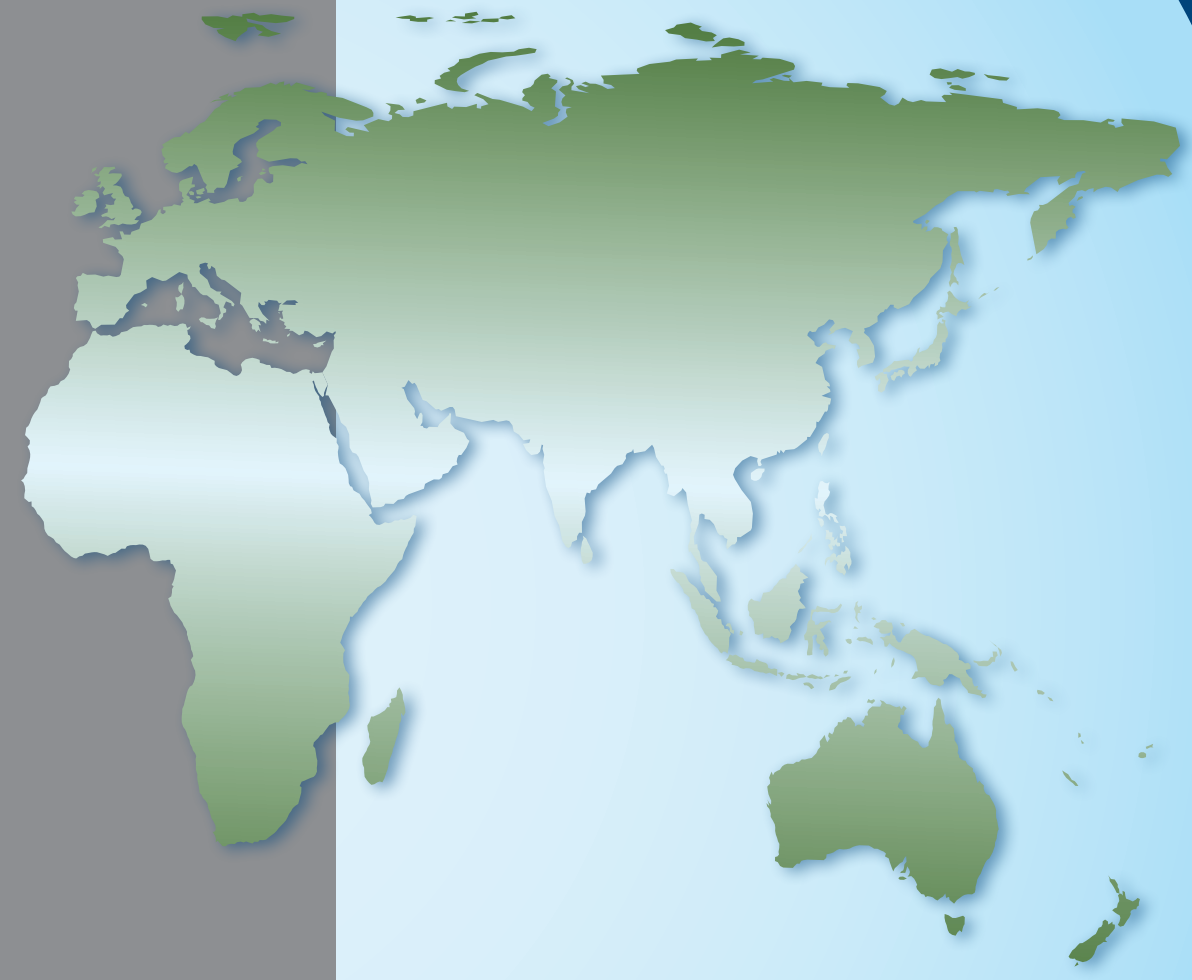
LUDWIG INSTITUTE FOR CANCER RESEARCH



2009 ANNUAL RESEARCH REPORT

LUDWIG INSTITUTE FOR CANCER RESEARCH

2009 Annual Research Report

2009 was a year of exceptional achievements for the Ludwig Institute for Cancer Research despite the financial and economic challenges around the world. These accomplishments are illustrated by more than 300 papers published in peer review journals; the increase in the Institute's overall research budget through the tireless efforts of our scientists in obtaining external grant funding; and the prestigious awards and distinctions received. They are testaments to the quality of the Institute's work and the commitment and dedication of its scientific staff.

