Department of Laboratory Medicine and Pathobiology

Faculty of Medicine

University of Toronto

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Message from the Department Chair:

The Faculty of the Department of Laboratory Medicine and Pathobiology has a unique knowledge base that is at the crossroads of basic biological science and clinical medicine. Many of our faculty teach and investigate the molecular mechanisms of human disease and have contributed significantly to biomedical education and research at the University of Toronto, across Canada, and at the international level.

Our graduate department reflects an essential departmental priority to enhance teaching and investigation of the cellular and molecular aspects of disease. Our faculty is well versed in studying human disease. Many of us employ experimental models of disease in our current research programs. We are very fluent in the handling and assessment of tissue, cells, and fluids from patients with disease. We take our inspiration and our ideas from our clinical material and, based on our understanding of the disease states, we develop realistic hypotheses to test within the context of the pathogenesis of human disease. We also use our clinical exposure to design courses in the pathogenesis of human disease. It is indeed noteworthy that many insights into the biology of normal cells came from studies on disease.

In the Graduate Department of Laboratory Medicine and Pathobiology, you will find excellent research programs on the nature and mechanisms of human disease. Since no single type of approach can give us answers needed to understand disease mechanisms, the interaction of scientists and clinicians with a broad variety of viewpoints provides for a powerful academic group to study pathogenesis of disease. This is the type of training program you will find in our research laboratories situated at the university and in the teaching hospitals and their research institutes. The challenge for our graduate students is to expand the knowledge base at the biochemical, cellular and molecular level and apply it rigorously and vigorously to unravel biological processes and the complex pathogenesis of human disease.

Today you will see the excellent work carried out by our dedicated graduate students. The oral and poster presentations are the result of hard work, creative experimental design, and careful analysis of the data. I wish to congratulate the graduate students, and especially the CLAMPS executive and program committee for their efforts in organizing this Research Day.

Avrum I Gotlieb, MD, CM, FRCPC
Professor and Chair
Department of Laboratory Medicine and Pathobiology
Faculty of Medicine, University of Toronto

Event Organizers:

CLAMPS Student Executives

Ben Beheshti	President
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George Foussias	Treasurer
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Chris Huggins	GSU Representative
Scott Bukovac	GSU Representative
Nima Nourhaghighi	Events Representative, Webmaster

Graduate Student Research Day Judges:

The organizers would like to extend their gratitude to the judges for their time and commitment in Research Day:

Dr. B. Bapat
Dr. M. Bendeck
Dr. R. Bremner
Dr. C. Catzavelos
Dr. G. Hannigan
Dr. P. Hamel
Dr. A. Hinek
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Dr. L. Lille (Parke-Davis)
Dr. M. McGavin
Dr. B. Mullen
Dr. J. Minta
Dr. J. Musser (Keynote Speaker)
Dr. S. Nag
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Dr. A. Seth
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Dr. M. Tsao
Dr. B. Yang
Dr. H. Yeger
Dr. M. Zielenska

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Graduate Student Research Day Programme:

08:00 - Poster Setup (MSB Stone Lobby)

08:45 - Opening Remarks (MSB3154)

09:00 - M.Sc. Oral Presentations (MSB3154)

10:30 - Refreshments Break (MSB3154)

10:45 - Ph.D. Oral Presentations (MSB3154)

12:15 - Lunch (MSB2173)

13:00 - Keynote Speaker: James M. Musser, M.D., Ph.D.,
Chief, Laboratory of Human Bacterial Pathogenesis
Rocky Mountain Laboratories
National Institute of Allergy and Infectious Diseases
National Institutes of Health, USA
"Molecular Genetic Basis of Group A Streptococcus Invasive Infection" (MSB2173)

14:00 - M.Sc. and Ph.D. Poster Presentations (MSB Stone Lobby)

17:00 - Awards for Oral/Poster Presentations

17:15 - Reception (MSB6303)

MSc Oral Presentations:

1. Retinoblastoma Protein in Retinal Development.

I.D. Burcescu[1,2], D.P. DiCiommo[1,3], B.L. Gallie[3,4], R. Bremner[1,2].
Vision Science Research Program, Toronto Western Research Institute[1]; Department of Laboratory Medicine and Pathobiology, Faculty of Medicine[2], Departments of Medical Genetics and Microbiology, Ophthalmology and Pathology[3], Cancer Informatics, Ontario Cancer Institute/Princess Margaret Hospital[4], University of Toronto, Toronto, Canada.

Human retinoblastoma tumours show disruption of both alleles of the retinoblastoma gene, RB. In humans, germline RB mutations (RB+/-) lead to an elevated risk for developing retinoblastoma tumours (which are RB-/-). The RB gene product, pRB, is required to allow cells determined to differentiate to exit the cell cycle. Our group has previously reported that ganglion cells in Rblox/RB-/- mouse retina express markers of differentiation, but fail to exit the cell cycle. We have also very recently shown that in the adult human retina, pRB is expressed exclusively in ganglion and Muller cells. This suggests that pRB is critical for the development of at least these two cell types in the retina. Using two recombinant virus expression systems, our goal is to reintroduce the RB gene into RB-/- explants and evaluate its effect on retinal neuroblast development. Knock out of two of the RB family of genes specifically predisposes to retinoblastoma in mice, providing an excellent system for our studies. However, such mice die between 12.5 and 15.5 days of gestation (E12.5-E15.5), well before the end-point of complete retinal differentiation. Thus, an investigation of the role of pRB in retinal differentiation beyond this time frame is impossible. To overcome this problem we have developed a retinal explant culture system that mimics *in vivo* development, including differentiation and cellular organization. For the purpose of gene delivery, we have successfully exploited two viral systems. One employs the long-term gene delivery capabilities of a replication-incompetent retrovirus, pLIA; the recombinant virus encodes alkaline phosphatase. We have successfully infected retinal neuroblasts with such retroviral vectors. The second viral system exploits Semliki Forest virus, wide host range; the recombinant virus encodes -galactosidase. We have used this novel approach to efficiently infect retinal explants. These two viral systems will enable us to deliver RB to the RB-/- retinas; we will then use immunocytochemistry to determine whether pRB can rescue the development of retinal cells. The cell cycle status can also be assessed by PCNA-staining and BrdU-labeling. This research represents a significant lead in our understanding of the role played by RB in retinal development.

2. Endothelial Cell NF-κB Signal Transduction Pathway is Primed for Activation in Regions Predisposed to Atherosclerotic Lesion Formation.

L. Hajra, A.I. Evans, M. Chen, S.J. Hyduk, T. Collins, M.I. Cybulsky.
Department of Laboratory Medicine and Pathobiology, Faculty of Medicine, University of Toronto.

Multiple genes regulated by NF- B participate in atherogenesis. Our goal was to investigate if NF- B signal transduction in endothelium contributes to the distribution pattern of atherosclerotic lesions. Regions with high and low probability (HP and LP) for atherosclerotic lesion formation were mapped in the ascending aorta of LDL receptor knockout ($LDLR^{-/-}$) mice. Silver nitrate staining showed that endothelial cells in the HP region were polygonal, in contrast to uniformly elongated LP region cells that were parallel to the direction of blood flow. Expression of p65, I B and I B was quantified by specific antibody staining, *en face* confocal microscopy and image analysis. In control C57BL/6 mice, these proteins were localized primarily in the cytoplasm and their expression levels were 5-18 fold higher in the HP regions (n=5, 3 HP and LP fields/mouse, $p<0.05$, paired t-test). NF- B activation, determined by nuclear translocation of p65 and phosphorylation of I B , was found in only 12% of HP region endothelial cells (40-65 cells/field). LPS treatment of C57BL/6 mice or feeding $LDLR^{-/-}$ mice a 1.25% cholesterol diet for 2 weeks resulted in significant activation of NF- B in the HP region (LPS: 88% of cells, diet: 23%, controls 12-13%, $p<0.05$, ANOVA, n=5). LPS- or cholesterol diet-induced NF- B activation was found in only 2 - 4% of LP region cells, which was lower than in the HP region of controls. Expression of NF- B target genes, E-selectin and VCAM-1, was also preferentially upregulated by LPS in the HP region, but not PECAM-1 and ICAM-2. The above data indicate that the NF- B signal transduction pathway in HP region endothelium was primed to respond to activation stimuli, including ingestion of an atherogenic diet.

3. Retroviral-Mediated Gene Transfer and Expression of the Multidrug Resistance Protein 1 (MRP1) for Hematopoietic Chemoprotection.

T. Juopperi[1], S. Kruth[2], C.-K. Chan[1], L. Li1, P. Woods[2], I.D. Dubé[1].
Department of Laboratory Medicine and Pathobiology, Faculty of Medicine, University of Toronto[1]; Department of Clinical Studies, Ontario Veterinary College, University of Guelph[2].

Multidrug Resistance Protein 1 (MRP1) is a member of the ABC superfamily of transporters that confers multidrug resistance. We hypothesize that the transfer and expression of the MRP1 gene to hematopoietic stem cells (HSCs) might provide protection from the myelosuppressive effects of chemotherapy. Our long-term goal in this project is to achieve hematopoietic chemoprotection in a canine model and develop conditions for human trials. We developed a retroviral producer cell line, MRP1-PG13, and optimized conditions for transfer of the human MRP1 cDNA into HSCs. We demonstrated that MRP1-PG13 is able to transfer MRP1 to K562 cells and that expression gives rise to drug resistance. To assess MRP1 transfer into canine HSCs, CD34+ cells were incubated with MRP1-PG13 supernatants on fibronectin in the presence of hematopoietic cytokines. PCR analysis of hematopoietic colonies (CFUs) derived from transduced CD34+ cells demonstrated proviral DNA in ~15% of HSCs. For *in vivo* studies, MRP1 transduced CD34+ cells were infused into two dogs. Blood and marrow samples obtained prior to drug challenge were provirus negative. Dogs were challenged with vincristine and multiple challenges were performed. MRP1 positive CFUs were detected by PCR in blood (4.0%) and marrow (0.9%) in one dog after three challenges. After a fourth challenge, 5.3% of marrow CFUs were provirus positive. Despite these results, myelosuppression was observed. We demonstrated that MRP1-PG13 is able to transfer MRP1 to canine HSCs *in vitro*. Furthermore, vector-containing cells can engraft and proliferate in response to drug challenge. Ongoing experiments are aimed at achieving higher levels of gene transfer, while optimizing regimens for engraftment and *in vivo* selection.

4. Dominant Negative Inhibition of ILK1 Signalling Blocks L6 Myoblast Fusion.

M. Miller, G. Hannigan.
Department of Laboratory Medicine and Pathobiology, Faculty of Medicine, University of Toronto, and Programme in Cell Biology, The Hospital for Sick Children.

A critical step in skeletal myogenesis occurs when myoblasts exit the cell cycle, fuse and form quiescent myotubes. Myotube formation and survival are regulated by cell-ECM interactions and growth factors. These stimuli regulate myogenesis by inducing cell signalling cascades through integrins, growth factor receptors and phosphoinositide-3-kinase (PI-3K). Integrin-linked kinase (ILK1) is a serine/threonine kinase that interacts with the $_{1}$ integrin cytoplasmic domain and mediates integrin and growth factor signalling. We hypothesize ILK1 signals contribute to skeletal myogenesis *in vitro* by regulating myotube formation through the PI-3K signalling pathway. We tested the *in vitro* myogenic model L6 myoblasts for ILK1 kinase activity response to ECM and insulin stimulus. ILK1 kinase activity is stimulated by ECM and insulin in L6 cells. Insulin activation of ILK1 is wortmannin-sensitive, showing PI-3K dependence. We stably transfected L6 cells with either wtILK1 or dominant negative ILK1 constructs and are examining effects on cell differentiation, proliferation and survival. L6 clones expressing wild type ILK1 exhibit enhanced myotube formation under non-differentiating conditions, while those expressing the dominant negative variant do not form myotubes. Dominant negative ILK1 also blocks biochemical differentiation in L6, as assayed by immunoblot using an anti-myogenin antibody. These data suggest that ILK1 is an important regulator of myogenesis *in vitro*. Current studies are focused on identifying specific upstream events and downstream targets of the ILK1 pathway in myogenesis.

5. Ectopic Expression of the p16^{INK4a} and p14^{ARF} Tumour Suppressors Mediates Growth Arrest of Malignant Human Gliomas.

C. Stewart, J.T. Rutka, P.A. Hamel.
Department of Laboratory Medicine and Pathobiology, Faculty of Medicine, University of Toronto.

The INK4a/ARF locus located on human chromosome 9 represents the second most commonly mutated gene in human cancers. Through alternative splicing, the gene encodes for two unrelated proteins which function as potent cell cycle inhibitors. p16^{INK4a} functions as an inhibitor of cyclin D/CDK4 complexes, thereby activating pRB to induce cell cycle arrest. Growth inhibition by p14^{ARF} is mediated by p53, which is activated by ARF interaction and subsequent impairment of MDM2. The characterization of p16^{INK4a} and p14^{ARF} as tumour suppressors is supported by their frequent inactivation in human malignancies such as gliomas. To determine their ability to arrest the growth of malignant astrocytomas, p16^{INK4a} and ARF were ectopically expressed in a panel of human glioma cell lines shown to be deficient in expression of these two proteins. We found that reconstituted expression of p16^{INK4a} via stable transfection inhibited proliferation of gliomas in a pRB-dependent manner. This correlated with the observation of G1 arrest and downregulation of E2F1 expression after infection with p16^{INK4a}-encoding adenovirus specifically in gliomas retaining wild-type pRB. Stable transfection of p14^{ARF}, on the other hand, potently inhibited the proliferation of gliomas harboring wild-type p53, but also exerted some growth suppressive effects in gliomas containing mutant p53. Furthermore, adenoviral infection of p14^{ARF} induced p53, MDM2 and p21^{Cip1} protein levels and G1 arrest in gliomas with wild-type p53, whereas no such effects were observed in the mutant p53-containing gliomas. Our results demonstrate a novel, p53-independent role for the ARF tumour suppressor in mediating cell cycle arrest of malignant human gliomas.

6. MRSA Phage Display Library.

D. Vaz, M. McGavin.
Department of Laboratory Medicine and Pathobiology, Sunnybrook and Women's College, Faculty of Medicine, University of Toronto.

Multiple drug resistance in some strains of *S. aureus* (MRSA), especially epidemic MRSA is a major cause of concern. We hypothesize that epidemic MRSA possess unidentified adhesins which contribute to epidemicity by promoting enhanced colonization. Phage display technology is being employed to identify the repertoire of adhesin molecules that can be expressed by epidemic MRSA. This makes use of an *Escherichia coli* filamentous phage (M13) to display foreign peptides in fusion with the coat protein, gpVIII. Genomic DNA of epidemic CMRSA-1 strain was sonicated, and the fragments were ligated to phagemid vector pG8SAET. Transformed *E. coli* cells were then infected with helper phage to yield the MRSA phage display library. The library was first screened using biotinylated fibronectin (b-Fn) to capture phage particles displaying Fn-binding sequences, as Fn binding proteins of *S. aureus* (FnbpA/B) have been previously well characterized. After two rounds of selection, greater than 80% of the captured phage contained DNA fragments corresponding to the Fnbp adhesin. Surprisingly, the majority (15/20) represented previously uncharacterized Fn-binding domains. The ability of these sequences to bind Fn was confirmed through the construction of glutathione S-transferase fusion peptides. Therefore, phage display represents an efficient means of directly isolating the ligand binding domains of microbial adhesins. Future work will focus on characterizing these novel Fn-binding domains, and on screening with biotin labeled surface proteins from keratinocyte cell culture. This will enable us to identify the repertoire of functional binding domains that promote colonization, and lead to the development of novel vaccine strategies.

Ph.D. Oral Presentations:

7. Platelet-Derived Growth Factor-Mediated Glycosaminoglycan Synthesis is Transduced via Akt-1,2 And Abrogates Apoptosis in Fetal Lung Fibroblasts.

N.J. Cartel, M. Post.
Programme in Lung Biology Research, Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, M5G 1X8; Department of Laboratory Medicine and Pathobiology, Faculty of Medicine, University of Toronto.

Proteoglycans (PG) are major components of the basal laminae. The PGs constitute a family of multi-domain core-proteins to which glycosaminoglycans (GAGs) are attached via O-glycosidic linkages to serine residues. GAGs mediate, at least in part, cell adhesion, structural organization, and cell proliferation during fetal development. PDGF-BB augmented the synthesis of all individual GAG types measured. PDGF-BB did not alter the proportion of total GAGs and did not affect the distribution of individual GAGs between medium and cell layer. PDGF-BB-stimulated GAG synthesis was abrogated by tyrphostin 9, a PDGF receptor-associated tyrosine kinase inhibitor, implying that the stimulatory effect is mediated via the PDGF -receptor. PDGF-BB-induced phosphatidylinositol-3 kinase (PI3K) activation and GAG synthesis were both abolished by the PI3K inhibitors, wortmannin and LY-294002. PDGF -receptor constructs, with cytoplasmic tyrosines mutated to alanine, further demonstrated that PI3K binding and activity is necessary for PDGF-BB-mediated GAG synthesis. A direct downstream kinase from PI3K is Akt kinase. Western blots demonstrated that Akt-1,2,3 are present in fetal lung fibroblasts but not in epithelial cells. Kinase assays showed that Akt-1,2 but not Akt-3 activity is increased in cells treated with PDGF-BB. Wortmannin blocked PDGF-BB-induced Akt activity as well as significantly diminished PDGF-BB-mediated GAG synthesis. Moreover, a dominant-negative Akt construct (Akt-AAA) abrogated Akt activity and GAG synthesis whereas a constitutively activated Akt construct (Akt-DD) activated Akt and stimulated GAG synthesis in the absence of PDGF-BB treatment. Furthermore, inclusion of the proteoglycan substitute, -D-Xyloside, abrogated apoptosis as measured by DNA laddering, TUNEL analysis and poly-ADP ribose polymerase assay. These data indicate that Akt kinase is an integral protein involved in GAG regulation in fetal lung cells.

