$, of 0.27 plus/minus 0.05 mM for the $PR_{D25N}$/substrate complex, and upper limits of the association and dissociation rate constants of $2 \times 10^4$ $M^{-1}s^{-1}$ and 10 $s^{-1}$, respectively. This association rate constant is not in the diffusion limit, which indicates that the association is controlled by a rare event, such as full opening of the protease flaps. In addition to these results on binding kinetics, information about internal protein dynamics of the substrate-bound $PR_{D25N}$ was obtained from the analysis of $^{15}N$ relaxation experiments. In the case of $PR_{D25N}$ bound to the peptide substrate, a larger number of flap residues were found to be flexible on the millesecond-microsecond timescale than has been observed for the protease bound to inhibitors which are significantly smaller than the peptide substrate.

Because the protease D25N construct is stable as a free homodimer in solution, we were able to carry out an extensive series of transverse relaxation dispersion measurements. This data enabled us to compare the slow (millesecond-microsecond) dynamics of the free and inhibitor bound proteins. The results obtained clearly confirmed the presence of such motions in the N- and C-terminal regions of the molecule, which contain the auto-catalytic loop and which form the main dimer interface. The flexibility observed in this region of the molecule allows one to rationalize how the N- and C-terminal strands of the protease, which form a highly ordered beta sheet in crystal structures, can separate and be available for cleavage. Cleavage at these the N- and C- terminal sites of the protease is required for maturation of the Gal-Pol polyprotein, which yields functional HIV proteins, including the protease itself. If cleavage is blocked the HIV cannot mature and reproduce.

**Structure and dynamics of the ribosomal proteins S4 and L11.**
Binding of protein S4 to 16S rRNA is critical for the subsequent binding of other ribosomal proteins. In addition S4 regulates its own translation and that of three other ribosomal proteins. Following our description of the solution structure of S4's mRNA binding domain (S4delta41, 159 residues), we showed that the structure of the C-terminal 158 residues of the intact protein is the same that of S4delta41. In contrast, the 45 N-terminal residues are highly flexible, but contain two conserved regions, $S_{12}RRL_{15}$ and $P_{30}YPP_{33}$, that adopt transiently ordered structures in solution. These transiently ordered conformations, predicted by NMR, were similar to those observed in the independently determined crystal structure of the 30S ribosomal unit from *T. thermophilus*.

During the course of the structural work on the S4 N-terminus, severe overlap problems were encountered in the NMR spectra resulting from chemical shift degeneracy and the high concentration of proline residues. To overcome these problems, we developed two novel experiments that used the carbonyl chemical shifts to remove signal degeneracy.

These experiments enabled us to make complete signal assignments of the N-terminal domain of intact S4.

$^{15}N$ relaxation data ($T_1$, $T_2$, and $\{H\}^{15}N$ NOE) have been collected on both S4 and S4delta41,. These data were analyzed using an anisotropic Model-free approach. In addition transverse relaxation dispersion measurements were also made. Taken together, the relaxation data indicate the C-terminal 159 residues of S4 reorient as an axially symmetric rigid body, suggisting that this portion of S4 and acts as a folding template upon which ribosomal RNA can organize.

**Structure/function study of VIAF, a protein that regulates apoptosis**
VIAF is a member of a conserved protein family that associates with animal IAPs (inhibitor of apoptosis proteins). VIAF itself substantially protects cells from Fas- and Bax-induced apoptosis, while coexpression of VIAF with suboptimal quantities of XIAP confers almost complete protection from these inducers. VIAF and XIAP activate JNK in a synergistic manner. Hence, VIAF is a novel cofactor which modulates the anti-apoptotic and signaling properties of the IAP family. In order to obtain a basis for understanding the function of VIAF at the molecular level, we have initiated a determination of the three-dimensional structure of VIAF using NMR spectroscopy.

Full length VIAF contains 239 residues, but two–hybrid-screening studies have shown that the C-terminal 128 residue region of the protein is necessary and sufficient for interaction with Op-IAP. Hence we have expressed the 15N/13C labeled C-terminal domain of VIAF, henceforth referred to as VIAFC in E.coli using the pET11 vector.

In the process of obtaining NMR backbone and sidechain signal assignments, a novel constant- time HSQC approach was developed to assign sidechain aromatic signals. A moderate-resolution structure of VIAFC, determined using over 1000 NOE constraints, has been obtained and shows that its three dimensional structure is surprisingly similar to that of phosducin, a protein of limited sequence homology. Five sets of different heteronuclear residual dipolar couplings have been measured, and are being used to refine and validate the structure determined using NOEs and coupling constants. After the three dimensional solution structure of VIAFC is completed, the region of VIAFC that interacts with XIAP will be determined.

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