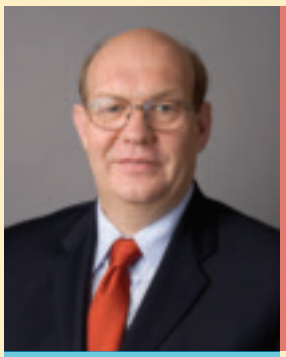
The Institute is constantly changing and 2009 saw two highly valued and long serving Branch Directors, Tony Burgess in Melbourne and Thierry Boon in Brussels end their tenure. The Branches they directed made major contributions to the work of the Institute. We are deeply grateful to them and delighted that in both cases they will continue to work at their respective Branches as Members of the Institute heading personal research groups. In addition, new outstanding members of scientific staff have been successfully recruited to a number of the Branches and changes to the future location of our Branch laboratories in Melbourne were announced as part of an exciting new initiative in cancer care in that city.

The research themes that the Institute is pursuing have remained relatively constant but are gradually evolving, evidenced by a sharpened focus on research driven by clinical questions to complement the outstanding basic research that has long been the backbone in our Branches. Although there are no restrictions as to which tumor types LICR investigators might study, areas of particular strength are melanoma, colon cancer, and brain cancer. In each of these areas, both fundamental and clinical based studies are being pioneered. Initiatives in these three areas have resulted in regular meetings of Institute scientists whose work impinges on these cancer types with a goal of fostering interaction and collaboration. It is encouraging to see these programs bear fruit and move forward.

The Institute is committed to improving cancer control through integrated laboratory and clinical research. We have done this over the years by seeking to develop novel therapeutic strategies based on our emerging understanding of cancer. Our work in this context has concentrated on immunotherapy in the form of monoclonal antibodies and cancer vaccines. Several of our antibodies are now being developed by commercial partners and are in a number of clinical trials around the world. The same is true for a variety of therapeutic vaccine formulations focusing on the use of proteins normally present only in the germ line but which are often re-expressed in cancer. Here the current focus is on overcoming tumor induced suppression of the immune system to enhance vaccine efficacy.

A new feature of the Institute's translational work is now emerging with small molecule drugs extending our reach into the therapeutic world. This field of research has gained increasing momentum including the establishment of a collaborative venture between the San Diego Branch and the LICR technology development effort in the New York office that supports basic researchers in the identification of experimental inhibitors that accelerate their basic research and generate lead compounds for drug development.

Cancer research is slowly making inroads into the human toll of the devastating set of diseases on which we work. The energy, creativity and drive of the Institute's scientists have ensured that, over almost four decades, LICR has made immeasurable contributions to this end. It is a source of great institutional pride and I applaud every staff member for meeting the challenges and making these contributions possible.