8. Transcriptional Repression by a Paired-Like Homeodomain Protein: Chx10.

K. Dorval, F. Ahmad, B. Bobechko, X.P. Zhu, R. Bremner.
Vision Science Research Program, Toronto Western Research Institute; Department of Laboratory Medicine and Pathobiology, Faculty of Medicine, University of Toronto.

Homeodomain proteins are master regulators of gene expression, activating and repressing genes that specify many developmental pathways. Inactivation of Chx10, a paired-like HD protein, causes blinding diseases in both mice and humans. Little is known about the mechanism HD proteins use to regulate gene expression. We have shown that Chx10 is a transcriptional repressor. Our goal is to elucidate the mechanism of transcriptional repression by Chx10. In order to build a repression model, it is essential to identify target genes and determine Chx10-DNA binding sites within target promoters. We have identified the photoreceptor (PR)-specific gene rhodopsin (rho) as a putative *in vivo* target for Chx10. Paired-liked HD proteins have been shown to bind A-T rich DNA sequences called P3-sites. The rho promoter contains several P3-like motifs such as Ret1, BAT-1 and Ret4. In band shift assays Chx10 bound only the Ret1 site. This result illustrates the ability of Chx10 to differentiate between similar DNA motifs, an important regulatory function for transcription factors. Surprisingly, rhodopsin was not repressed in transient transfection assays, but the PR-specific arrestin promoter, which also contains P3-like motifs, was efficiently regulated by Chx10. Deletion analysis of the arrestin promoter indicated the Chx10-regulatory region spans bases -107 to +112. Future band shift assays will identify Chx10-binding sites within the arrestin promoter. A comprehensive analysis of the nucleotides within and surrounding Chx10 binding sites of PR-specific promoters will identify a consensus sequence. Collectively, this data will establish a model of transcriptional repression for Chx10.

9. Submicroscopic Deletions Close to the *ABL* and *BCR* Genes are Associated With Reciprocal 9;22 Translocations In 8% of Philadelphia-Positive Leukemias and Implicate the Presence of a Novel Tumour Suppressor Gene(s) Mapping to this Region.

E. Kolomietz[1,2], J. Al-Maghrabi[1,2], S. Brennan[3], J. Karaskova[2], B. Beheshti[1,2], J.A. Squire[1,2,3].
Department of Laboratory Medicine and Pathobiology, Faculty of Medicine, University of Toronto[1]; Division of Cellular and Molecular Biology, Ontario Cancer Institute[2], and the Banting Cancer Cytogenetic Program[3], the University Health Network, Toronto, Canada.

During routine dual-color *BCR*/*ABL* fluorescence *in situ* hybridization (FISH) analysis of CML patients, we observed that a subset of the patients demonstrated an atypical hybridization pattern. A retrospective D-FISH study of 250 cases of CML patients in chronic and blast crises and 13 cases of Ph+ ALL patients indicated that 21 CML patients (8.4%) and 1 ALL patient (7.7%) exhibited an atypical hybridization pattern consistent with deletion of the two regions: proximal to the rearranged *ABL* and distal to *BCR* gene on the 9q+ derivative chromosome. The spanning *BCR* probe is approximately 300 kb extending from exon 1 of *BCR* to an area telomeric to the M-bcr region. The *ABL* probe spans a 400 kb region extending from an area telomeric to the last *ABL* exon to a region centromeric to the common breakpoint in *ABL*. The deletions probably involve several hundred kilobases of DNA. Analysis of clinical outcome data has demonstrated that patients with the deletions have a different disease phenotype associated with poorer prognosis. Cytogenetic analysis indicated that the atypical hybridization pattern is associated with the standard (9;22) translocation as well as complex or variant translocations. The coincident detection of cryptic deletions associated with what appeared to be reciprocal translocation suggests that deletion most likely arose simultaneously with translocation and may be associated with Alu sequences that map close to the translocation breakpoint region. Analysis of sequence data from distal 3' *BCR* region has identified several putative peptide structures, which could represent candidate tumour suppressor genes in this region. We postulate that such deletions can lead to loss of one or more tumour suppressor genes that map close to *BCR* and/or *ABL* and that the associated haploinsufficiency may result in a modification of the disease phenotype.

10. Myocardial Over-Expression of Human Inducible Nitric Oxide Synthase [iNOS] in Transgenic Mice Leads to Sudden Death and Heart Failure.

I.N. Mungrue, X. You, R. Gros, M. Husain, D.J. Stewart.
The Toronto General and St. Michael's Hospitals, Department of Laboratory Medicine and Pathobiology, Faculty of Medicine, University of Toronto.

Increased cardiac iNOS expression is known to accompany cardiomyopathy, myocarditis, infarction, and rejection. However, the effects of enhanced iNOS activity on cardiomyocyte survival and function are poorly understood. We have generated mice that harbor an -MHC promoter-directed tetracycline-responsive transcriptional activator (MHC-tTA) and a bi-directional tTA-responsive transgene for human iNOS and gal [iNOS gal]. Transfections of iNOS gal in CHO cells that stably express tTA confirmed doxycycline-regulated [DOX] iNOS expression & gal activity. Pro-nuclei injections produced 2 lines, 240 & 365, which have transmitted iNOS gal in an autosomal fashion. Breedings between MHC-tTA+ and 240+ mice have resulted in only 17 of 110 progeny positive for both transgenes (expected 25%), in the absence of DOX. Given that near equal numbers of non-binary genotypes [NBG] were born, a ~50% lethality in MHC-tTA+/240i+ embryos is suggested ($p<0.01$). While initially normal in appearance, a striking incidence of sudden death (7/16) was observed in MHC-tTA+/240i+ mice by 25 weeks compared to NBG ($p<0.05$). Unlike NBG, MHC-tTA+/240i+ mice display DOX-suppressible cardiac iNOS expression (mRNA, protein, immuno-histology) and *lacZ* activity. Preliminary analyses reveal that MHC-tTA+/240i+ mice, compared to age-matched NBG, show a trend towards increased +dP/dT (9488 ± 1027 vs. 5807 ± 1035, p=0.1), a significant increase in LVEDD (4.13 ± 0.11 vs. 3.71 ± 0.23 mm, p=0.05), and a small increase in myocardial nitro-tyrosine staining. Of note, 1 MHC-tTA+/240i+ animal developed severe overt heart failure and 2 demonstrated cardiomegaly. These data suggest that MHC-tTA-driven iNOS expression is cardiotoxic. This model will allow the exploration of iNOS-specific inhibitors as a novel therapeutic strategy for cardiac disease.

11. Expression and Regulation of the Endothelial Nitric Oxide Synthase (eNOS) Gene During Murine Development.

A.M. Teichert, C. Law, P.A. Marsden.
Division of Nephrology, Department of Medicine and Department of Laboratory Medicine and Pathobiology, Faculty of Medicine, St. Michael's Hospital and the University of Toronto.

Our previous work has addressed the transcriptional control pathways implicated in the regulation of the human eNOS gene (JBC 1998, BBActa 1998). Based upon these studies we were motivated to develop an insertional murine transgenic model with which to address the *in vivo* applicability of *in vitro* findings, especially in the setting of development and disease. Multiple eNOS promoter/reporter (-galactosidase) transgenic murine lines were created revealing that *in vivo* expression of the transgene in adult mice recapitulated the known expression profile of eNOS. Expression was uniform across founders, highly specific to the endothelium, robust even at the single copy level and independent of genomic integration site (Am J Physiol. 2000). We have completed a comprehensive assessment of the expression profile of an eNOS reporter transgene during early and late murine development, focusing upon both embryonic and extraembryonic fetal tissue (20 litters, multiple founders). As in the adult, eNOS expression is remarkably endothelial and endocardial restricted during mammalian development. In contrast to early endothelial ontogeny (VEGF-R1, R-2), eNOS is a relatively late endothelial marker (ED 10.5) that is strongly expressed in major blood vessels of the cardiovascular tree and maturing solid organs. An exciting finding from these studies implicates a novel role for NO in the developing musculoskeletal system. Late-stage embryos (ED 14.5 and onwards) strongly express the transgene in the myocytes of the developing proximal limb. Moreover, transient transfection of eNOS promoter/luciferase reporter constructs into murine C2C12, an eNOS (+) skeletal muscle cell line, demonstrated strong promoter activity and conservation of functionally important cis-DNA elements (n=3). Given that eNOS (-/-) mice evidence limb reduction defects and that *in vitro* studies support a role for NO in mediating the transition of myocytes to myotubes we posit an important contribution of eNOS in developing as opposed to adult skeletal muscle. In summary, the study of eNOS gene transcription has provided a unique opportunity to examine endothelial gene expression in the later stages of the developing mammalian cardiovascular system and uncovered conserved transcriptional paradigms in skeletal myocytes and vascular endothelium.

12. Characterization of the Human Kallikrein Gene Locus and Discovery of Six New Genes.

G.M Yousef, E.P Diamandis.
Department of Laboratory Medicine and Pathobiology, and Department of Pathology and Laboratory Medicine, Faculty of Medicine, University of Toronto, Mount Sinai Hospital, Toronto, Ontario, M5G 1X5, Canada.

Context: Kallikreins are a subgroup of serine proteases with diverse physiological functions. In addition to PSA, growing evidence indicates that other kallikreins are involved in malignancy. In mouse and rat, kallikreins are encoded by large multigene families. The human kallikrein gene family was thought to have only three members: the tissue kallikrein (KLK1), the human glandular kallikrein (KLK2), and prostate specific antigen (PSA or KLK3). We hypothesized that the human kallikrein multigene family might be larger than previously expected.
Materials and methods: We studied an area on chromosome 19q13.3, which harbors the human kallikrein gene locus. Restriction enzyme analysis, bacterial artificial chromosome (BAC) library screening, long PCR, and other informatics tools were used to determine the direction of transcription and distances between genes. Computer programs were used to predict new kallikrein genes; these genes were further confirmed by expressed sequence tag (EST) search, 5' and 3' rapid amplification of cDNA ends (RACE) technology, PCR, and other techniques.
Results: We constructed the first detailed map of the human kallikrein gene locus, describing the order of the kallikrein genes along the chromosomal region and the direction of transcription. We also identified six new kallikrein genes. Based on our preliminary experiments, some of these newly discovered genes are differentially expressed in certain malignancies.
Conclusion: We have constructed the first detailed map of the human kallikrein gene family, comprising 14 members, of which six were discovered by our group. Similarly to PSA, some of these kallikreins may prove to be valuable cancer biomarkers.

M.Sc. Poster Presentations:

1. Identification of Numerical Chromosomal Changes Detected by Interphase FISH in High-Grade Prostrate Intraepithelial Neoplasia (HPIN) as a Predictor of Carcinoma.

J. Al-Maghrabi, L. Vorobyova, A. Toi, J. Sweet, S. Jothy, J. Trachtenberg, W. Chapman, M.A.S. Jewett, D. Benerjee, J.A. Squire.
Department of Laboratory Medicine and Pathobiology, Division of Cellular and Molecular Biology, Ontario Cancer Institute, Department of Surgical Oncology, Princess Margaret Hospital, Department of Pathology, The Toronto General Hospital, University Health Network, Department of Pathology, Sunnybrook and Women's College Health Science Centre, Faculty of Medicine, University of Toronto, Toronto, Canada.

Context: HPIN is the most likely precursor of prostate cancer. About half of all patients with HPIN show carcinoma in the second subsequent follow-up biopsies. There are currently no available clinical, immunohistochemical or morphological criteria that are predictive of this progression or association.
Design: Dual-colour interphase FISH analysis was performed on formalin fixed paraffin-embedded prostate material (5µm) from biopsies of 1) 16 HPIN patients with persistent HPIN and/or benign lesions on the follow-up biopsies, and 2) 12 HPIN patients with progression to prostate carcinoma on the follow-up. We utilized commercially available enumeration probes for chromosomes 4, 7, 8, 10. The criteria for chromosomal gain and loss was >8% of cells with >2 signals and >50% of cells with <2 signals respectively.
Results: Thirty-three percent of the patients in the second group displayed numerical chromosomal aberrations. Only 12.5% of the patients from the first group had chromosomal abnormalities. All aberrations were detected in the form of chromosomal gain. Overall, the commonest aberration was gain of chromosome 8, followed by gains of chromosomes 7 and 10. No anomalies were seen in the adjacent hyperplastic or normal prostate glandular epithelium.
Conclusion: These preliminary results indicate that although no single numeric chromosomal abnormality could be assigned as a predictor of HPIN progression to carcinoma, the overall numeric chromosomal abnormalities in chromosomes 7, 8, and 10 were more common in HPIN patients that progress to carcinoma. Although currently there is no statistically significant difference between the two study groups examined ($p>0.05$), ongoing work with a larger sample size may permit us to show a statistical difference.

2. Lysosomal Membrane Proteomics Initiative.

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Lysosomes are subcellular organelles that represent the end-stage of the endosomal pathway. They are responsible for the digestion of complex macromolecules to forms that can be reutilized by the cell. Even though the lysosomal membrane plays important roles in transporting material to and from the lysosomal lumen and assembling mature lysosomes from late-endosomes, its protein complement is poorly characterized. We have initiated studies to assemble a proteomic map of the lysosomal membrane. Purification of lysosomes involves a single intra-peritoneal injection of Triton WR-1339 into rats, which artificially decreases the density of the lysosomes in liver. The lysosomes are collected away from contaminating organelles through density gradient centrifugation and are enriched 84 fold using beta-hexosaminidase A as the lysosomal marker. The lysosomal membrane proteins are fractionated based on solubility and separated by two-dimensional polyacrylamide gel electrophoresis. Peptide mass mapping using MALDI-TOF or *de novo* amino acid sequencing using the Q-TOF mass spectrometer then identifies the protein spots. We have developed a digestion, extraction, and desalting technique that provides excellent signal-to-noise ratio, and is suitable for routine peptide sequencing. To this point we have identified several proteins in a sodium carbonate fraction and a non-ionic detergent fraction of the lysosomal membrane, three of which appear to be unique, as they have no protein database entries.

3. Molecular Cytogenetic Analysis of Medulloblastomas Using Conventional Banding, Comparative Genomic Hybridization and Spectral Karyotyping.

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Medulloblastomas are malignant, invasive embryonal tumours with predominantly neuronal differentiation and comprise 20% of pediatric brain tumours. Cytogenetic analysis has shown that alterations of chromosome 17, particularly the loss of 17p and the formation of i(17q), as well as the gain of chromosome 7 are the most common change among medulloblastomas. Comparative Genomic Hybridization (CGH) studies have largely confirmed these cytogenetic findings and have also identified regions of amplification, commonly at bands 2p24 (*MYCN*) and 8q24 (*MYCC*), as well as novel amplifications at 1p32-p34, 3p21.2-p22, 4q12-13, 5p15.3 and 11q22.3. The advent of more sophisticated multi-coloured FISH techniques such as Spectral Karyotyping (SKY) now permits complete recognition of all aberrations including extremely complex rearrangements. With SKY, the origins of marker chromosomes as well as hidden chromosomal alterations, that may have otherwise gone undetected by conventional cytogenetic methods can now be easily detected. Here we report a retrospective analysis of 27 medulloblastoma cases analyzed using combinations of classical banding analysis, fluorescence *in situ* hybridization (FISH), CGH and SKY to comprehensively analyze the chromosomal aberrations present in this tumour group and to identify a common structural rearrangement(s) that characterizes the tumour. Our data confirms previous reports of consistent chromosomal gains of chromosomes 17q and 7, however we also demonstrate the frequent involvement of chromosomes 3, 5, 6, 10, 13, 14, 18 and 22 in both structural and numerical rearrangements.

4. Porous Titanium Alloy Substrates: A Model For the Study of the Effect of Pore Size on Proteoglycan Synthesis and Accumulation by Articular Chondrocytes *in vitro*.

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To determine whether material geometry effects cartilagenous tissue formation *in vitro*, bovine articular chondrocytes were plated on porous Ti6Al4V discs of different pore sizes (average pore size; small = 13μm, intermediate = 43μm, and large = 68μm) and grown in culture for 4 weeks. Light microscopical examination of histological sections of the composite showed that cartilagenous tissue formed on all discs of each pore size. The cartilagenous tissue formed on discs of the smallest pore size had a proteoglycan content of 164.50 * 4.99 μg/disc (mean * SD) which was significantly greater than the proteoglycan content of the cartilaginous tissue formed on the discs of the intermediate or large pore sizes. The pore size of the disc did not influence the size of proteoglycans synthesized by the chondrocytes (Kav * SD: 13μm = 0.28 * 0.03; 43μm = 0.29 * 0.03; 68μm = 0.27 * 0.04) and were similar in size to those synthesized by chondrocytes in *ex vivo* cartilage culture (Kav = 0.27). The cartilagenous tissue formed on discs of the smallest pore size had a DNA content of 8.79 * 0.2μg/disc (mean * SD) and was more cellular than the cartilagenous tissue formed on the discs of the intermediate or large pore sizes. However, after normalizing for cellularity, the amount of proteoglycan accumulated per cell was similar in the tissues formed on the discs of different pore sizes (GAG/DNA * SD: 13μm = 18.73 * 0.4; 43μm = 19.81 * 4.2; 68μm = 20.48 * 4.8). These results suggest that material geometry, as defined by pore size, does not affect cartilage formation but can affect the amount of tissue that forms.