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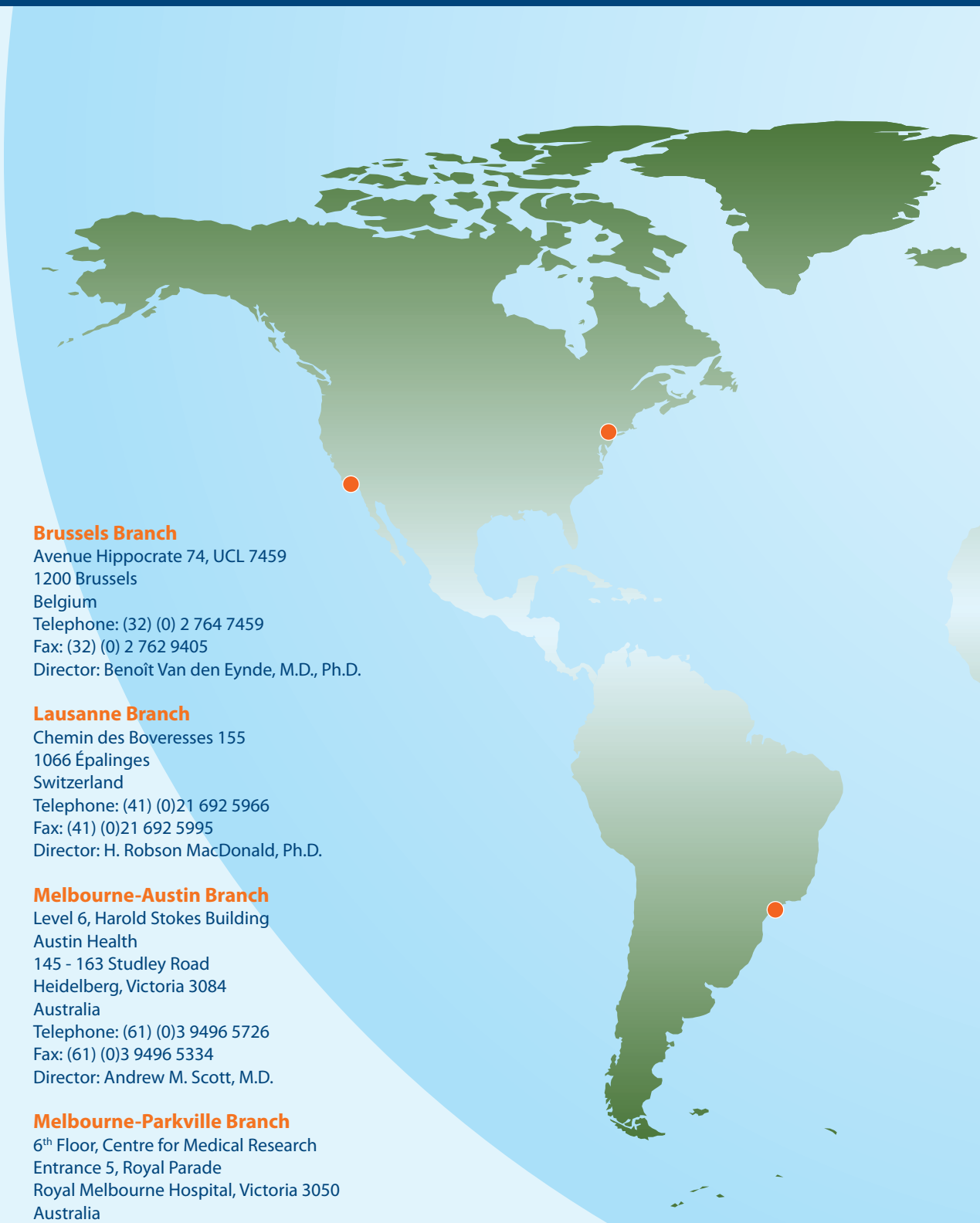