5. Measurement of Myelin Basic Protein in the Circulation of MS Patients.

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Multiple sclerosis (MS) is a neurological disorder of the central nervous system (CNS). MS usually causes sudden neurological symptoms such as numbness, paralysis, walking difficulties, and vision loss. These symptoms occur due to demyelination of the nerve fiber, thus losing its ability to conduct impulses. It is characterized by inflammatory lesions within the CNS and these lesions are multifocal areas of demyelination. Currently the diagnosis and monitoring of the progression of MS relies on neuro-imaging, clinical observations, and tests of the cerebral spinal fluid (CSF). There is a need for a surrogate marker for the diagnosis and monitoring of disease activity. The marker must be present in an easily accessible body fluid to facilitate frequent testing. Myelin basic protein (MBP) is one of the major proteins of the myelin sheath and constitutes 30% of total CNS myelin protein. It has been demonstrated that MBP is completely absent from the centre of most lesions, however, the mechanism of this loss has not been elucidated. MBP has been detected in the cerebral spinal fluid of MS patients and it is postulated to be present in the blood and urine of these patients. We anticipate that MBP will serve as a useful marker protein for monitoring MS once it can be accurately measured within the circulation. Our methodology employs affinity purified polyclonal antibodies covalently attached to microparticles to facilitate the detection of MBP in the serum or plasma of MS patients. The microparticles constitute a diagnostic test methodology relying on the principle of turbidimetry.

6. A Rapid and Sensitive ELISA for the Detection of MBP Autoantibodies in Human Plasma.

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Context: The presence of MBP autoantibodies and their clinical utility in Multiple Sclerosis (MS) is controversial. In order to address this issue, we developed a plasma assay to measure MBP autoantibodies. If circulating MBP autoantibodies can be reliably detected in plasma, this assay would offer a simple, rapid and non-invasive means for the diagnosis and monitoring of the progression of MS.
Objective: To measure the titers of MBP autoantibodies in the plasma samples of MS patients by ELISA.
Methodology: A direct ELISA has been developed for the *in vitro* measurement of IgG and IgM classes of autoantibodies against MBP in human plasma. Microtitre wells, coated with purified bovine MBP constitute the solid phase. Diluted patient plasma is added to the paired wells, followed by a purified anti-human IgG/M HRP conjugated antibody. The substrate-chromogen is added and optical density is read at 450nm.
Results: Using three samples covering the range of the standard curve, a mean intra-assay precision (%CV) at $<6.2\%$ and mean inter-assay precision at $< 7.9\%$ was achieved. The linearity of the reference standard curve was achieved from 0-200 U/ml. The antibody response was specific for MBP. No cross-reaction was observed against PLP, NSE , and S100B. Of the clinically definite MS patients tested (n=24), 96% exhibited elevated MBP autoantibodies (IgG) and the levels paralleled the clinical course.
Conclusion: The assay is simple, rapid, has high sensitivity for the detection of circulating MBP autoantibodies and may be of use in diagnosing and monitoring relapsing episodes in MS.

7. Inducible Transgene Expression Systems for Analysis of Gene Functions in Lung Epithelia.

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Inducible transgene expression systems are invaluable tools for understanding gene functions in complicated organs, such as the lung. To develop a lung epithelium-specific inducible system we used an expression cassette based on DNA regulatory elements of the human cytokeratin 18 (K18) gene to express the transcriptional regulator, rtTA of the tetracycline inducible system. The expression pattern of keratin 18 is tightly regulated and restricted to the simple epithelium of a number of internal organs, including the lung, liver, kidney and intestine. The expression pattern of keratin 18 is very similar to that of the CF gene product - Cystic Fibrosis Transmembrane conductance Regulator (CFTR). Cystic fibrosis is the most common lethal inherited disease in the caucasian population. An inducible expression system of CFTR will be useful in studies to further our knowledge of potential CFTR gene therapy and to pursue the understanding of CF pathophysiology. Reporter gene expression under a tetracycline-inducible promoter in cultured cells of human lung epithelial origin is greater when the cells are co-transfected with a K18 promoter-based rtTA expression plasmid, pK18rtTA, than with a CMV promoter-driven rtTA expression plasmid. Transgenic mice were generated with pK18rtTA and the temporal and spatial expression patterns were determined. Transgenic mice were bred with a *lacZ* reporter transgenic line (from Jackson Laboratories) and cell specificity of the *lacZ* reporter expression was determined by X-gal staining. Using this system the expression of a gene of interest can be targeted to the lung epithelia and controlled temporally, without pleiotropic effects, by the administration of doxycycline.

8. Transcriptional Regulation of Human Cytokeratin 18 Gene in Lung Airway Epithelial Cells.

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The human keratin 18 (K18) gene contains seven exons that code for an acidic type I intermediate filament protein that is normally coexpressed with its heteropolymeric partner, keratin 8 (K8), in differentiated epithelial cells of the lung and other internal organs. In transgenic mice, the 10-kb genomic fragment containing the human K18 gene reveals that there is sufficient genetic information for appropriate tissue-specific, copy number-dependent, and integration site-independent expression. Recent transgenic analysis, by Dr. Chow et al. from our lab, indicates that a specific combination of regulatory elements in the distal 5'-flanking region of the K18 gene, including the minimal promoter and intron-1, can direct efficient *lacZ* reporter gene expression in lung airway epithelial cells. However, regulatory elements that are essential for modulating the expression of K18 in a tissue-restricted manner and the transcription factors responsible for mediating this activity are not well understood. Thus, the present research concerns investigating transcriptional regulation by identifying and characterizing the interaction between lung epithelium-specific nuclear transcription factors and their cognate DNA binding domain within the K18 5'-flanking region. Within this context, cis-acting elements were mapped and isolated by transient transfection analysis of K18-promoter gene derivatives in different cell lines. Presently, three regions have been targeted as bait for the *in vivo* one-hybrid system. Subsequent screening of a human lung library for the isolation of biologically relevant DNA-BP are being generated. Further confirmation of the protein-DNA interaction will be made using *in vitro* assays. Additionally, co-transfection and transgenic mice analysis of the transcription factor will assist in not only providing a definitive biological role for this factor but also enable us to improve our gene expression cassette to target therapeutic gene expression in airway epithelial cells.

9. Investigation of Kinases Phosphorylating Microtubule Associated Protein, LC-3 in the Regulation of Fibronectin mRNA Translation.

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Intimal cushion formation in the fetal ductus arteriosus (DA) requires fibronectin (FN)-dependent smooth muscle cell (SMC) migration. Nitric Oxide (NO) mediates increased FN translational efficiency in DA SMC through enhanced phosphorylation and binding of LC-3, a microtubule-associated protein, to an AU rich element in the 3' UTR of FNmRNA. While the MAP kinase kinase inhibitor PD98059 decreased FN synthesis as assessed by metabolic labeling and gelatin-sepharose purification, western blotting showed no reduction in NO-mediated phosphorylation of LC-3 related to PD98059. *In vitro* assays also failed to demonstrate LC-3 phosphorylation by ERK-2. PD98059 did, however, decrease FN mRNA levels indicating that a MAP kinase may regulate the transcription or the stability of FNmRNA. NO can induce cGMP-dependent protein kinase G (PKG) activity, but FN synthesis was not decreased by a cGMP dependent kinase inhibitor. *In vitro* assays showed phosphorylation of LC-3 by PKA and PKC, but H89, a PKA inhibitor, and chelerythrine, a PKC inhibitor, did not decrease FN synthesis, although a slight decrease was observed with staurosporine, another PKC inhibitor with tyrosine kinase inhibitory activity. These results and that NO-mediated FN mRNA translation related to LC-3 phosphorylation is not related to PKG, PKA or PKC. Other candidate kinases including tyrosine kinases and casein kinases are being investigated based on sequence analysis of phosphorylation sites in LC-3.

10. Identification and Molecular Characterization of a Novel Member of the Siglec Family.

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Using the positional candidate gene approach, we have identified a novel member of the sialic acid-binding Ig-like lectin (Siglec) family, which belongs to the immunoglobulin superfamily (IgSF). We characterized the genomic structure of this gene, determine its chromosomal localization, its homology to other members of the siglec family, and its tissue expression profile. The novel siglec gene is comprised of seven exons, with six intervening introns. The coding region consists of 1,392 nucleotides, and produces a 463 amino acid protein. Further, we have localized this gene to 19q13.4, 43.19 Kb more telomeric than KLK-L6 (a member of the kallikrein gene family) through genomic sequencing data and restriction mapping with EcoRI. This novel siglec shows a high degree of homology to many members of the siglec family, including siglec-7 (80%), Siglec-5 (65%), and CD33 (64%). This high degree of homology is also conserved in the extracellular Ig-like domains. Through RT-PCR, we have examined the expression of this novel siglec in a large number of tissues, and have found relatively high expression in bone marrow, placenta, spleen, and fetal liver. Based on its homology to CD33, we speculate that this gene may also have some utility as a target for immunological antineoplastic therapy.

11. Characterisation of the Human Splicing Factor hPrp3p.

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Nuclear RNA splicing occurs in an RNA-protein complex, termed the spliceosome. U4/U6 snRNP is one of four essential small nuclear ribonucleoprotein (snRNP) particles (U1, U2, U5 and U4/U6) present in the spliceosome. The human splicing factor hPrp3p plays a major dynamic role in promoting the U4/U6 snRNA association at the early stages of U4/U6 snRNP assembly, as well as releasing U4 snRNA during spliceosome activation. Regions of hPrp3p may be involved in protein-protein or protein-RNA interactions that are critical to the assembly and disassembly of the spliceosome during RNA splicing. To identify the putative hPrp3p domains involve in nuclear localisation, hPrp3p mutants were constructed and expressed in HeLa cells. The nuclear and cell extracts were analysed by Western blot and immunostaining. The experiments suggest that the putative nuclear localisation signals (NLS) of the hPrp3p are necessary for its transport to the nucleus but their deletion only partially stops hPrp3p from going to the nucleus. We speculate that hPrp3p interacts with other(s) splicing factors such as hPrp4p in order to reach the nucleus. We are also working with yeast systems. A yPrp3 knock-out yeast strain was built to study whether the human Prp3 is able to complement the function of its yeast homologous. Other experiments such as immunoprecipitation are being carried out to analyse molecular interactions of hPrp3p and hPrp4p *in vitro* and *in vivo*.

12. Regulation of RGS Proteins By Phosphorylation: A Possible Mechanism in Insulin Regulation Of RGS4.

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The RGS (Regulator of G-protein Signaling) proteins are negative regulators of heterotrimeric G-protein signaling and function by enhancing the inherent GTPase activity of G subunits that are activated by G-protein coupled receptors (GPCRs). RGS4, a GTPase activating protein (GAP) for Gi and Gq _ is expressed in brain, heart, and pancreas tissues as well as in pituitary and adrenal cell lines. Recently our lab has shown through *in vitro* phosphorylation and in-gel kinase assays that ERKs 1/2 can phosphorylate his-tagged RGS4 but not his-tagged GAIP. Also immunoprecipitated RGS4 from insulin stimulated PC12 cells showed a reduced GTPase activity compared to unstimulated cells. Based on the above data, we hypothesize that the phosphorylation of RGS4 may alter GAP activity and/or create binding sites for new protein-protein interactions that may reduce GAP activity. To study the role of phosphorylation by MAPK, three mutants of putative MAPK sites have been made. In-gel and *in vitro* kinase assays will be utilized to verify the successful targeting of the phosphoacceptor sites. The mutant proteins will be functionally compared to wild-type RGS4 in GAP assays and protein interaction studies. To look at protein interaction, GST-RGS4 recombinant proteins have been made. To identify which insulin stimulated pathways are critical for regulation of RGS4 GTPase function and to define the relationship between RGS4 phosphorylation and GTPase activity, specific inhibitors of insulin activated kinases will be compared in GAP assays. Phosphorylation of RGS proteins is a new level of RGS regulation. The effect of insulin stimulation on RGS4s GAP activity suggests cross-talk between GPCRs and RTKs and represents a novel regulatory mechanism of RGS proteins by insulin.

13. Identification of BRCA2 Interacting Proteins.

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Germline mutations in the tumour suppressor gene, BRCA2, are found to be present in nearly half of all hereditary breast cancers and represent an elevated risk for this disease. The functional rationale for the increased risk resulting from these mutations still remains unclear. The BRCA2 protein is speculated to be involved in maintaining the integrity of the genome due to its association with the processes of DNA repair and recombination. To aid in the understanding of this gene, novel interacting protein(s) are being identified. We are utilizing a yeast two hybrid system with a vector containing different BRCA2 sequences, minus the c-jun like transactivation domain as the bait for a human mammary gland and HeLa cDNA library. The different bait molecules will also be used to determine the region(s) of BRCA2 involved in binding in other studies, such as p53. Vectors have been generated using the Pas-2 vector system which fuses the BRCA2 fragments in frame to Gal-4 DNA-binding domain. Results of screening will be discussed.

14. N-cadherin Mediated Cell-Cell Adhesion in the Arterial Wall.

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Smooth muscle cell migration in the arterial wall occurs during normal growth and development as well as during atherosclerosis and restenosis. In order for this to occur, smooth muscle cells must modulate interactions with surrounding matrix and cells. Smooth muscle and endothelial cells both express the cell-cell adhesion molecule N-cadherin. N-cadherin localizes to adhesion plaques on the cell surface of cultured smooth muscle cells, whereas the protein is diffusely distributed over the plasma membrane of endothelial cells. In other cell types, N-cadherin is important in the regulation of contact inhibition of cell migration, but the functions of this protein in vascular cells has not been determined. The goal of this study was to determine how the smooth muscle cell distribution of N-cadherin, and its related catenin proteins, are modulated when these cells are in contact with endothelial cells and when they assume a migratory phenotype during repair processes. Whole mount arterial preparations were prepared for *en face* examination and immunostained with anti-N-cadherin antibody. The protein localized to the fenestrae of the internal elastic lamina, an area of smooth muscle-endothelial contact. During neointimal formation after balloon injury, N-cadherin in intimal smooth muscle cells localized to the midpoint between the basal and apical surface of the cell. Strongest staining was observed for the luminal smooth muscle cells. These results indicate that the modulation of N-cadherin may be involved in regulating smooth muscle cell migration during arterial responses to injury.

15. NADPH Oxidase as the Molecular Oxygen Sensor in Pulmonary Neuro-Epithelial Bodies.

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Whole animal studies of the respiratory effects of intra-tracheal administration of oxidase inhibitor (DPI): In order to assess the physiological function of NEBs in the control of breathing, we administered an inhibitor of the putative molecular oxygen sensor in NEBs, namely NADPH oxidase, to newborn rabbits (4 day old). Hypoxic and normoxic ventilatory response of animals after the intra-tracheal administration of the drug was compared to that of the age matched control animals that underwent the exact procedure except that their intra-tracheal infusion solution did not contain the DPI. Furthermore, in order to evaluate the contribution of vagus nerve, which innervates pulmonary NEBs, vagotomy was performed in the same animals after a 20-minute washout period following DPI infusion. The ventilatory measurements in response to vagotomy, post-vagotomy administration of DPI, and hypoxia were compared against the aged-matched control group. Analysis of data is underway.
In vivo anterograde DiI tracing of vagal afferent nerves innervating pulmonary neuroepithelial bodies: Previous studies by Lauweryns in the late 80s, in which the results of supra- and infranodose vagotomy were compared, had indirectly shown that NEBs were innervated by sensory branches of vagus nerve. In order to directly investigate the NEBs vagal innervation we have used a tracing method of the sensory branches of the vagus nerve into the pulmonary tissue. 7-day old rabbits were anesthetized with an anesthetic drug combination (Ketamin+Cylasin+Atropin). Right Nodose ganglion was exposed through a ventral midline incision under surgically sterile condition. Bidirectional neural tracer DiI (dioleyl tetramethyl indocarbocyanine methanosulfonate, 25 mg/ml in methanol) in volumes of 10-100 μl was injected into the nodose ganglion. The incision was closed and 6 weeks were allowed for the fluorescent dye to be transported and to accumulate in the peripheral terminals of vagal afferent axons. After 6 weeks, animals were sacrificed and their lungs and brains were dissected, fixed, and sliced for fluorescent microscope analysis. A laser-scan confocal microscope is used to visualize the DiI-labelled vagal afferent nerves in lung. This technique can be used in conjunction with double staining for the NEBs to study their innervation pattern.

16. Epigenetic Regulation and Allelic Diversity in the Human Endothelin Converting Enzyme-1 (ECE-1) Gene.

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Vascular tone, perturbation of which is implicated in cardiovascular disease, is modulated by vascular endothelium through the release of vasoactive substances such as endothelin-1 (ET-1). PreproET-1 undergoes cleavage by ECE-1, a type II metalloendopeptidase, to yield the mature form of ET-1. Our laboratory has isolated 5 ECE-1 mRNA species from endothelial cells that evidence unique 5'-termini, ECE-1A, B, C, D, and E. These variants arise from alternate usage of 5 different promoters, give rise to proteins differing at their N-termini, and are expressed in a tissue- and cell-specific manner. The ECE-1C variant is expressed broadly across cell types, while the ECE-1A variant is endothelial cell-restricted. We hypothesize that cell-specific expression of the ECE-1 variants is regulated, in part, by DNA methylation. Preliminary results using the sodium bisulfite genomic sequencing method demonstrate that the ECE-1A promoter is largely unmethylated in endothelial cells, but densely methylated in non-expressing cells. Furthermore, we demonstrated that the ECE-1C promoter harbors a highly polymorphic alternating purine-pyrimidine sequence of the d(CA)n type (18 alleles from 200 chromosomes, heterozygosity value=0.91). We proposed that the allelic diversity in the ECE-1C promoter is responsible for differential transcription of the ECE-1C variant in different individuals. Transient transfections of cultured vascular endothelial cells with promoter/reporter luciferase constructs have demonstrated that promoter activity is strongly influenced by the allele present, providing a potential molecular mechanism for interindividual differences observed in the expression pattern of ECE-1 mRNA and protein. This is an important finding with respect to human disease since ECE-1C promoter activity may depend on the inheritance of a particular allele.

17. The *in vivo* Effects of AhR Ligands on Young Female Rats.

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Cigarette smoke is a major source of environmental toxins and recognized as one of the risk factors for postmenopausal osteoporosis and compromised reproductive function. Although the mechanism by which this association occurs is less clear, it is known that benzo(a)pyrene (BaP) and 7,12-dimethylbenz(a)anthracene (DMBA), are found in significant amounts in the tar fraction of cigarette smoke. These compounds are also known to be prototypical polycyclic aromatic hydrocarbons which bind the aryl hydrocarbon receptor (AhR). More recently, resveratrol, an anti-fungal agent found in red wine, was shown to be a natural antagonistic AhR ligand. We hypothesize that AhR ligands, such as BaP and DMBA, are responsible for the toxic effects associated with cigarette smoking. Our proposed mechanism by which BaP:DMBA exerts this association is via an indirect toxic effect on bone and a direct effect on the ovary. Preliminary studies using western blot analysis revealed that the rat liver, kidney, lung and ovary were sensitive to the toxic effects of BaP:DMBA DNA adducts generated by CYP1A1 *in vivo*. Furthermore, this effect could be diminished or reversed with resveratrol. Based on our findings, we have conducted a 17-week study using young female Sprague-Dawley rats to investigate the long-term effects of AhR ligands specifically on osteogenic and ovarian function, and to determine if resveratrol is able to protect against the adverse effects of AhR ligands. The objective of our experiment design was to mimic the situation of premenopausal women who are exposed to AhR ligands. Quantitative analysis of urinary pyridinium crosslinks and serum osteocalcin are currently being studied to investigate the chronic effect of BaP:DMBA on bone resorption and bone formation.

18. An *in vivo* Study on the Effect of AhR Ligands from Cigarette Smoke and Resveratrol from Red Wine in Rats.

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Epidemiology studies have shown that cigarette smoking has detrimental effect on the bone. It is believed that the binding of aryl hydrocarbon receptor (AhR) ligand to AhR is responsible for the bone loss. Benzo[a]pyrene (BaP) and 7,12-dimethylbenz[a]athracene (DMBA) are two AhR ligands found in cigarette. Resveratrol is an AhR antagonist, which is found in red wine. We hypothesize that BaP/DMBA have a direct effect on bone loss and that resveratrol, through its antagonistic property, can prevent AhR-induced bone loss. To investigate a direct bone loss due to AhR, we used an aged rat model independent of ovarian function. The removal of the ovaries eliminated the ovarian function as a variable. However ovariectomy would lead to a loss of estrogen; which has been shown to cause bone loss. To prevent this, we re-introduced estrogen back into the rats. In order to determine the direct effects of BaP/DMBA, two dose-dependent studies on BaP/DMBA and resveratrol were examined. Western blots were used to evaluate tissue response. From these studies, we determined that 250ug/kg of BaP/DMBA and 1250ug/kg of resveratrol as the dosages to use in this study. Nine-month old Sprague-Dawley rats were sub-cutaneously injected with BaP/DMBA and/or resveratrol for 15 weeks. Osteocalcin (a marker for bone formation) and pyridinium crosslinks (a marker for bone resorption) will be used to measure for increases in bone turnover. Osteocalcins, synthesized from osteoblasts, will be measured by a sandwich EIA assay. Pyridinium crosslinks, from the degradation of collagen, will be measured by a competitive enzyme immunoassay. The increase in bone turnover would indicate an increase in bone loss.

19. The Effects of Hyaluronan on Chondrocyte-Matrix Interaction.

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Articular cartilage, composed of chondrocytes and extracellular matrix, is a thin resilient layer covering the joint. In North America, joint diseases, such as osteoarthritis and rheumatoid arthritis, and joint injuries from sports affect a large percent of the population. In these two scenarios, cartilage is damaged and fails to lubricate joint movement. An abundant component of the matrix is hyaluronan, a repeating disaccharide of glucuronic acid and N-acetyl-D-glucosamine. This macromolecule, belonging to the glycosaminoglycan family, is believed to play an important role in maintaining a unique three-dimensional structure of a functional joint. This study was designed to investigate the roles of hyaluronan in mediating chondrocyte-matrix interactions. Hyaluronan was digested with hyaluronidase. To suspension chondrocyte cultures, various concentrations of hyaluronidase were added under different conditions. Degradation of hyaluronan induced chondrocyte aggregation in agarose-coated wells. In addition, chondrocyte attachment to normal tissue culture plates, collagen-coated plates, fibroblast-like chondrocytes and cartilage sections were observed with hyaluronidase treatment. In the case of tissue culture plates, treated chondrocytes attained an elongated morphology. Chondrocyte interactions with other matrix molecules were also examined. Suspension chondrocyte cultures were treated with hyaluronidase then stained for matrix molecules. Aggrecan level was analyzed with Alcian blue staining, while type II collagen and link protein were detected by immunohistochemistry and FACS analysis. Introduction of hyaluronidase to suspension culture decreased chondrocyte interactions with aggrecan, type II collagen and link protein. Our results indicated degradation of hyaluronan disrupts chondrocyte interaction with the rest of the matrix. This suggests that hyaluronan affects the stability of chondrocyte-matrix interaction.

20. Effect of Atorvastatin (Lipitor™) on VLDL-apoB Overproduction in an Insulin Resistant Hamster Model.

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A novel animal model of insulin resistance, the fructose-fed Syrian golden hamster, was employed to investigate the effect of atorvastatin, a potent HMG-CoA reductase inhibitor, on the overproduction of VLDL in the insulin resistant state. Fructose feeding for a period of two weeks induced significant hypertriglyceridemia and hyperinsulinemia, and the development of whole body insulin resistance. To investigate the effect of atorvastatin on VLDL-apoB overproduction in fructose-fed hamsters, we performed a series of *in vivo* feeding experiments. Two groups of three hamsters were fed a fructose-enriched diet for 14 days to induce the state of insulin resistance, followed by a fructose-enriched diet supplemented with 40mg/kg atorvastatin for 7 to 14 days. Fructose feeding in the first two weeks caused a significant increase in plasma total cholesterol and triglyceride in both groups. However, there was a significant decline in plasma triglyceride levels following supplementation of the fructose-enriched diet with atorvastatin (Day 1, 1.7+/-0.4 mmol/L; Day 14, 4.7+/-0.2 mmol/L; Day 21, 1.5+/-0.3 mmol/L; Day 28, 1.5+/-0.5). This decline was not observed in control animals receiving a fructose-enriched diet without the drug (Day 1, 1.4+/-0.5 mmol/L; Day 14, 3.8+/-0.8 mmol/L; Day 21, 3.3+/-0.4 mmol/L; Day 28, 3.0+/-0.4 mmol/L). In addition, experiments with hepatocytes isolated from both groups of hamsters revealed an average 37% decrease in VLDL-apoB production in hepatocytes isolated from hamsters fed atorvastatin compared to the control group. Experiments studying the effect of atorvastatin feeding on intracellular apoB stability showed no significant difference in the intracellular stability of apoB in both groups. However, there was a significant decrease in the levels of apoB secreted from atorvastatin fed hamsters compared to control hamsters, suggesting that atorvastatin was not affecting the synthesis of apoB, rather increasing it's degradation. Taken together, these data suggest that atorvastatin treatment appears to ameliorate the VLDL-apoB overproduction observed in a fructose-fed, insulin resistant hamster model.

21. The Neuroprotective Effects of Insulin May Operate Via Interactions with the Akt Signalling Pathway.

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Stroke, an interruption of blood supply to some or all of the brain, is a leading cause of mortality and disability. Stroke pathogenesis is often viewed as necrotic, the consequence of massive release of the excitatory neurotransmitter glutamate, however, recent work has suggested that apoptosis, a distinct cell death progression, may play an extensive role. The hormone insulin has been shown to protect against stroke induced cell death, and although the underlying mechanism is unknown, one putative explanation is that the activated insulin receptor causes a downstream translocation of the serine/threonine kinase Akt from the cytosol to the plasma membrane, thereby activating the peptide and setting in motion a cell survival cascade. Primary cultured mouse cortical neurons have been used to establish an *in vitro* model of stroke based upon exposure to varying concentrations of glutamate. Cell viability has been assayed via cytofluor analysis of propidium iodide fluorescence, and increasing cell death has been shown to occur with increasing glutamate concentrations. Analysis of glutamate treated cells has also revealed the presence of altered nuclear morphology and DNA strand breaks, as evidenced by Hoechst 33258 and TUNEL staining, indicating that apoptosis plays a role in the death of these cells. To examine insulin effects on Akt, HEK cells were transiently transfected with GFP tagged wild type Akt, and translocation from the cytosol to the plasma membrane following insulin administration was seen via confocal microscopy. Future efforts will determine whether insulin can limit the cell death and stigmata of apoptosis witnessed with glutamate toxicity, and will work to clarify the role of insulin's stimulation of the Akt survival pathway.

22. Murine Colon Carcinogenesis: Alterations in Cell Adhesion Before Morphologic Evidence of Neoplastic Changes.

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Colon cancer is the second cause of cancer death in North America. It is characterised by a series of morphologically visible stages, known as the adenoma-carcinoma sequence, with the accumulation of genetic mutations affecting cell proliferation and apoptosis. Recently, however, it has become more apparent that other molecules, such as cell adhesion molecules, are linked with these processes and thus, may also in part play important roles in colon cancer development. Most studies on adhesion molecules have focused on the late stages of colon cancer, such as invasion and metastasis. However, it is not known whether alterations in cell adhesion consistently occur before morphologically visible changes are observed in the transformation of the colonic epithelium. One strong candidate marker is the CD44 family of cell adhesion molecules that consist of diverse structural and functional isoforms that are generated by alternative splicing and glycosylation. Specific CD44 isoforms are already overexpressed at the polyp stage of colonic neoplasia. We hypothesize that alterations in CD44 expression occur as early as the pre-malignant stage of colon cancer and can thus be used as a predictive marker of colon cancer development. Using a murine model of colon cancer, induced by the carcinogen azoxymethane (AOM), changes in CD44 RNA transcript and protein expression are being investigated, before morphologically apparent neoplasms can be observed grossly or histologically. Changes in CD44 protein expression are being investigated using immunohistochemistry and western blot analyses. Changes in CD44 transcript expression are being investigated by reverse-transcriptase polymerase chain reaction (RT-PCR).

23. Apigenin Inhibits Cell Cycle Progression and Induces Apoptosis in Human Neuroblastoma.

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Neuroblastoma (NB) is a paediatric cancer of the peripheral nervous system. Apigenin, a dietary flavonoid, has anti-tumour promoting, anti-proliferative and anti-angiogenic effects. To investigate the effects of apigenin on human NB, NUB-7 and LAN-5 cell lines were treated with 0-200mM apigenin. After treatment for 24 hours NUB-7 and LAN-5 displayed marked morphological changes with increasing dose of apigenin, as determined by phase contrast microscopy. Cell numbers were significantly reduced in NUB-7 (75% at 100mM) and LAN-5 (90% at 200mM) in monolayer culture. Long term treatment with 60mM apigenin abrogated colony formation of NUB-7 cultured in soft agarose. Apigenin (10-150 μM) induced decreased AlamarBlue fluorescence in NUB-7 and LAN-5 indicating a dose dependent increase in cytotoxicity. Cytotoxicity inversely correlated with fetal bovine serum concentrations in the culture medium, suggesting that components in serum may protect against the cytotoxic effects of apigenin. 100mM apigenin induced an increase in TUNEL positive staining of fixed cells by 14.3 fold in NUB-7 and 7.4 fold in LAN-5. Apigenin induced an increase in the 85 kDa poly (ADP-ribose) polymerase cleavage product in both cell lines further indicating induction of apoptosis. Propidium iodide staining and FACS analysis demonstrated that 100mM apigenin induced an increase in G0/G1 (47.3%) and G2/M (12.7%) fractions in NUB-7, while LAN-5 showed an increase in G0/G1 (14.3%) and G2/M (58.96%), indicating that apigenin likely targets different cell cycle related signaling pathways in NB. These results indicate that apigenin may have therapeutic potential in human NB.

24. Effect of Atorvastatin on Hepatic Production of Atherogenic Lipoproteins in Insulin Resistance Syndrome/Metabolic Syndrome X.

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We have investigated the potential hypolipidemic effects of atorvastatin, an HMG-coA reductase inhibitor, in patients with insulin resistance syndrome/metabolic syndrome X. Twenty patients were recruited for the study and baseline measurements of specific parameters were obtained. The group was then randomized, in a double-blinded manner, into two different sub-groups. Group A received atorvastatin (80mg) and group B received placebo daily for two months. At the end of the first treatment period, all patients were re-analyzed for the same parameters. The second treatment period then began with group A taking a placebo and group B taking atorvastatin (80mg) daily for 60 days. All parameters were re-measured at the end of the study for each patient. Treatment with atorvastatin resulted in a statistically significant reduction in total cholesterol (41%), LDL-cholesterol (55%) and apolipoprotein B (40%). Plasma triglyceride levels were also reduced but did not reach statistical significance. Interestingly, changes in apoB levels correlated with changes in lipid profiles. Measurement of c-peptide levels suggested a reduction in insulin production rate following atorvastatin treatment. Mean LDL-particle diameter significantly increased after treatment with atorvastatin resulting in the presence of less atherogenic particles. Atorvastatin also significantly changed the distribution of the LDL-particle size profile. At baseline, LDL-particles were predominantly found in the small dense LDL-subclass; atorvastatin treatment resulted in a shift in the profile to the larger and more buoyant LDL-subclass. Atorvastatin treatment did not produce consistent changes in the generation of apoB degradation fragments. Overall, the data suggests that atorvastatin significantly reduces the hepatic production of apoB and apoB-containing lipoproteins and also induces a significant decrease in the density of LDL particles produced (resulting in more buoyant and less atherogenic particles) in patients diagnosed with insulin resistance/metabolic syndrome X.

25. The $T_{1/2}$ in the Blood Compartment and Subsequent Tissue Reassortment of Fluorescently Labeled Peripheral Blood Leukocytes.

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Leukocyte migration and recirculation via the blood vasculature and lymphatic system is the putative basis of immune surveillance. To study the physiological aspects of this dynamic process fluorescent (fluorescein-5-isothiocyanate (FITC), and 5-(and-6) carboxyfluorescein diacetate succinimidyl ester, (CFSE)) or radio-labels (Chromium and Indium) were employed to mark peripheral blood leukocytes (PBLs). The labeled PBLs were returned via the venous circulation of sheep and tracked in the blood, lymph, peripheral lymph nodes and spleen. The emigration kinetics out of the blood were studied over a two week period observing the initial disappearance and later recirculation-dependent disappearance of labeled PBLs. 4.96 ± 1.16% of the labeled cells were recovered per 10^9 blood leukocytes at 40 hours post infusion (n=6). In lymph the 40 hour recovery per 10^9 cells was one-tenth the recovery in blood. The tissue distribution of labeled cells was determined 8 hours post infusion (n=4), and 5 days post infusion (n=5). These data indicate that the overwhelming majority of lymphocytes in a resting lymph node are part of the recirculating lymphocyte pool and are "in transit" rather than residential. (Supported by MRC, CANADA).

26. Temperature-Sensitive Stability Defect of the Cell Surface Associated ΔF508 CFTR.

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Cystic fibrosis (CF) is caused by the dysfunction of CF Transmembrane Conductance Regulator (CFTR), which is a cAMP-stimulated chloride channel at the plasma membrane. The consensus notion is that the most common mutation, deletion of phenylalanine 508 (F508) is misfolded and degraded at the endoplasmic reticulum (ER), compromising its accumulation at the cell surface. While partial correction of the ER-retention was achieved with chemical chaperones in cultured cells, low level of functional expression was documented at the apical surface of epithelia in homozygote F508 mice, suggesting that the intracellular retention of the F508 CFTR is not complete. To further elucidate the reasons behind the inability of F508 CFTR to efficiently accumulate at the plasma membrane, the biological half-life and structural stability of the complex-glycosylated F508 CFTR, following its escape from ER, were determined. Here we show that the complex-glycosylated F508 CFTR has a temperature-sensitive stability defect *in vivo*. The accelerated degradation of the mutant is conceivable due to a global decrease of its conformational stability, as demonstrated by *in situ* protease susceptibility and SDS-resistant thermoaggregation. Thus besides the impaired processing, the F508 mutation compromises the stability of the spontaneously or pharmacologically rescued mutant CFTR. Therefore therapeutic efforts to correct the folding defect should be combined with the stabilization of F508 CFTR at the cell surface in CF.