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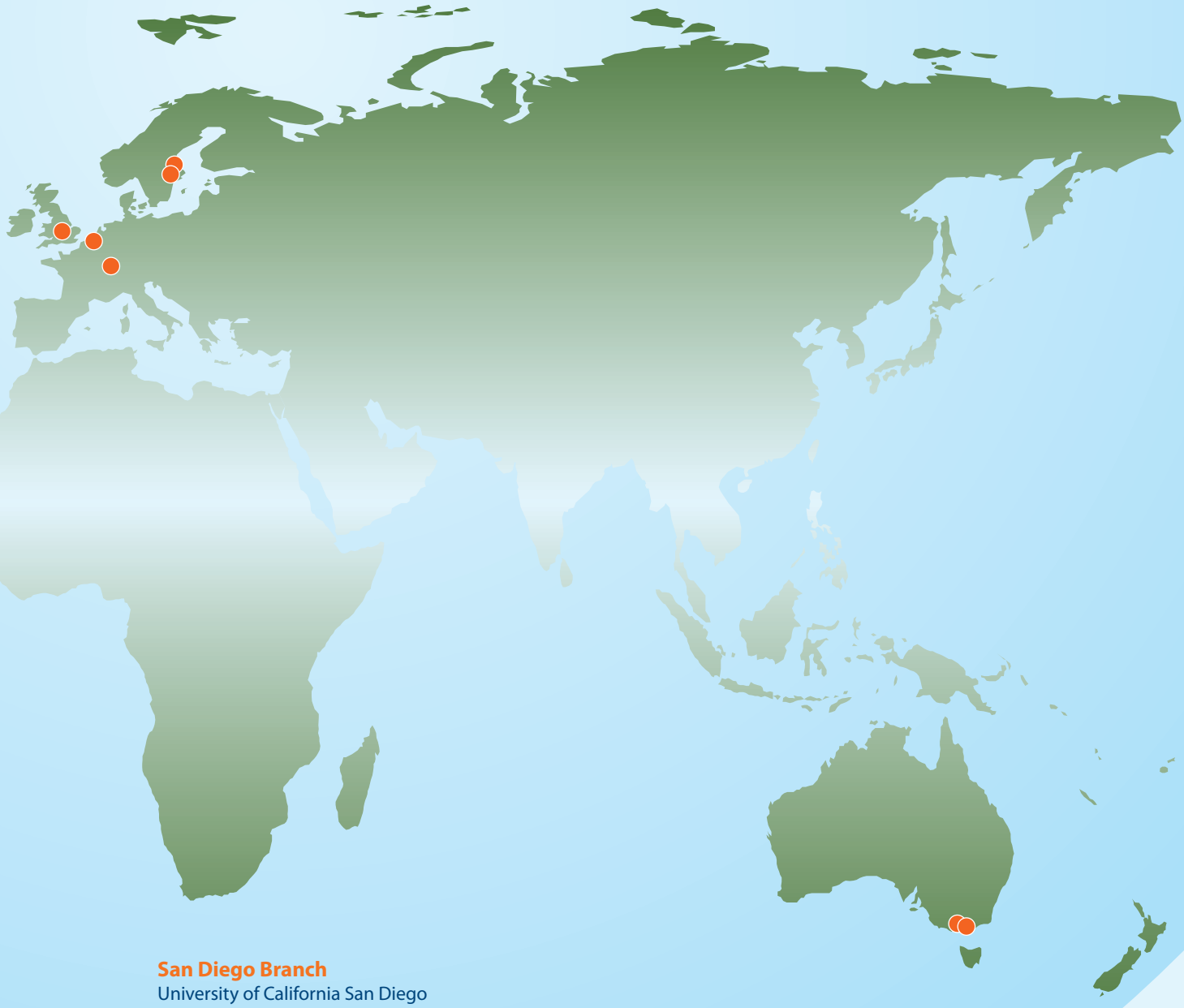

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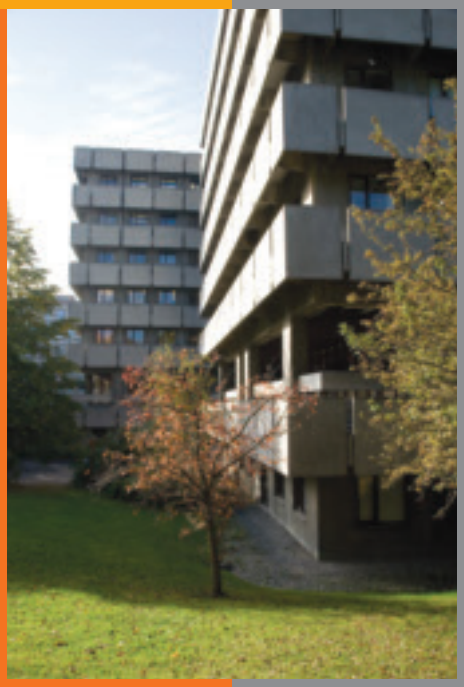