27. Characterization of the C6 Sulphate Binding Site in the Active Site of Hexosaminidase A.

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The lysosomal enzyme Hex A is responsible for the degradation of GM2 ganglioside, a glycosphingolipid found in the gray matter of the brain. The enzyme consists of two nonidentical subunits respectively referred to as alpha and beta. Mutations in the former result in Tay-Sachs disease and in the latter, Sandhoff disease. The genes encoding for these polypeptides are evolutionarily related, thus both subunits are able to hydrolyze neutral substrates such as N-oligosaccarides and artificial substrates such as 4-MUG (4-methylumbelliferone-beta-N-acetylglucosamine). However, it is only the alpha subunit that is also capable of hydrolyzing negatively charged substrates such as 4-MUGS (4-MUG-6-sulphate) and more importantly, GM2. Thus, although both subunits are associated with distinct active sites, dimerization is required for either subunit to become catalytically active. Using 3D modeling of the evolutionarily related enzyme chitobiase, we identified two residues (Arginine 424 and Asparagine 423) that are hypothesized to interact with the C6 sulphate group of 4-MUGS. The following mutations were designed in order to examine the effects of altering these two residues on the ability of the alpha subunit to hydrolyze 4-MUGS: Arginine424Lys, Arginine424Glutamine, Asparagine423Aspartic Acid. Transient transfections with normal CHO cells indicated that the resulting Hex S protein (an alpha dimer) was not efficiently able to hydrolyze 4-MUGS, with the R424Q mutation showing no activity. Similar results were observed when the alpha cDNAs corresponding to each of the mutations were cotransfected with an inactive beta sequence (resulting in mutated Hex A). Further work is needed in order to further understand this apparent decrease in the hydrolysis of 4-MUGS as a consequence of mutating these two residues.

28. Mutational Studies of an Essential Glutamate Residue in Folylpolyglutamate Synthetase: Role in ATP Binding and a Conformational Change Induced by Folate Binding.

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Site-directed mutagenesis studies were performed on an essential glutamic acid residue, Glu143, in the *Lactobacillus casei* folylpolyglutamate synthetase (FPGS) and the structurally equivalent residue, Glu123, in the *Escherichia coli* FPGS. Glu143 is positioned near the P-loop in the same position as acidic residues that interact with the Mg^{2+} of Mg-ATP in proteins, such as p21ras, MurD and G-proteins that are structurally similar to FPGS and have a mononucleotide fold. *L.casei* FPGS mutant proteins E143A, E143D and E143Q and all had less than 1% of the activity of the wild type enzyme. The mutant proteins failed to complement the methionine auxotrophy of the *E.coli folC* mutant SF4, showing that Glu143 is essential for the function of the enzyme. ATP photolabeling studies suggested that the *L.casei* FPGS mutants could bind ATP normally. Equilibrium dialysis studies with *L.casei* FPGS mutant proteins and fluorescence studies with the *E.coli* mutant protein showed that they bound folate and pteroate substrates with the same affinity as the wild type enzymes. The *E.coli* E123Q mutant protein bound –^{32}P ATP, as well as wild type FPGS did, but no binding of –^{32}P ATP to the mutant enzyme could be detected. The mutant protein had higher ATPase activity than the wild type enzyme. The presence of dihydropteroate or tetrahydrofolate increased the affinity of ATP binding, suggesting that a conformational change occurred after binding of these substrates. An increase in affinity for –^{32}P ATP was observed with the E123Q mutant using equilibrium dialysis but not with a non-equilibrium spin column assay, showing that the mutant was defective in performing this conformational change.

29. New Insight into Nicotine's Role in Development of the Cardiovascular Disease.

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Nicotine has been linked to development of occlusive thromboangitis obliterans (Buerger disease) in young smokers, as well as to the sudden arterial occlusion in heavy smokers with preexisting atherosclerosis. Since, most arterial occlusions occur in result of progressive intimal thickening, the present studies have been aimed at elucidation whether nicotine and its active metabolite, cotinine may stimulate proliferation, migration and extracellular matrix deposition by coronary artery smooth muscle cells (CA SMC). Our results indicate that nicotine and cotinine (tested in concentrations 10^{-3}M to 10^{-7}M) do not induce any cellular effect which can justify development of progressive arterial lesions. On the contrary, they inhibited migration and proliferation of cultured CA SMC and substantially decreased their deposition of fibronectin and elastin. Moreover, nicotine caused occlusive collapse of the carotid arteries in organ cultures. Our data indicate that such occlusive collapse of the whole artery can be linked to the fact that nicotine and cotinine impaired normal arrangement the actin cytoskeleton, which in turn, lead to a rapid lose of the characteristic shape, inhibition of the cell movements, proliferation, and disturbances in the intracellular transport and secretion. We suggest that these effects of nicotine and cotinine may lead to weakening of arterial walls and to inward eutrophic remodeling that may explain their sudden collapse and occlusion.

30. Intracellular Traffic of Verotoxin Subunits.

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Verotoxin-1 (VT1), produced by certain strains of enterohaemorrhagic *Escherichia coli*, is etiological agent of haemorrhagic colitis and haemolytic uremic syndrome. VT1 is an AB_5 toxin consisting of an enzymatic A-subunit and a receptor binding B-subunit pentamer. Upon binding to the cell surface receptor, globotriaosylceramide (Gb_3), in certain cell lines VT1 is internalized and transported in a retrograde manner to the Golgi, ER, and nuclear envelope. From the ER, the A-subunit is thought to translocate to the cytosol where it inhibits protein synthesis. Fluorescein-labelled VT1 B-subunit has been shown to target the nucleus and ER in highly toxin-sensitive tumour cells as well as in butyrate-treated cell lines (Arab & Lingwood, 1998, J. Cell. Physiol. 177: 646-660). VT1 holotoxin has been shown to increase some steady-state mRNA transcript levels in endothelial cells (Bitzan et al., 1998, J. Clin. Invest. 101: 372-382). These findings indicate a possible nuclear effect of the holotoxin, in addition to classical protein synthesis inhibition. To determine the intracellular location of the A and B subunits various combinations of fluorescent holotoxin were constructed. These fluorescent VT1 holotoxins retain full cytotoxicity before dissociation and after reconstitution. Confocal microscopy of toxin treated SF-539 human astrocytoma cells shows the A-subunit is internalized to a perinuclear region and within the nucleus, as previously reported for the B-subunit. Streptolysin-O permeabilized Vero cells revealed the presence of processed A-subunit in the cytosol while holotoxin was found in the cytosol of permeabilized SF-539 and HeLa cells. These results indicate that not only internalization route but processing pathways of the toxin may be cell-type dependent and the A-subunit may have a role other than protein synthesis inhibition.

31. Expression and Regulation of Matrix Metalloproteinases (MMPs) and Tissue Inhibitors of Metalloproteinases (TIMPs) in the Hematopoietic Microenvironment.

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The hematopoietic microenvironment is a complex structure comprised of stem cells, progenitor cells, growth factors and extracellular matrix (ECM) molecules. Stem cells and progenitor cells interact specifically with microenvironment present in the bone marrow. Matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) are key regulators of ECM turnover. In addition, MMPs and TIMPs have growth modulatory activities for a variety of cell types. We hypothesize that MMPs and TIMPs are expressed and regulated by cytokines and growth factors in a cell specific manner. The general aims of this study are to analyze the expression and function of MMPs and TIMPs in different cellular components of the hematopoietic microenvironment. We have analyzed the expression of functional gelatinases (MMP-2, MMP-9) and their inhibitors (TIMP-1, TIMP-2) in long-term bone marrow cultures (LTBMC) by zymography, reverse zymography and Western blotting. Bone marrow cells constitutively express MMP-9 and TIMP-2 whereas expression of MMP-2 and TIMP-1 is correlated with the growth of the stromal cell population. Isolated bone marrow stromal cell cultures were established to study cytokine regulation of MMPs and TIMPs. MMP-9 and MMP-2 expression was induced by PMA, TNF- and IL-1 . PMA, IL-1 and EGF induced TIMP-1 expression in stromal cells approximately 2-fold over the control. Stromal cells did not express TIMP-2. Combinations of PMA/TNF- and GM-CSF/ IL-1 had synergistic effects on the induction of MMP-9 and TIMP-1 expression. Cytokine-induced expression of MMP-9 and MMP-2 was inhibited by protein kinase inhibitors, thus suggesting the involvement of a tyrosine kinase signaling pathway.

32. Dual Role of $CD4^-CD8^-$ T Cells in Preventing Graft-vs-Host and Promoting Graft-vs-Leukemia Responses.

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Anti-leukemia effects can be achieved by infusing patients with allogeneic lymphocytes from healthy donors, however the infused lymphocytes can also respond to major (MHC) and minor histocompatibility antigens and cause graft-versus host disease (GVHD). Using a transgenic system, we have previously demonstrated that reconstitution of immunodeficient mice with single class I locus mismatched splenocytes leads to indefinite GVHD-free survival of the recipient animals, and produces an anti-lymphoma response. In the present study we aimed to validate these findings in non-transgenic animals and to identify the underlying mechanisms. Non-transgenic mice were lethally irradiated and reconstituted with single class I locus mismatched splenocytes, and co-injected with a B cell lymphoma (A20) syngeneic to the host. These animals survived indefinitely tumour-free and without GVHD. To determine the fate of donor T cells, FACS analysis was performed following reconstitution. The total number of anti-host $CD8^+$ cells increased 10-20 fold within days after reconstitution, and then quickly decreased. Interestingly anti-host $CD4^-CD8^-$ (double negative, DN) T cells significantly increased throughout the experimental period to over 30 fold on day 150. When DN T cells were co-injected with A20 tumour cells into naïve mice, the mice survived tumour-free for more than 3 months, whereas the control animals injected with A20 alone succumbed to lymphoma at 30-40 days without any clinical signs of GVHD. *In vitro* suppression assays with DN T cell clones show that these cells can mediate the suppression of A20 tumour cells. Furthermore, these DN cells can kill syngeneic anti-host T cells *in vitro*, suggesting a role for the DN cells in preventing GVHD. In conclusion, injection of single class I MHC locus mismatched lymphocytes does not cause GVHD, but results in an expansion of DN T cells in the periphery of the recipients. These DN T cells can eliminate both anti-host $CD8^+$ T cells and leukemia cells, thereby preventing GVHD and promoting anti-leukemia.

Ph.D. Poster Presentations:

33. Protein Dependent Excessive Phosphatidylcholine Core Aldehyde and Lysophosphatidylcholine Production During High Density Lipoprotein Oxidation.

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High density lipoproteins (HDL) are rich in phospholipids and these play an important role in the antiatherogenic properties of this lipoprotein. Peroxidation is believed to reduce the effective plasma HDL level to compromise its physiological function. However the oxidation products, that are potentially responsible for this action have not been identified. In separate experiments, we subjected human HDL, trypsinized HDL and HDL lipid liposomes to oxidation by the $ONOO^-$ donor, 3-morpholinosydnonimine (SIN-1). We determined the yield and composition of the phosphatidylcholine (PtdCho) oxidation products using normal phase liquid chromatography with on-line electrospray mass spectrometry (LC/ES/MS) The basal level of PtdCho mono- and di-hydroperoxides increased similarly in HDL, HDL lipid liposomes and in trypsinized HDL during 6 hours of oxidation. The core aldehydes (C5 and C9) of PtdCho were not detectable at zero time, but increased with time to a significantly higher concentration in HDL, compared to liposomal HDL and trypsinized HDL (16.5 $\pm$ 1.2μg/ml, versus 4.2 $\pm$ 0.4 μg/ml and 1.6 $\pm$ 0.6μg/ml respectively, $p<0.004$, $p<0.0021$). The PtdCho isoprostanes iso PGE2/D2, iso-PGF2 were also detected at baseline. After 6 hours of oxidation they had accumulated in similar amounts in HDL, HDL lipid liposomes and in trypsinized HDL. The basal level of lysoPtdCho increased 300% in HDL, compared to 80% in trypsinized HDL, and 25% in HDL lipid liposomes (55.5 $\pm$ 11.2μg/ml, versus 19.2 $\pm$ 2.4 μg/ml and 22.4 $\pm$ 11.2μg/ml, respectively; $p <0.0016$, $p<0.0027$). We conclude that the formation of PtdCho hydroperoxides and Ptdcho isoprostanes occurs independently of HDL proteins. In contrast the accumulation of PtdCho core aldehydes and lysoPtdCho are increased by residual activity of HDL-bound enzymes.

34. Expression of HGF and met in Wilms Tumours.

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HGF and its receptor met are important regulators of tubulogenesis in normal kidney. HGF and met expression were examined in a series of homotypic and heterotypic Wilms tumours. By *in situ* hybridization, high levels of met expression were observed in tumour blastema and epithelium relative to negligible levels in surrounding stroma. Met protein expression using immunohistochemistry showed similar results. Western blot analysis revealed the met 145kD beta chain and 170kD precursor form. Immunohistochemically, HGF protein was expressed in blastema and epithelium; Western blot analysis showed the 69kD alpha chain. HGF and met protein were also co-expressed in the heterotypic rhabdomyoblastic and smooth muscle elements. Staining with the MIB-1 monoclonal indicated a high proliferative fraction in blastema and epithelium relative to low levels in stroma. Interestingly, the proliferative index corresponded with differential patterns and levels of HGF and met expression in Wilms tumours. These observations lead us to postulate a potential autocrine/paracrine growth pathway governed by HGF and met in Wilms tumours.

35. Protein-Protein Complexes in Myelin.

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Myelin is produced as an extension of the oligodendrocyte plasma membrane in the central nervous system. The two major myelin proteins are myelin basic protein (MBP) and proteolipid protein (PLP). Other proteins include 2-3-cyclic nucleotide 3-phosphodiesterase (CNP) and myelin-associated glycoprotein (MAG). Despite the abundance of biochemical and molecular knowledge accumulated thus far, no single function has been definitively assigned to any of the myelin proteins. However, recent information provided with the use of non-ionic detergents such as TritonX-100, has furthered our understanding of myelin protein interactions and organization. These studies have shown the differential extraction of myelin proteins and the cytoskeletal proteins actin and tubulin. We studied proteins associated in Triton X-100 soluble extracts by immunoprecipitation and Western blotting. A significant fraction of the PLP, MBP, MAG, CNP and actin were present in the soluble fraction. By immunoprecipitating with both anti-PLP and anti-MBP antibodies we have demonstrated that PLP, MBP and MAG are complexed together. CNP and actin were not associated with this complex. The inclusion of 25mM $MgCl_2$ in the buffer, but not 300mM KCl, dissociated PLP from the MBP-MAG complex. This suggests that PLP is associated with this complex by electrostatic interactions. Since MAG is only present in the paranodal loops and periaxonal membrane of CNS myelin, this complex must come from these regions. Further studies of the complex to determine additional associations, and studies of the insoluble fraction for protein associations may reveal the structural and functional significance of several myelin proteins.

36. Incretin Antagonists Define Contrasting Roles for GLP-1 and GIP in the Control of Glucose Homeostasis *In Vivo*.

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Glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide I (GLP-I) have been identified as incretins, gut-derived peptides secreted in response to nutrient ingestion that potentiate glucose-stimulated insulin secretion. The physiological importance of incretins for glucoregulation is exemplified by studies of mice with defects in incretin receptor function. Mice with a targeted disruption in the GLP-1 receptor gene (GLP-1R -/-) exhibit increased levels of fasting glycemia and abnormal glycemic excursion following both oral or intraperitoneal glucose challenge. In contrast, GIPR-/- mice exhibit abnormal glycemia after oral glucose loading, but fasting and post IPGTT glucose is normal in the absence of GIPR signaling. As knockout mice may exhibit subtle developmental and adaptive mechanisms that modify interpretation of physiological phenotypes, we have examined the differential roles of GLP-1 and GIP using the GLP-1 receptor antagonist exendin (9-39) and GIP immunoneutralizing antisera in +/+ and -/- mice *in vivo*. Treatment with exendin 9-39 produced a significant increase in blood glucose levels following either oral or i.p. glucose in wild type mice, but had no effect on blood glucose levels in the GLP-1R -/- mice. Administration of anti-GIP antisera produced significant increases in blood glucose levels in both wild type and GLP-1 R -/- mice following oral glucose. In contrast, administration of anti-GIP antisera had no effect on blood glucose levels following i.p. glucose loading. The effect of chronic exposure to the incretin antagonists was also examined *in vivo*.

37. Chromosomal Aberrations in Early Stage Prostate Cancer Patients and Virus-Immortalised Cell Lines Identified by Combined CGH, SKY, and Allelotyping Techniques.

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We have utilised comparative genomic hybridization (CGH) with confirmatory interphase nuclei fluorescence *in situ* hybridization (FISH) to examine early stage prostate cancer (CaP) patients for recurrent chromosomal copy number changes suggestive of tumour initiation and progression. In addition, combined conventional and spectral karyotyping (SKY) techniques and allelotype analysis were used to assess numerical and structural chromosomal alterations in three cell lines derived from normal human prostatic epithelium, and three cell lines derived from primary human prostate tumour epithelium, immortalized with the Large T antigen gene of simian virus 40 (SV40) or the E6 and E7 transforming genes of human papillomavirus (HPV) 16, respectively (Macoska et al, Cancer Genetics & Cytogenetics, In Press). CGH and interphase FISH results suggested gain of chromosome 8q and loss of 8p in the patient cohort. Interestingly, allelotyping identified loss of 8p sequences in two of the three primary prostate tumour-derived cell lines, and SKY analysis revealed that the loss of 8p sequences was directly due to isochromosome 8q formation and/or other structural alterations of chromosome 8. This provides evidence that 8p loss in primary human prostate tumours may, in some cases, result from complex structural rearrangements involving chromosome 8. Moreover, the data reported here provides direct evidence that such complex structural rearrangements sometimes include isochromosome 8q formation.