Director's Message

The organization of the Branch favors the numerous interactions and synergies that exist among the six research groups which share their respective expertise in related fields. Four of the research groups continued their study of tumor immunology focusing efforts on the mechanisms used by tumors to resist or escape immune rejection by T lymphocytes. The aim is to identify strategies to overcome such mechanisms and thereby increase the success rate of cancer immunotherapy. Based on recent work on galectin-3 inducing anergy of human tumor-infiltrating lymphocytes, Pierre van der Bruggen's group demonstrated, in a preclinical model, the efficacy of galectin-3 ligands *in vivo* to promote tumor rejection by reversing T-cell anergy. This important benchmark opens the way to the use of galectin-3 ligands in humans. Nicolas Van Baren's group is developing a clinical trial in melanoma patients combining a peptide vaccine with an experimental carbohydrate drug binding galectin-3. Results obtained in Thierry Boon's group studying a mouse model of skin graft indicated that local injection of cytokines may also induce rejection by boosting the activity of infiltrating lymphocytes. These findings will be tested in humans in a clinical trial developed by the therapeutic vaccination group. My own research group previously identified expression of indoleamine 2,3-dioxygenase (IDO), a tryptophan-degrading enzyme, as a major mechanism of tumoral immune resistance. Current work aims at finding new IDO inhibitors that can be used clinically. A study performed in collaboration with Olivier Michielin from the Lausanne Branch has identified promising chemical scaffolds. This program is run in conjunction with the small molecule group recently established at the San Diego Branch. My group is also studying the processing of tumor antigens and recently discovered the unexpected role of a cytosolic metallopeptidase, named insulin-degrading enzyme, in the breakdown of intracellular proteins – exemplified by MAGE-3 – and production of an antigenic peptide from this protein.

The other two groups followed research lines focused on cytokines and cytokine-receptor signaling. Stefan Constantinescu's group continued the study of mutated JAK2, which is oncogenic in polycythemia vera and other hematological malignancies, and demonstrated the critical contribution of pseudokinase domain residues in activity of mutated, but not wild-type JAK2. This opens up the possibility of targeting these pseudokinase domain residues with small molecule inhibitors to block activity of mutated JAK2 while leaving

wild-type JAK2 untouched. Jean-Christophe Renauld's group worked on the function of interleukin-9 and interleukin-22, both discovered at the Branch, and focused on new JAK1 mutations associated with some human acute lymphoblastic leukemias. These mutations increase sensitivity to type I interferons, which might be considered for therapy of these tumors.

2009 was the last year Thierry Boon held the position of Director. He stepped down on December 31st after 31 years. During his tenure, major scientific breakthroughs were achieved including identification of the first human tumor antigens recognized by T lymphocytes. Thierry will remain part-time at the Branch in the role of scientist and group leader. I am very honored to succeed him as Branch Director and would like to take this opportunity to thank him for the way he nurtured a group of scientists, now senior staff at the Branch, who not only became internationally renowned individuals in their field but also developed an exceptional sense of collaboration, resulting in strong scientific synergies and a genuine pleasure in working together. Over the years, this spirit inspired interactions between the Branch and our host institutions, the de Duve Institute and the Université catholique de Louvain, with whom our partnership is strong. I am indebted to Thierry for his humane view of management, which allowed me to inherit a Branch in very good shape not only at the scientific level but also at the human and institutional level.

Benoît Van den Eynde

CYTOKINES IN IMMUNITY AND INFLAMMATION

Jean-Christophe Renauld, M.D.

The group studies the biology of interleukin-9 (IL-9) and IL-22, two cytokines discovered at the Branch. IL-9 is associated with allergic/Th2 responses. Previous studies highlighted the role of this cytokine in lung inflammatory models such as asthma. Recent work showed that IL-9 also acts on immune responses in the intestinal mucosae by promoting mucus production by goblet cells, and also by inducing an IL-13-dependent hyperplasia of Paneth cells, a major source of antimicrobial peptides. It might represent a key player in anti-parasite responses. Beside its protective role in intestinal parasite infections, IL-9 promotes oral-antigen induced anaphylaxis as shown in a mouse model of repeated oral-antigen induced anaphylaxis in OVA-sensitized animals. In contrast to parenteral-antigen induced murine systemic anaphylaxis, which is mediated by both IgG- and IgE-dependent pathways, and can occur independently of IL-9/IL-9R signaling, oral-antigen induced intestinal and systemic anaphylaxis is strictly IgE-mediated and requires IL-9/IL-9R signaling.

Beside its production during TH2-associated immune responses *in vivo*, recent observations point to a preferential production of IL-9 by TGFß/IL-6-induced TH17 cells or a new subset called TH9 cells that differentiate in the presence of TGFß and IL-4 and produce mainly IL-9. Experiments characterizing the effect of IL-9 in a TH17 inflammatory model provided conflicting results. IL-9R-deficient mice showed aggravated symptoms in the classical TH17-dependent model of experimental autoimmune encephalitis, suggesting IL-9 plays an anti-inflammatory role in such models, potentially via Treg stimulation.