38. Synergistic Interaction Between the Paired-Like Homeodomain Protein, Alx4, and the HMG-Box Protein, LEF-1, Modulates N-CAM Promoter Activity.

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The patterning of developing tissues and organs during embryogenesis is mediated by inductive processes between opposing epithelial and mesenchymal cell layers. Signaling between these layers governs organogenesis from initiation of tissue development to terminal differentiation of organ-specific cell types. Considerable progress has been made towards identifying the molecules and mechanisms that mediate the patterning of developing tissues. We and others recently isolated a paired-like homeodomain protein, mouse *aristaless-like-4 (Alx4)*. During murine embryogenesis, Alx4 expression is restricted to mesenchymal cells in developing bones, limbs, hair, whiskers, teeth, and mammary glands. The development of all these tissues is also dependent on expression of the HMG-box transcription factor, lymphoid enhancer factor, LEF-1. Several domains of Alx-4 are predicted to facilitate protein-protein interactions, including a WW/SH3 protein binding domain, the paired-like homeodomain, a proline rich region, and the paired-tail motif. These domains are important in specifying the transcriptional activities of Alx-4. Given the requirement for LEF-1 activity during formation of several tissues expressing Alx4, we demonstrated previously that Alx4 and LEF-1 expression overlap spatially and temporally in developing hair follicles during mouse embryogenesis. Since LEF-1 is known to bind positive and negative transcriptional regulators, we hypothesized that LEF-1 may form physical complexes with Alx4 resulting in altered expression of target genes. We demonstrate the interaction between Alx4 and LEF-1 and further show that these two factors can simultaneously bind to specific sites in the promoter region of the cell adhesion molecule, N-CAM. LEF-1 and Alx4 alter transcription from the N-CAM promoter and, when expressed in primary mammary mesenchymal cells, Alx4 inhibits the expression of N-CAM protein.

39. Identification of Differentially Expressed Genes in Rhabdomyosarcoma Subtypes.

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Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in children. RMS arises from primitive mesenchymal cells showing signs of skeletal muscle differentiation. There are 2 major histologic subtypes of RMS, namely embryonal (RMS-E) and alveolar (RMS-A). RMS-E and RMS-A show different clinical features as well as prognosis. In terms of genetic changes, the loss of heterozygosity at chromosomal region 11p15.5 is commonly seen in RMS-E, while the t(2;13) or t(1;13) translocation is present in most RMS-A. To increase our understanding of the 2 apparently distinct molecular pathways, we employed representational difference analysis (RDA) and cDNA array to identify differentially expressed genes involved in each subtype. RDA is a PCR-based subtractive hybridization technique in which differences between 2 populations of genetic materials can be identified. cDNA array is a hybridization-based screening method where multiple known genes were screened for expression. With RDA, 2 novel sequences were isolated. Increased expression of these sequences in RMS-A tumours were confirmed by semi-quantitative RT-PCR. Biological significance of these sequences are to be determined. cDNA array screening suggested that 2 genes, paxillin and laminin receptor, were expressed at higher level in RMS-A. Both of them have implications in the metastatic potential of various tumour cells. Their expression in RMS subtypes is currently being studied by immunostaining. Identification of these genes can lead to further understanding of RMS tumourigenesis and provide specific targets for new therapy.

40. Chemokine/Chemoattractant-Mediated Affinity Regulation of alpha4beta1 Integrin Modulates Leukocyte Adhesion to VCAM-1 Under Shear Flow.

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Modulation of integrin affinity or avidity by chemokines and chemoattractants may provide a mechanism for leukocyte adhesion to endothelial adhesion molecules, such as VCAM-1, at different stages of emigration. Changes in integrin affinity may be rapid relative to avidity and therefore may be necessary for leukocyte arrest at an inflammatory site. Using a parallel plate flow chamber, we investigated fMLP and stromal derived factor-1 (SDF-1) stimulation of leukocyte adhesion to VCAM-1 under static and shear flow conditions. In static adhesion assays, both fMLP and SDF-1 induced alpha4beta1 integrin-mediated adhesion of formyl peptide receptor transfected U937 cells (U937-FPR) on VCAM-1. This adhesion was maximal after 2 minutes, declined to unstimulated levels by 20 minutes, and was blocked by monoclonal antibody HP2/1 which recognizes the alpha4 subunit. Alpha4beta1 integrins can also mediate rolling interactions on VCAM-1. At 1 dyne/cm^2 shear stress, U937-FPR cells either rolled indefinitely or tethered briefly on VCAM-1 (site density 300/µm^2) with very few cells arresting. However, if either fMLP or SDF-1 were coimmobilized with VCAM-1, U937-FPR cells accumulated and arrested efficiently (1.1 ± 1.1, 18.6 ± 2.9, and 17.5 ± 1.6 cells/field on VCAM-1 alone, VCAM-1/fMLP, and VCAM-1/SDF-1, respectively, n=3, $p<0.05$). The presence of soluble VCAM-1 in the assay buffer inhibited both fMLP and SDF-1 mediated arrest implicating increased alpha4beta1 integrin affinity. fMLP-mediated induction of high affinity alpha4beta1 integrins was confirmed by transient binding of soluble VCAM-1 in flow cytometry experiments. Our results demonstrate the importance of integrin affinity modulation during the capture of rolling leukocytes on VCAM-1.

41. The Role of Matrix Metalloproteinases in a Spontaneously Demyelinating Mouse Model.

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Multiple sclerosis (MS) is a demyelinating disease of the central nervous system. The ND4 transgenic mice are used by our laboratory as a model for MS. This line has 70 copies of a myelin proteolipid protein (DM20) cDNA incorporated into the genome. These mice develop consistent clinical signs (tremors, wobbly gait) as they age. The matrix metalloproteinases (MMPs) are a family of endoproteinases that degrade various components of the extracellular matrix. We have examined the expression of MMP mRNA in brain tissue of ND4 transgenic mice. Stromelysin-1 mRNA was elevated above normal levels starting at 1 month (i.e. before onset of clinical symptoms) suggesting a causative role for MMPs in this process. To examine the role of MMPs in the demyelination of ND4 mice overexpressing DM20, we have crossed these mice with a line of transgenic mice which overexpress TIMP-1 (tissue inhibitor of metalloproteinases) in brain. Preliminary results indicate that overexpression of TIMP-1 can attenuate clinical signs in these mice. We have also used the anti-cancer drug paclitaxel (Taxol) as another approach to examine the role of MMPs in the progression of demyelinating disease. Paclitaxel has been shown to decrease AP-1 binding activity and MMP mRNA expression in IL-1 stimulated chondrocytes (Hui et al., 1998). Paclitaxel treated ND4 mice showed improvement clinically and showed increased amounts of myelin when examined histologically. Levels of stromelysin-1 were elevated in paclitaxel treated mice indicating a possible role for this MMP in remyelination. Thus, it is possible that MMPs may play a dual role in demyelinating disease.

42. PI3-Kinase Binds to and Modulates the $GABA_A$ Receptor.

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Phosphoinositide 3-kinase (PI3-K) is a lipid kinase that is involved in diverse cellular signaling pathways. Activation of PI3-K has recently been shown to regulate the function of voltage-gated calcium channels, suggesting a role for this kinase in modulating neuronal activity. In the present work, we have investigated the physical and functional interaction of PI3-K with the $GABA_A$ receptor, the principal receptor mediating synaptic inhibition in the CNS. We found that recombinant PI3-K, as well as the N-terminal SH2 domain of the p85 subunit of PI3-K, could pull down $GABA_A$ receptors from hippocampal slice homogenates. Pre-treatment of slices with the protein tyrosine phosphatase inhibitor pervanadate dramatically increased the amount of $GABA_A$ receptor precipitated. Thus PI3-K appears to complex with $GABA_A$ receptors *in situ* in a tyrosine phosphorylation-dependent manner. *In vitro* binding of purified proteins revealed that the major intracellular loops in the 1, 2 and 2 subunits of the $GABA_A$ receptor all show weak constitutive binding to PI3-K. The protein tyrosine kinase c-src can phosphorylate both 2 and 2 loops *in vitro* but such phosphorylation only increases 2 loop-PI3-K binding. Point mutations identified tyrosine residues 396 and 403 in the $GABA_A$ receptor 2 loop as critical determinants for this binding. Consistent with a functional importance of the physical association, perfusion of purified PI3-K into hippocampal CA1 cells in slice preparations resulted in potentiation of $GABA_A$ receptor-mediated inhibitory postsynaptic currents. These results suggest that PI3-K complexes with $GABA_A$ receptors in a tyrosine kinase-dependent manner, and this binding may permit PI3-K modulation of the receptors.

43. P53 Mutation in the Development of Breast Cancer.

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Mutation in the tumour suppressor gene p53 is one of the most frequently identified events in human cancers. In order to understand the role of p53 mutation in the pathogenesis of breast cancer it is important to identify at what histologic stage mutation first occurs. We have previously shown that when a mutation is present in invasive breast cancer the same mutation can also be found in all surrounding areas of ductal carcinoma *in situ* (DCIS) but in no areas of hyperplasia or normal breast epithelium studied. More recently we have studied cases of DCIS alone, not associated with invasive cancer, to determine the spectrum of types of DCIS that can harbor a p53 mutation. From the files of Mount Sinai Hospital between 1993 and 1998, 94 cases of DCIS were identified. The histology from all cases was reviewed and the areas of DCIS were histologically typed and graded. A block from each case was selected and immunohistochemically stained with an anti-p53 antibody (D07 clone, Novocastra). Eleven cases stained strongly, all were of a solid histological pattern containing cells of nuclear grade 2 and 3. To date, mutations have been found in 8 of these immunohistochemically positive cases. The cases in this series are representative of clinically detected DCIS rather than just the large lesions reported in the literature. This study indicates that mutation in the development of breast cancer occurs prior to invasion. This could have important implications for prevention and treatment.

44. Identification of a Novel Interactor of the Armadillo Domain of the Adenomatous Polyposis Coli Tumour Suppressor Protein.

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Adenomatous polyposis coli (APC) is an important tumour suppressor protein that is mutated in most cases of familial adenomatous polyposis (FAP) and in the majority of sporadic colorectal cancers. APC interacts with diverse cellular proteins such as -catenin, through distinct structural motifs. Interestingly, proteins that interact with the armadillo (ARM) domain of APC remain unknown. Therefore, to further elucidate APC function, we searched for potential interactors of the armadillo domain. We performed a two-hybrid screen of a HeLa cDNA library, using the APC-ARM region (spanning amino acids 437-771) as "bait". After screening 10^6 human cDNA clones, 13 positives were obtained. DNA sequence analysis revealed that one particular clone, (5-12), was represented 4 times. The association of APC with 5-12, was confirmed *in vivo*, by co-immunoprecipitation (I.P.) experiments carried out in HCT116 and SW480 colorectal cancer cell lines. 5-12 was also observed to co-I.P. with -catenin, suggesting possible formation of a trimeric complex of APC, -catenin, and 5-12. This may have important implications for the Wnt signaling pathway and is presently under further investigation. Importantly, we have observed that clone 5-12 is overexpressed in FAP colonic adenomas (preneoplastic lesions) harboring APC mutations. This supports a role for the 5-12 gene in early development of colorectal cancer. The upregulation of the 5-12 gene in FAP adenomas suggests that this gene may be a target of the -catenin mediated Wnt signaling pathway, which becomes aberrantly activated in absence of functional APC. These results identify a novel interactor of the APC-ARM domain, which may participate in regulation of APC tumour suppressor function.

45. Virulence Mechanisms of the Zoonotic Pathogen *Streptococcus iniae.*

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Streptococcus iniae is an important fish pathogen in the aquaculture industry, causing meningoencephalitis and death, and has recently been reported to cause fulminant soft tissue infections in fish handlers following percutaneous injury. A distinct clone capable of invasive disease in fish and zoonotic infection in humans has been identified by pulsed-field gel electrophoresis (PFGE). In contrast, isolates from non-diseased fish were genetically diverse. This finding suggests the existence of virulence determinants, not present in all strains, which contribute to pathogenicity. Since *S. iniae* causes acute cellulitis in humans following skin puncture, an established murine model of subcutaneous streptococcal infection was used to assess the virulence of disease and non disease-associated strains. Animals injected with 10^6 CFU of the disease associated *S. iniae* strain 9117 showed a median weight loss of -3.25 g (range –1.5 to –4.5 g), whereas mice infected with 10^6 CFU of the non-disease associated strains 9041, 9059, 9066 and 9098 exhibited weight gain comparable to controls (+1.5 g; range –1.0 to 4.0 g), $p < 0.05$. Bacteremia could be established within 24 h in mice infected with 9117, at an inocula as low as 10^2 CFU. In contrast, non-disease-associated strains never established bacteremia despite inocula of 10^6 CFU and the recovery of viable organisms at the injection site. Ongoing studies are exploring enhanced resistance to phagocytic clearance as a potential phenotypic marker for this clonal population.

46. Transduction of Cyclin-Dependent Kinase (CDK) Inhibitor Proteins to Growth Arrest Glioma Cells.

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Astrocytomas are rapidly proliferating and highly invasive neoplasms. Recent studies have used adenovirus- and retrovirus-mediated gene delivery in attempts to attenuate the growth characteristics of these tumours. Despite the ability of viruses to elicit the desired effect, they are inefficient gene delivery vehicles. In the present study, we used TAT fusion proteins to treat astrocytoma cells. TAT protein is a product of the HIV genome with the inherent ability to cross cellular membranes. Sequence encoding the 11 amino acid TAT leader responsible for membrane transduction was incorporated into a His-tag expression vector. Various cyclin-dependent kinase (CDK) inhibitors were subcloned into this vector in order to generate TAT fusion proteins. Early work has focussed on delivery of $p16^{INK4A}$ which is homozygously deleted in 30-60% of high grade astrocytomas and has been shown to induce growth arrest in glioma cells upon reconstitution both *in vitro* and *in vivo*. TAT-$p16^{INK4A}$ was expressed, purified and applied to U343, U251 and U87 cells in culture at a final concentration of 2.5μM. Administration of FITC-labeled TAT-$p16^{INK4A}$ to cells in culture facilitated detection of protein using fluorescence microscopy within 15 minutes post-application. Seventy two hours after protein addition morphological changes characteristic of growth arrest were observed in these cells. FACS analysis was performed to confirm G1 arrest. We have since purified TAT-$p14^{ARF}$, TAT-$p21^{cip1/waf1}$ and TAT-$p27^{kip1}$ proteins and are in the process of examining their effects both individually and in combination in astrocytoma cells.

47. The Role of Axial Strain in Vascular Remodeling.

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Arterial tissues remodel in response to shear forces associated with blood flow and to circumferential tensions associated with blood pressure. These remodeling responses have been extensively characterized and their respective mechanotransducer mechanisms are being actively pursued. Arteries are also under considerable lengthwise stretch (40-60%); however, biological responses to axial stretch have never been investigated. We have surgically placed interposition grafts into the adult rabbit carotid artery that reduced its axial stretch from its initial value of 62±2% to a decreased value of 34±2%. To assess developmental differences in subsequent remodeling, interposition grafts were also placed in the immature 6-week-old rabbit carotid artery that reduced axial stretch from 51±3% to 30±3%. In both adult and immature vessels, no significant differences were observed in the axial stretch measured immediately following off-loading surgery and those measured 5 weeks and 12 weeks following off-loading surgery, thus these arteries were unable to re-establish axial stretch. However, massive axial growth of off-loaded arteries was observed and extreme vessel tortuousity was evident by 5 weeks following surgical off-loading. It remains unclear how vessels can be both tortuous and under axial stretch at the same time. These data indicate that resting levels of axial stretch are required to prevent arterial instability that produces grossly abnormal vascular anatomy characterized as extreme tortuosity.

48. Separate Domains Confer $GABA_A$ Receptor Cell Surface Expression and Function.

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$GABA_A$ (-Amino Butyric Acid subtype A) receptors mediate rapid inhibition in the CNS and are normally expressed on the surface of neurons. These heteropentameric transmembrane receptors have been shown to have varying subunit combinations while the major combination in the mammalian brain has been determined to consist of $_1$, $_2$ and $_2$ subunits. However, while much is known about the receptor, little is known about the mechanism underlying its surface expression. Our lab has previously shown that the $_2$ subunit is crucial for surface expression, while the $_2$ subunit is involved in the pharmacological regulation of the $GABA_A$ receptor. Whether the region of the $_2$ subunit required for surface expression also determines the function and responsiveness of the receptor is unknown. We hypothesized that domains in the $_2$ subunit controlling receptor function and expression were distinct. $_2$/ $_2$ fusion chimeras were generated by PCR and cotransfected into HEK293T cells with the $_1$ subunit to simulate native $GABA_A$ receptor subunit combinations. Surface expression of the $_1$ subunit when cotransfected with various $_2$/ $_2$ fusion chimeras and various subunit combinations were analyzed by immunoassays. From these assays we found that only $_1$ and $_2$ combinations were expressed on the cell surface. Interestingly, only $_2$/ $_2$ chimeras containing the major intracellular loop of the $_2$ subunit were expressed on the surface when co-transfected with the $_1$ subunit- implicating the loop as the minimal requirement for receptor surface expression. Analysis of receptor function by electrophysiology surprisingly showed that the N-terminal domain of the $_2$ subunit was required for proper functioning of the receptor. These results imply that surface expression of these chimeras is not sufficient for receptor function and that different domains of the $_2$ subunit confer surface expression and function of the $GABA_A$ receptor.