IL-22, originally identified as a gene induced by IL-9 in T lymphocytes, is mainly produced by TH17 lymphocytes, although innate cell populations such intestinal NK-like cells and LTi-like cells also contribute to production of this cytokine during inflammatory processes such as inflammatory bowel disease (IBD), psoriasis and rheumatoid arthritis. IL-22 plays a protective role in mouse IBD models such as Dextran sulfate-induced colitis. By contrast, IL-22-deficient mice are partially protected against collagen-induced arthritis, indicating this cytokine can have both pro- and anti-inflammatory activities. Its activity in inflammatory responses is modulated by a specific antagonist, the IL-22 binding protein, which binds IL-22 with high affinity and whose 3D-structure has been characterized in collaboration with Igor Polikarpov's group in São Paulo, Brazil. The signaling pathway mediating IL-22 activities has also been investigated. IL-22 represents one of the most potent activators of STAT3 in intestinal epithelial cells and keratinocytes. Of particular note, the interleukin-22 receptor (IL-22R) does not recruit STAT3 via a classical phospho-Tyr/SH2 domain interaction but the C terminus of IL-22R recruits the coiled-coil domain of STAT3 in a tyrosine-independent manner. This represents a new and unique mode of activation of STAT3 by a cytokine receptor.

The role of IL-9 in tumorigenesis has previously been shown in various models. In many cases it turned out that IL-9-dependent tumor cells can acquire the ability to proliferate autonomously through a series of ill-defined genetic events. Recently, it was discovered in mouse models that overexpression and activating mutations of JAK1 represent efficient mechanisms leading to cytokine-independent growth with constitutive STAT activation. In collaboration with Tartaglia's group, activating mutations of JAK1 in human acute lymphoblastic leukemias (ALL) were found. Expression of JAK1 mutants alone fails to trigger STAT activation, but co-expression of the IL-9Ralpha chain promoted JAK1 mutant phosphorylation and STAT activation. IL-9Ralpha homodimerization is involved in this process, as IL-9Ralpha variants with mutations of

the JAK-interacting BOX1 region not only failed to promote JAK1 activation but also acted as dominant negative forms reversing the effect of wild-type IL-9Ralpha. Coimmunoprecipitation experiments also showed formation of IL-9Ralpha homodimers. Similar results were obtained with the IL-2Rß chain, confirming JAK1 mutants need to associate with cytokine receptors to activate STAT transcription factors.

In a recent study, a type I IFN transcriptional signature in JAK1 mutation-positive human ALL samples was found suggesting these mutants not only activate growth-promoting pathways that depend on IL-9R or IL-2R, but also antiviral pathways. Expression of these activating JAK1 mutants in murine hematopoietic cell lines *in vitro* recapitulated this signature in absence of IFN, and strongly potentiated the *in vitro* response to IFN. Typically, proliferation of cell lines expressing the JAK1(A634D) mutant was abrogated by type I IFNs. Different JAK1 mutations differentially potentiate responses to type I IFNs or to IL-9. This suggests the type of mutation influences specificity of the effect on distinct cytokine receptor signaling. An *in vivo* leukemia model showed that cells expressing mutants such as JAK1(A634D) are hypersensitive to the anti-proliferative and anti-tumorigenic effect of type I IFN, suggests that type I IFNs could be considered potential therapy for ALL with JAK1 activating mutations.

IMMUNOTHERAPY ANALYSIS

Thierry Boon, Ph.D.

A mouse model of skin grafts was developed that recapitulates what happens in cancer patients, where T lymphocytes often infiltrate the tumor without rejecting it. The study of this model aims at identifying methods to overcome anergy of such infiltrating T cells. Another line of research, performed in collaboration with Jean-Christophe Renauld's group, focuses on control of interleukin-9 (IL-9) secretion and contributed to the recent description of a new subset of T helper cells, putatively named TH9.