49. Regulation of Iron Uptake in Hep G2 Cells.

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Non-transferrin-bound iron (NTBI) is a low molecular mass form of plasma iron and is an important source of intracellular iron. Rate of NTBI uptake in human hepatocellular carcinoma cells (Hep G2) is linearly related to cellular iron concentration and is reversible by chelation. Protein Kinase C (PKC) is activated by negatively charged phospholipids, phorbol esters and calcium ions. In K562 cells, phorbol esters have been shown to upregulate NTBI transport and increase phosphorylation of the iron regulatory protein (IRP) which is involved in maintaining iron homeostasis. We hypothesized that under the control of intracellular iron, activated PKC affects NTBI uptake through phosphorylation of IRP in Hep G2 cells. In this study we have shown that intracellular iron affects PKC, and chelation with deferoxamine reverses this effect. The iron responsive element (IRE) present in transferrin and ferritin (proteins involved in iron homeostasis) mRNA is bound by IRP for maintaining iron homeostasis. Phosphorylation increases the RNA binding activity of IRP. Therefore, as the first step towards proving that PKC phosphorylates IRP under the control of iron, we have shown the presence of the IRE-IRP complex in Hep G2 cells. Experiments with and without PKC activators will be conducted in the future. Secondly, it has been suggested that NTBI uptake occurs due to membrane damage caused by free radicals generated by long term iron loading. On the other hand, short term loading with gallium (which does not redox cycle) also increases NTBI uptake in macrophages. To prove that NTBI uptake does not occur due to membrane damage, we have first shown that long term gallium loading is non toxic to Hep G2 cells. Iron uptake studies on long term gallium loaded cells were then conducted, which demonstrated an increase in NTBI uptake with 500 μM gallium, suggesting that free radical damage is not involved.

50. Identification of the Motifs in Aggrecan G1 and G2 Domains that Inhibit Product Secretion.

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The large aggregating chondroitin sulfate proteoglycans include aggrecan, versican, neurocan and brevican. The core protein contains a signal peptide, an amino-terminal globular domain known as G1 domain, a carboxyl-terminal globular domain known as G3 domain and a large sequence situated between G1 and G3 for modification by glycosaminoglycan (GAG) chains, mainly chondroitin sulfate (CS) side chains. Aggrecan has some additional gene structures. Aggrecan contains a G2 domain which is structurally similar to the two tandem repeats of the G1 domain and link protein. One Inter-globular domain (IGD) is also situated between the G1 domain and G2 domain of agrecan. A large extended region for glycosaminoglycan (GAG) chain attachment is situated between G2 and G3. This extended region is further divided into two domains: keratan sulfate (KS) and CS binding domains. All members of aggrecan family contain an amino-terminal fragment known as G1 domain, which is composed of an immunoglobulin (IgG)-like motif and two proteoglycan tandem repeats. Previous studies indicated that the G1 domains of aggrecan and versican inhibited product secretion. This study was designed to identify the motif in the G1 domain of aggrecan which inhibits product secretion. We generated a number of recombinant genes containing different motifs of the G1 domain alone or in combination with other aggrecan domains or subdomains and expressed these constructs in COS-7 cells. We observed that the inhibition of product secretion occurred through the tandem repeats of the G1 domain while the IgG-like motif had no negative effect on secretion. Link protein, which is structurally similar to the G1 domain of aggrecan, is well secreted to culture medium. Further studies indicated that it was the first tandem repeat of aggrecan G1, but not the second tandem repeat, that inhibited product secretion. The G2 domain of aggrecan is structurally similar to the tandem repeats of the G1 domain, and we further demonstrated that the first tandem repeat, but not the second tandem repeat, of G2 also inhibited product secretion.

51. Identification of a GA-Rich Sequence as a Protein Binding Site in 3'UTR of Chicken Elastin mRNA with a Role in the Developmental Regulation of Elastin Message.

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Synthesis of aortic elastin peaks in the perinatal period and then is strongly down-regulated with post-natal development and growth. We have previously shown that decreased stability of elastin mRNA contributes to this developmental decrease in chick aortic elastin production, and that destabilization of elastin message is correlated with decreased protein binding to a large, GC-rich region of secondary structure in the 3'untranslated region (3'UTR) of elastin mRNA. Using deletion constructs and sense and antisense competition assays, we identify the major protein binding site in the 3'UTR of elastin as a GA-rich sequence (UGGGGGAGGGAGGGAGGGA) which we have designated the G3A motif. This motif is present in the 3'UTR of elastin from several species. Binding proteins are present in both nuclear and cytoplasmic extracts, and their abundance is associated with tissues producing elastin and correlated with circumstances in which elastin mRNA is stable. Correlations of protein binding with both predicted and experimentally assessed RNA structure indicate that this sequence is crucial for the formation of a stable secondary structure in elastin mRNA. These results suggest that the conserved GA-rich sequence regulates elastin mRNA stability through stabilization/destabilization of specific 3'UTR structures in elastin mRNA.

52. Effect of Microtubule Disruption on Endothelial Integrity, Focal Adhesion, and Actin Microfilaments, and Endothelial Repair.

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An important mechanism for initiation and progression of atherosclerosis is dysfunction and/or loss of intact endothelial monolayer, which is required for normal blood vessel function. Endothelial cell actin microfilaments and microtubules are essential for endothelial integrity and efficient repair. To date, we and others have shown that microfilaments are crucial in maintaining the structural integrity of the endothelium while microtubules are responsible for directional cell migration. I tested the broad hypothesis that microtubules interact with actin microfilaments and with focal adhesion complexes to regulate endothelial integrity, using intact *in vitro* porcine aortic endothelial cell monolayers. I investigated interactions between microtubules and both actin microfilaments and focal adhesion proteins by disrupting microtubule organization using Colchicine. To observe effects of microtubule disruption on distribution of focal adhesion proteins, immunofluorescence was used to localize actin and microtubules, focal adhesion kinase (FAK), talin, and vinculin, followed by quantitation and analysis with scanning confocal laser imaging system. Western analysis and densitometry was also used on soluble and non-soluble fractions to observe effects of microtubule disruption on levels of focal adhesion proteins. I found an increase in peripheral FAK, talin, and vinculin 6 hours following microtubule disruption in confluent monolayer. This increase was reversed at 24 hours, and I saw a decrease in focal adhesion proteins as cells began to lose their cell-cell and cell-substrate adhesion and shape. Western analysis and densitometry confirm these results. These studies support the hypothesis that microtubules interact with microfilaments and focal adhesion complexes to regulate endothelial integrity.

53. Involvement of a Novel Gene XIN in Cardiac Hypertrophy.

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A long-term characteristic of cardiac hypertrophy is the reversion of the heart to an embryonic pattern of gene expression. Transcription factors such as GATA-4, which are active during cardiac development have been shown to play important roles. It is believed that by looking at factors involved in cardiac development in the embryo, one may gain insight into mechanisms responsible for cardiac hypertrophy in the adult. A novel gene Xin has recently been cloned in the chick and mouse (mXin); and has been shown to be required for cardiac morphogenesis and looping. Sequence analysis of the Xin gene suggests that it may act as a transcriptional regulator. Four clones have been identified in a human hypertrophic heart cDNA library constructed in our laboratory, which show similarity to the mXin cDNA sequence. From this, it is speculated that Xin may play a role in both cardiac development and hypertrophy. Characterization of the human Xin gene is currently underway. Full-length sequencing, chromosome mapping and tissue expression studies will be performed. Functional studies will be carried out in murine C2C12 myoblasts since this cell line is well established in our laboratory. Anti-sense strategies and micro-array technology will be utilized. mXin co-localizes with connexin-43 and N-cadherin at the intercalated discs in the mouse heart. Intercalated disc remodelling and gap-junction reorganization have been demonstrated in cardiac hypertrophy. It is hoped that further study of Xin in this context will reveal a novel signalling pathway involving these factors in cardiac hypertrophy.

54. Secretion, Purification and Characterization of Biologically Active Herring Antifreeze Protein in *Pichia pastoris.*

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Antifreeze proteins and antifreeze glycoproteins are structurally diverse molecules that share a common property in binding to ice crystals and inhibiting ice crystal growth. Type II fish antifreeze protein of Atlantic herring (*Clupea harengus harengus*) is unique among known antifreeze proteins and antifreeze glycoproteins in its requirement of a metal cofactor for antifreeze activity. Expression of herring antifreeze protein in *Escherichia coli* resulted in production of inclusion bodies, which required further denaturation and refolding. In this study, we utilized yeast *Pichia pastoris* as a eukaryotic expression host. The coding sequence of herring antifreeze protein was cloned into the pGAPZ _A vector to generate recombinant herring antifreeze protein (WT), and two fusion proteins with a C-terminal peptide containing only six-histidine tag (WT-6H), or a *myc* epitope with a polyhistidine tag (WT-myc6H), respectively. The recombinant proteins were targeted for secretion using the yeast -factor secretion signal. After integration into *P. pastoris* strain X-33 or protease-deficient strain SMD1168H, the recombinant proteins were expressed constitutively using the promoter of the gene encoding glyceraldehyde-3-phosphate dehydrogenase. We found that all of the recombinant proteins were secreted into the culture media and exhibited antifreeze activities. Furthermore, our studies demonstrated that expression at lower temperature increased the yield of the recombinant herring antifreeze protein dramatically. These data suggest that *P. pastoris* is a useful system for the production of soluble and biologically active herring antifreeze protein required for structural and functional studies.

55. Expression of a Winter Flounder Skin-Type Antifreeze Polypeptide in Transgenic Plant *Arabidopsis thaliana* and its Protective Effects.

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Recent discovery of winter flounder skin-type, intracellular antifreeze polypeptides (wfsAFPs) has raised an important question regarding the functional differences between these polypeptides and their analogues, liver-type, extracellular antifreeze polypeptides (wflAFPs). To investigate the protective function of wfsAFPs, gene of wfsAFP-2 was introduced into *Aradopsis thaliana* by *Agrobacterium*-mediated transformation. Transgenic plants were selected by kanamycin resistance and advanced to homozygosity. The wfsAFP-2 mRNA and protein expression levels varied dramatically in the transgenic plants grew at 5°C, and the protein levels directly correlated with the mRNA levels. The wfsAFP-2 protein levels decreased significantly while the mRNA levels remained after the transgenic plants grew at 5°C were moved to room temperature for one week, probably due to an increased susceptibility of the AFP to proteolytic degradation at higher temperature as it became less helical. Leaf extracts from transgenic plants grew at 5°C showed significant antifreeze activity thus confirmed the expression of functional wfsAFP. Transgenic plant lines grew at 5/2°C (day/night temperatures) all exhibited significantly greater growth than the wild type plants, indicating certain hypothermic protective effect of the wfsAFP-2 transgene. However, there was little effect of the expression of wfsAFP-2 on the freezing tolerance of cold-acclimated tissue of the transgenic plant lines, as determined by electrolyte leakage.

56. Regulation of Pit-1 Function by Protein Interactions.

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The mammalian anterior pituitary is an important endocrine gland regulating growth, sexual development, thyroid activity, milk production and adrenal gland function. A pituitary specific transcription factor, Pit-1, is expressed during development and is critical for the differentiation of three pituitary cell types – somatotropes, lactotropes and thyrotropes. In the fully differentiated pituitary, Pit-1 maintains the expression of the hormones secreted by these cells. Pit-1 function is determined by interaction with other transcription factors, and by hormone/growth factor signals in both the developing and mature pituitary gland. We are interested in the mechanisms of Pit-1 function, especially identifying novel interaction with other transcription factors, and defining how specific hormone signals regulate transactivation by Pit-1. Using the promoter of the prolactin gene (a Pit-1 target) as a model, we have demonstrated that the MAP kinase regulated ETS-domain factor, ERF, specifically inhibits prolactin gene transcription by interfering with Pit-1/DNA interaction at ETS/Pit-1 composite sites. Site-directed mutations of the ETS binding core in these composite sites can prevent ERF inhibition, while Pit-1 transactivation is not affected. At present, I am characterizing the potential protein-protein interactions of ERF with Pit-1 and the effect of MAP kinase activation on Pit-1 target genes in the presence of ERF. These studies will provide new insights as to how transcriptional activation by Pit-1 is determined by specific protein interactions. Moreover, we will obtain a better understanding of the role of this critical regulator in the development and function of the pituitary gland.

57. Lack of Steroid Hormone Response Elements Within the 2.5kb Promoter Region of Normal Epithelial Cell-Specific (NES1) Gene.

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The normal epithelial cell-specific 1 gene encodes for a secreted serine protease. It is found to be down-regulated in breast cancer and acts as a tumour suppressor gene. In order to understand the role of NES1 gene in breast cancer pathogenesis, we investigated the effect of steroid hormones on the expression of NES1 gene in breast carcinoma cell line T-47D. The T-47D cells were stimulated with 10^{-8}M of 17 -estradiol, 5 -dihydrotestersterone (DHT), and norgestrel. Using semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) and Western blotting, we found that NES1 was up-regulated by DHT and 17 -estradiol, but not norgestrel, both at the mRNA and protein level. To investigate the mechanism underlying the hormonal regulation, the promoter region of the NES1 gene was functionally characterized. A 2.5kb fragment of the NES1 gene promoter was cloned in front of a chloramphenicol acetyltransferase (CAT) reporter gene. This construct was transiently expressed in breast carcinoma cell lines T-47D and ZR-75-1. The production of the CAT protein was not increased upon 17 -estradiol and DHT stimulation (at a concentration ranging from 10^{-6} to 10^{-8}M). Our results indicated that there were no functional estrogen or androgen response elements in this 2.5kb region of the NES1 gene promoter. It is likely the up-regulation of the NES1 gene by steroid hormones is not due to direct interactions between the steroid hormone receptor complexes and NES1 gene promoter. Other indirect mechanisms may be considered to explain the modulation of NES1 expression by steroid hormones.

58. HSP70-Sulfoglycolipid Binding: A Novel Therapeutic Point of Intervention for Inhibiting Bacterial/Host and Sperm/Egg Binding.

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A novel and highly conserved function of heat shock/stress proteins of the 70 kDa (hsp70) family is their ability to bind the sulfated galactolipids (SGL), 3'sulfogalactosyl ceramide (SGC) and 3'sulfogalactosyl glycerolipid (SGG). SGL are found throughout the body and have been implicated in spermatogenesis and sperm/egg binding. *In vitro* studies have suggested that these lipids may function as receptors for outer membrane associated and cell surface expressed hsp70s. Recombinant hsp70 family members from the bacterial pathogens, *Chlamydia trachomatis*, mycoplasma, *Helicobacter pylori* and *Haemophilus influenzae* all bind specifically SGC/SGG. The recombinant sea urchin receptor for sperm, possessing 45% homology with the murine testis specific hsp70.2 gene product, P70, also binds SGC and SGG. DnaK, the most widely studied intracellular chaperone also possess SGC/SGG binding specificity. To investigate the molecular mechanism(s) of sulfated glycolipid recognition by hsp70 we have used a series of sulfated galactolipid derivatives and recombinant P70 (rP70) as our model system. Thin layer chromatography (TLC) and receptor ELISAs demonstrated rP70 to bind specifically 3'and 4' sulfated galactolipids and not any other sulfated or sialic acid containing lipid. Binding to 3'SGL was dependent upon a ceramide based backbone, whereas binding to 4'SGL was observed with either a ceramide or alkyhydrocarbon backbone. Binding of rP70 was not observed to the sulfated sugars heparin or 3' sulfate galactose. Soluble analogues of SGC have been designed by substituting the acyl chain with adamantane and by generating a neoglycoconjugate of SGC and bovine serum albumin. Adamantyl-SGC can inhibit rP70/SGL binding in the μM range. Proteolytic cleavage and truncation analysis demonstrated that the SGL binding site is localized within amino acids 300-387 of the highly conserved N-terminal ATPase domain of rP70. Site directed mutagenesis showed arginine 345 of rP70 to be critical for SGC binding, less so for SGG. Substituting phenylalanine 198 with alanine alters the binding specificity of rP70. Mutations in DnaK (YND147,149,151AAA) also eliminate SGL recognition. Elucidating the molecular mechanisms of SGL recognition by hsp70 provides the basis for the rational design of specific protein, glycolipid and neogylcoconjugate compounds which may be effective in inhibiting the hsp70 adhesin function.

59. Long-Term Depression of AMPA Receptor-Mediated Synaptic Transmission by Insulin Through Receptor Internalization.

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Modification of synaptic strength is central to information transfer between neurons in the central nervous system. Although the underlying mechanisms remain hotly debated, it has recently been proposed that a rapid change in number of functional receptors in the postsynaptic domains, as a result of insertion and/or removal of the receptors, may be an efficient means of controlling synaptic efficacy. This has gained support from recent studies on synaptic plasticity in several experimental preparations. By immunostaining and colormeric assay on HEK cells transfected with GluR1 or GluR2 AMPA receptor subunits, we found that the AMPA subtype glutamate receptor, which mediates fast synaptic transmission at most excitatory synapses in the CNS, undergoes constitutive endocytosis via a clathrin-mediated pathway, and that this process can be swiftly enhanced by insulin in a GluR2 subunit-dependent manner. The facilitated endocytosis reduces the number of AMPA receptors on the postsynaptic membrane, and hence results in a long-term depression of AMPA receptor-mediated synaptic transmission. These results provide the first evidence for a rapid change in number of postsynaptic receptors by modulating the clathrin-mediated receptor internalization, thereby suggesting that the regulation of receptor trafficking in the postsynaptic neurons is an important mechanism for synaptic plasticity in the mammalian CNS.