Female CBA mice do not reject male skin grafts, even though they are able to mount a specific anti-H-Y cytolytic T cell response. Several approaches were tested to break this tolerance. Immunization with male lymphoblasts did not induce rejection of established skin grafts, but repeated local injections of a low dose of IL-12, combined with IFNα, caused rejection in all mice. This also occurred when IL-12 was combined with ligands against Toll-Like Receptors 3, 7 or 9. Like IFNα, IL-1α, IL-18 and IL-2 were incapable of inducing rejection on their own, but synergized effectively with IL-12. To follow lymphocyte infiltration into the graft after cytokine injections, adoptive transfer of transgenic T cells was utilized bearing a receptor specific for a H-Y antigen. When transferred to a female CBA before grafting, naïve transgenic CD8 T cells did not infiltrate or reject the graft. But a low dose of IL-12 injected locally stimulated infiltration of the graft, whereas IFNα had no effect. Results suggest local delivery of cytokine combinations may enhance efficacy of anti-tumoral vaccination.

Contrary to naïve transgenic CD8 T cells, adoptive transfer of activated transgenic CD8 T cells leads to CD8 T cell infiltration of the grafts. Skin grafts are rejected when transferred CD8 T cells are stimulated *in vitro* with H-Y peptide pulsed B-blasts, whereas no graft rejection is observed when CD8 T cells are stimulated *in vitro* with antibodies against CD3 and CD28. There were attempts to identify the critical difference between CD8 T cells stimulated with the specific antigen and those stimulated with antibodies. Comparison of expression levels of cell surface markers by FACS analysis revealed that a CD8 T cell population stimulated with the H-Y peptide is composed of a larger proportion of CD62L-negative effector memory T lymphocytes, known to circulate in the periphery, whereas the population stimulated with anti-CD3 is composed of more CD62L-

positive T lymphocytes, known to home to secondary lymphoid tissues. The functional avidity of these two activated CD8 T cell populations was tested by their capacity to secrete IFN-γ in response to various concentrations of H-Y peptide. No apparent avidity difference between the two populations was detected. The gene expression profiles of the two activated CD8 T cell populations are being compared by RNA microarray.

In an attempt to understand the anergy of tumor-infiltrating lymphocytes in patients, tumor samples in a series of metastatic melanoma patients vaccinated with MAGE-3.A1 tumor-specific antigen, were analyzed for expression of a series of genes coding for tumor-specific antigens, differentiation antigens and genes putatively associated with immunosuppression. Tumor samples for twenty-six patients who did not show any tumor regression were compared with fourteen patients who showed significant tumor regression. For the gene coding for transforming growth factor beta 1 (TGF-β1), the ratio of expression of non-regressors to regressors was 1.6, for TGF-β2 it was 2.4 and for TGF-β3 it was 1.1. One tumor cell line analyzed produced the TGF-ß2 polypeptide (latent form) and showed SMAD2 phosphorylation, indicating TGF-ß2 is active. This cell line was unique in the fact that it was not able to support proliferation of autologous anti-tumoral cytolytic T cells. These results suggest TGF-β1 and β2 may participate in an immunosuppressive environment protecting the tumor against immune rejection.

Since its discovery, IL-9 has been considered a TH2 cytokine but more refined analyses using intracellular cytokine staining of naïve CD4 T cells activated *in vitro* indicated that IL-9 does not fit the TH2 paradigm. In a publication by Marc Veldhoen and Brigitta Stockinger, to which the group contributed by producing the first antibody suitable for intracellular IL-9 detection, IL-9 producing cells were selectively induced when CD4 T cells were stimulated in the presence of TGF-ß and IL-4, suggesting existence of a novel T helper subset putatively designated "TH9". There is evidence that "TH9" cells also develop when *in vivo* primed T cells are re-stimulated *in vitro* with the priming antigen in the presence of TGF-β and IL-4, indicating this cytokine milieu can completely orient an established immune response to selective IL-9 production. Given the importance of this finding, a search for factors other than IL-4 that could stimulate IL-9 production in the presence of TGF-ß was begun. TGF-β combination with IL-1a, IL-1b IL-18, and IL-33 had equivalent IL-9 stimulating activities in all mouse strains tested, including IL-4- and IL-4-R-deficient animals. As IL-9 levels were much lower in *TH2* and *TH17* cultures, these results identify TGF-β/IL-1 and TGF-β/IL-4 as the main control points of IL-9 synthesis. IL-13 and IL-17 were stimulated more by IL-1 alone than IL-9 and inhibited (IL-13) or not further stimulated (IL-17) by addition of TGF-β. Moreover, IL-9 and IL-17 productions differed in their dependence on IL-2. Blockade of IL-2R by anti-IL-2Ra Ab PC61 inhibited IL-9 production in TGF-β/IL-1 cultures by ± 75 % but rather stimulated, although not significantly, that of IL-17. IL-1 and IL-23 have been reported to initiate a strong IL-17 production by T cells even in the absence of other stimuli. This synergistic interaction was confirmed for IL-17 but IL-9 remained undetectable under these conditions. Together these data lend additional proof to the existence of unique control patterns of IL-9 production.