60. The Post-Transcriptional Regulation of Preproendothelin-1 in Vascular Endothelial Cells.

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The regulated turnover of cytokine and proto-oncogene mRNAs, typically with half-lives of 15-30 minutes, is primarily mediated by AU-rich elements within their 3'-untranslated regions (3'-UTRs). We endeavored to study this process in endothelial cells utilizing the preproendothelin-1 (ppET-1) transcript, which encodes endothelin-1 (ET-1), a potent vasoconstrictor peptide. We have recently demonstrated that the labile ppET-1 transcript, with a half-life of 15-30 minutes, is stabilized 3-fold in a model of verotoxin-mediated endothelial cell activation (Bitzan, M. et al, JCI, 1998). We therefore hypothesize that ppET-1 is regulated post-transcriptionally by a conserved AU-rich element (ARE) found within its 3'-UTR. To characterize the functional importance of these elements, the human ppET-1 3'-UTR was cloned downstream of a luciferase reporter and its expression characterized utilizing transient DNA and RNA transfection assays. Both methods demonstrated that the presence of the ppET-1 3'-UTR decreased steady state expression of the reporter three-fold and revealed surprising translational properties. Taken together, this suggests that specific elements within the 3'-UTR possess regulatory cis-RNA elements. Seven deletion and seven linker scanner mutants of the ppET-1 3'-UTR have been generated and preliminary results suggest a requirement for multiple domains in ARE-dependent transcript turnover. An assay to address the functional role of these elements within the context of a full-length human ppET-1 cDNA and the involvement of a newly described AUF-1-associated proteasome-dependent activity on regulating ppET-1 stability is under investigation. Future studies will biochemically and functionally characterize potential trans-factors that may bind key regulatory regions of the ppET-1 3'-UTR. Identification of regulatory cis-RNA elements is integral to understanding ppET-1 expression in the vessel wall and offers both potential pharmacological targets and novel tools in therapeutic gene transfer technologies.

61. A Study of the Bone Morphogenetic Protein Pathway in Human Gliomas and Medulloblastomas.

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The bone morphogenetic proteins (BMPs) are members of the TGFb family of growth factors. BMPs bind to cell surface serine-threonine kinase receptors called activin-receptor-like kinases (ALKs) and mediate signaling via the Smad effector molecules. Ligand binding to ALK receptors phosphorylates and activates Smads. Activated Smads translocate to the nucleus where they activate gene transcription. Studies have shown that several BMPs and their receptors are expressed in both the developing and adult brain. BMPs mediate a wide variety of events in the central and peripheral nervous systems including proliferation, apoptosis and differentiation of neuronal and glial progenitor cell populations. Disturbances in the BMP pathway may be relevant to the pathogenesis of CNS neoplasias. To determine whether components of the BMP pathway are intact in human glial and neuronal tumours, we examined human glioma (U87, U251, U343, U373, SF-126, SF-188, SF-539, XF-498) and medulloblastoma (DAOY, PFSK) cell lines for components of the BMP pathway. We have shown that Smads 1, 4, and 5 are differentially expressed in the cell lines examined by Western blotting. We have also found differential expression of the ALKs (ALKs 1-6) in various tumour cell lines by RT-PCR. These data suggest that alterations in Smads and ALK expression may lead to aberrant BMP signaling and this may be relevant in the pathogenesis of gliomas and medulloblastomas.

62. An efficient Method to Generate Neutralizing hu-mAbs: Application of hu-PBL-SCID Mice and scFv Phage Display Libraries.

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The establishment of single chain antibody phage display library (scFv-lib) technique has opened a new way for generating recombinant human monoclonal antibodies (hu-mAbs) by-passing the hybridoma technology. Most of scFv-libs have been constructed from naive human peripheral blood lymphocytes (hu-PBL) and often result in hu-mAbs which exhibit both low antigen (Ag) binding affinity and non-neutralizing activity. We have previously demonstrated that human immune responses can be established in severe combined immunodeficiency mice engrafted with hu-PBLs (hu-PBL-SCID) after the depletion of the mice's NK cells and macrophages. In order to improve the efficiency of the scFv-lib technique, we constructed scFv-lib of 10^8 members from the splenocytes of hu-PBL-SCID mice immunized with either whole Respiratory Syncytial Virus (RSV) proteins or human TNF- . RSV and hu-TNF- were used as model Ags in this study. This approach has enabled us to generate a panel of human monoclonal antibodies that exhibit both high binding affinities ($\sim 10^8$ M^{-1}) for the fusion glycoprotein of the RSV long strain A2 and strong virus neutralizing activities *in vitro*. Sequence analysis of isolated anti-RSV-F human scFvs revealed that they had originated from different V_H families, and that specific hu-Ab maturation had taken place in the mice. Three distinct anti-hu-TNF- clones with binding affinities of 10^8 M^{-1} have also been isolated. Together, the results suggest that: (1) hu-PBL-SCID mice can be effectively used to generate hu-Ig gene repertoire for the cloning of highly specific hu-mAbs, and (2) the combination of hu-PBL-SCID mice and scFv-lib can be applied widely to generate large repertoires of hu-Abs against both foreign (i.e. RSV) and self-Ags (i.e. hu-TNF- , in progress) for both research and therapeutic purposes. With simplicity and efficiency, this approach represents a significant step toward the generalization of hu-mAb production.

63. Regulation of Human Proglucagon Gene.

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Proglucagon is the precursor of glucagon, GLP-1, and GLP-2. Glucagon and GLP-1 have proved to play important roles in regulating and maintaining normal blood glucose level. GLP-2 was found to be an intestinal growth factor. The regulation of rat proglucagon gene had been extensively studied, but little is known about the regulation of the human proglucagon gene. Preliminary evidence suggests that important differences exist between the regulation of rat and human proglucagon gene. Transient transfection studies demonstrated that sequences between -5.7kb and –3.3kb are required for islet-cell specific expression of the human proglucagon gene. Shorter human proglucagon constructs can be expressed in intestinal cell lines, although the longest constructs yielded the highest expression. However as little as 300bp of rat proglucagon promoter can drive expression of the reporter gene in both the islet and intestinal cell lines. In order to test whether *in vitro* results adequately reflect normal human islet cells, two transgenic mice containing either a 1.6kb or a 5.7kb human proglucagon promoter-reporter gene have been generated. Both RT-PCR and immunohistochemistry showed that the 1.6kb human proglucagon promoter-reporter transgene is expressed in the brain and intestine but not in the pancreas which are in agreement with our *in vitro* transient transfection results. We are currently analyzing the tissue specific expression patterns of the transgenic mouse carrying the 5.7kb human proglucagon promoter. Both our *in vitro* and *in vivo* experiments have yielded important insight into the regulation of the human proglucagon gene expression.

64. Reorganization of Endothelial Adherens Junctions in Response to Shear Stress.

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Endothelial cells exhibit profound changes in cell shape in response to altered shear stress. These shape changes may require disassembly/reassembly of the adherens junction protein complexes that mediate cell-cell adhesion. To test whether changes in shear stress induced redistribution of these proteins, we exposed confluent porcine aortic endothelial cells to a shear stress of 15 dynes/cm^2 for 8.5, 24, and 48 hours using a parallel plate flow chamber. Confluent static monolayers were used as controls. The cells were fixed and stained with a monoclonal anti-VE-cadherin antibody, or polyclonal antibodies to -catenin, -catenin or plakoglobin. Under static conditions, endothelial cells were cuboidal in shape, whereas after 8.5 hours of shear, the cells were stellate and attachment to neighboring cells was discontinuous. After exposure for 24 or 48 hours, cells were elongated with their major axis aligned in the direction of flow. Under static conditions, staining for all proteins was intense and peripheral, forming a continuous band around the cells at cell-cell junctions. By 8.5 hours of shear stress, staining was less intense and occurred only at sites of cell-cell attachment. After 24 or 48 hours of shear stress, staining for VE-cadherin, -catenin and -catenin was intense and peripheral, forming adherens plaques that colocalized with the ends of stress fibers that inserted between cells along the lateral membrane. Staining for plakoglobin was not observable after 24 hours of shear stress, but returned after 48 hours in a distribution similar to that of the other proteins. Western blot analysis indicated that during the transitions from a cuboidal to elongated morphology (after 8.5 hours), protein levels of VE-cadherin, -catenin and plakoglobin decreased, while -catenin levels increased. As shape change reached completion (24 – 48 hours), all protein levels were upregulated except for plakoglobin, which remained below control levels. Our results imply that shear stress induces partial disassembly of adherens junctions during shear-induced changes in endothelial cell shape.

65. Positive Gene Regulation by the Retinoblastoma Protein.

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The retinoblastoma protein (pRB) is a multifunctional transcriptional regulator. Recent evidence has demonstrated a role for pRB in positive gene regulation. Specifically, we have shown that pRB is required for IFN- -induction of the murine MHC class II A locus. Of particular interest, is my discovery of three IFN- -specific, pRB-dependent, DNase I hypersensitive (HS) sites upstream of A . These sites demonstrate an exciting role for pRB in transcriptional activation through chromatin remodeling. Our goals are to locate a pRB-responsive element (RbRE), within the A HS sites, and to characterize the proteins that interact with these sites. In order to locate the RbRE, I am building deletion reporter constructs of the upstream A region. Stable integration of these constructs into $RB^{+/+}$ and $RB^{-/-}$ mouse embryonic will narrow down a region that is specifically pRB-dependent. Within this region, elements can be analyzed for homology to known protein-binding sites. Next, proteins that bind these elements will be identified through DNase I footprinting and confirmed using bandshift assays. Should important protein-binding sites not show homology to any known DNA sequences, proteins will be isolated using affinity purification, or yeast one-hybrid assays. IFN- -induction of the A locus is an excellent system in which to study positive gene regulation by pRB. It represents a novel example of a role for pRB gene regulation in immunity, and it demonstrates a role for pRB in modifying chromatin structure. Overall, this research has broad applications, and could lead to the development of strategies that induce immune defences against pRB-defective tumour cells.

66. Retinoic Acid Induces the ETS1 and its Target Genes in Osteoblastic Cells.

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Bone development is a complex process, which involves temporal and site specific expression of transcription factors. The *ets* family of transcription factors have been shown to play an essential role in bone formation. *In situ* hybridization experiments have shown that Ets1 is expressed in mesenchymal cells at sites where bone development occurs. PU.1 knock out mouse features osteopetrosis due to the lack of osteoclast differentiation. In mouse, Ets2 over expression causes skeletal deformities such as osteomalacia and delayed calvarial closure. Retinoic acid (RA) is a natural morphogen, which enhances the function of mature osteoblasts. Previously, it was shown that RA induces the Ets1 mRNA expression in P19 cells. In this study we demonstrate that RA increases the expression of Ets1 mRNA in osteoblastic cells. This induction is observable after 4hr and can be sustained for at least 14 days in culture as demonstrated by Northern blot analysis. Interestingly, of the *ets* transcription factors tested (Ets1, Ets2, Elk1 and Fli1) only Ets1 mRNA was induced upon RA treatment. Act D inhibits the induction of Ets1 mRNA expression caused by RA which suggests that RA regulation of Ets1 gene may in part be transcriptional. The promoter region of Ets1 contains four retinoic acid response element (RARE) half-sites. We have found that RAR expression can only be detected in the MC3T3-E1 cells in presence of RA, whereas RAR expression can be detected without RA. Through competitive gel retardation assay we have been able to show that RAR , RAR , and RXR _ can bind to at least one of these RARE half sites. Transient transfection of MC3T3-E1 cells revealed that RA induces the transcriptional activity of Ets1-promoter luciferase construct containing ~700 bp of the identified Ets1 regulatory region. These results indicate that RA upregulates the Ets1 gene expression, suggesting that some of the osteoblast specific genes may be regulated through Ets1. To identify potential RA-inducible targets of Ets1 in MC3T3-E1 cells, we monitored the relative expression levels of 72 genes by array hybridization with probes derived from MC3T3-E1 cultures grown with and without RA. We identified 6 genes namely, SGP-1, Stra3/Lefty, apolipoprotein D, Osteopontin, Hsc70 and connexin 43, whose expressions were induced by more than 20 units in the presence of RA. Of these genes, Stra3/Lefty and Osteopontin contain two consensus Ets binding sites in their promoters. It is likely that RA induces the expression of Stra3/Lefty or Osteopontin genes through induction of Ets1 transcription factor.

67. The Role of Protease Activity in Controlling the Adhesive Phenotype and Virulence of *Staphylococcus aureus*.

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Fibronectin (Fn)-binding proteins (FnBPs) of *S. aureus* are expressed maximally during exponential growth, promoting colonization of host tissues. We hypothesize that in colonized tissue, a secreted serine protease (Ssp) promotes the spread of infection by degrading cell-surface FnBP. To test this hypothesis, the *ssp* gene of *S. aureus* RN6390 was replaced with a mutant allele containing an inserted erythromycin resistance cassette (*erm*). Although the mutant SP6391 did not express Ssp, it retained the characteristic growth-phase dependent loss of cell-surface FnBP, and exhibited slightly enhanced virulence in a tissue abscess model of infection. However, Northern blot analyses and homology searches of the *S. aureus* strain COL genome subsequently revealed that *ssp* is transcribed as part of an operon, in which downstream open reading frames encode a cysteine protease (*ssp*B), and a 12.9 kDa protein (*ssp*C) of unknown function. Furthermore, the *erm* insertion in SP6391 was non-polar, allowing transcription and expression of both *ssp*B and *ssp*C. Therefore, a second mutant was constructed (SP6392) by insertion of a non-replicating plasmid within *ssp* through targeted homologous recombination. This resulted in loss of transcription of the *ssp* operon, and a pleiotropic phenotype, which included loss of protease activity, constitutive expression of FnBP, and a significant reduction in size of tissue abscess. We conclude that the adhesive phenotype of *S. aureus* is controlled by the *ssp* operon; a process which is necessary for tissue invasion. Furthermore, our data suggest that SspC is a global regulator of protease expression, and experiments are in progress to test this hypothesis.

68. Molecular Evidance of Hepatic Insulin Resistance in a Fructose Fed Hamster Model.

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We have employed Syrian golden hamsters to study the molecular mechanisms of apoB-containing lipoprotein overproduction in an insulin resistant model. Feeding the hamsters with a high fructose diet results in peripheral insulin resistance. This has been previously documented in our laboratory using an euglycemic hyperinsulinemic clamp method. It has been shown that fructose-feeding can interfere with insulin signal transduction causing insulin resistance and type 2 diabetes. However, little is known regarding the molecular interactions that influence the balance of signal processes following fructose feeding. Using hepatocytes isolated from fructose- and normal chow-fed hamsters we have attempted to study the molecular mechanisms underlying the insulin resistance following high fructose feeding. Immunoprecipitation and Western blotting analysis revealed that in hepatocytes isolated from fructose fed hamsters, tyrosine phosphorylation of insulin receptor and insulin receptor substrate-1 decreased 62.98 ± 11.1% and 75 ± 21.42% respectively compared to those of control hepatocytes. Further studies showed that in hepatocytes isolated from fructose-fed hamsters, the level of protein-tyrosine phosphatase-1B (PTP-1B), a specific phosphatase of insulin receptor and IRS-1, increased significantly (33.91 ± 6.5%, $p < 0.05$) compared to that of control cells. PI3 kinase assays using two different antibodies were also conducted to further evaluate possible impairment of downstream signal transduction. PI 3 kinase assays using an anti-phosphotyrosine antibody showed a 25.27± 3.3% ($p < 0.03$) reduction of activity in hepatocytes isolated from fructose-fed hamsters. However, a similar assay using an antibody against the P85 subunit of PI3 kinase showed no difference in activity, supporting the notion that high fructose feeding may cause impairment of insulin signal transduction upstream of PI3 kinase. In summary, high fructose feeding induces hepatic insulin resistance by increasing the level of PTP-1B, causing enhanced dephosphorylation of both insulin receptor and IRS-1.

69. Application of ESTs to the Zebrafish: A New Vertebrate Model for Human Development and Disease.

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The generation of expressed sequence tags (ESTs) have proven to be a rapid and economical approach to identify and characterize expressed genes. We have generated 3,514 ESTs from a three-day old embryonic zebrafish heart cDNA library. Of these, 57.6% matched to known genes, 14.2% matched only to other ESTs and 27.8% showed no match to any ESTs or known genes. A total of 1,044 unique known genes were used to analyze the gene expression patterns in the zebrafish embryonic heart. These were categorized into seven categories based on functions of the known genes. The largest class of genes represented those involved in gene/protein expression (24.0% of known transcripts). This was followed by genes involved in metabolism (17.7%), cell structure/motility (17.2%), cell signaling and communication (8.4%), cell/organism defense (7.5%), and cell division (3.9%). Novel genes constituted the remaining 14.0%. Comparison of the ESTs within the database resulted in the generation of 240 unique clusters (consisting of 1,489 ESTs), while 2,025 ESTs did not cluster. This estimates the number of unique transcripts identified thus far to be 2,265. Comparative analysis of gene expression profiles between the four-chambered fetal human and the two-chambered embryonic zebrafish hearts indicated that there are significant differences in numerous transcripts within several of the functional categories. Furthermore, the establishment of an EST-based resource of data and clones from the zebrafish provides a valuable substrate for physical mapping of the zebrafish genome. To this end, we have mapped 69 zebrafish embryonic heart ESTs using radiation hybrid mapping and comparison of map positions between zebrafish and human identified new synteny groups. This comparative analysis will be useful in defining the boundaries of conserved chromosome segments between zebrafish and humans, which will facilitate the transfer of genetics information between the two organisms and improve our understanding of vertebrate evolution.

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