REGULATION OF T LYMPHOCYTE FUNCTION IN TUMORS

Pierre van der Bruggen, Ph.D.

The group studies the anergy of tumor-infiltrating lymphocytes, resulting from exposure to galectin-3 produced by tumors, and how this anergy can be reversed by galectin-3 ligands. Vaccine-specific regulatory T cells detected in cancer patients vaccinated against MAGE-A3 were also analyzed.

Human CD8 tumor-infiltrating T lymphocytes (TILs), in contrast

with CD8 blood cells, show impaired IFN-γ secretion upon *ex vivo* re-stimulation. The decreased IFN-γ secretion has been attributed to reduced mobility of T cell receptors upon trapping in a lattice of glycoproteins clustered by extracellular galectin-3. Treatment of TILs with N-acetyllactosamine (LacNAc), a galectin ligand, restored this secretion. Why do galectin-3 ligands improve human TIL function? The working hypothesis is that TILs have been recently stimulated by antigen and the resulting activation of T cells could modify expression of enzymes of the N-glycosylation pathway and change the structure of N-glycans exposed at the cell surface, as shown for murine T cells. It was surmised that the recently activated TILs, compared to resting T cells, harbor a set of glycans that are either more numerous or better ligands for galectin-3. Galectin-3 is abundant in many solid tumors and carcinomatous ascites, and can bind to surface glycoproteins of TILs and form lattices that would reduce TCR mobility. This could explain the impaired function of TILs. The release of galectin-3 by soluble competitor ligands would restore TCR mobility and boost IFN-γ secretion by TILs. This hypothesis was supported by the observation that $CD8^+$ TILs treated with an anti-galectin-3 antibody had increased IFN-γ secretion.

Galectin competitor ligands, e.g., disaccharides, lactose and LacNAc, are rapidly eliminated in urine, preventing their use *in vivo*. Other compounds that could block interactions between galectin-3 and glycoproteins are under development. A plant-derived polysaccharide, which is in clinical development, detached galectin-3 from TILs and boosted their IFN-γ secretion. $CD8^+$ TILs and $CD4^+$ TILs were treated with this polysaccharide and secreted more IFN-γ upon *ex vivo* re-stimulation. In tumor-bearing mice vaccinated with a tumor antigen, injections of this polysaccharide led to tumor rejection in half the mice, whereas all control mice died. In non-vaccinated mice, the polysaccharide had no effect on its own. These results suggest that a combination of galectin-3 ligands and therapeutic vaccination may induce more tumor regressions in cancer patients than vaccination alone. The group intends to pursue clinical trials involving the use of these agents in combination with anti-tumoral vaccination.

Another blockage could be the presence of regulatory T cells which have the ability to dampen immune responses. During the course of an analysis of anti-vaccine T cell responses of melanoma-bearing patients, who were injected with different vaccines containing a MAGE-3 peptide presented to T cells by HLA-DP4 molecules, 197 anti-MAGE-A3.DP4 T cell clones from ten patients were isolated. About five percent of them appeared to be regulatory T cells, characterized by high CD25 and FOXP3 expression in the resting state and an unmethylated intronic sequence of *FOXP3*. Upon stimulation, these cells secrete active TGF-β but none of the following cytokines: IFN-β, IL-2, IL-4, IL-5 or IL-10. These clones have a clear suppressive activity *in vitro* partly attributable to secretion of active TGF-β. This work is the first to describe the presence of anti-vaccine regulatory T cells not only on the basis of markers such as FOXP3, but also on the basis of suppressive activity *in vitro*. FOXP3 seems of doubtful value as a unique marker for regulatory T cells, due to its transient expression in some activated non-regulatory T cells and in some resting T cells without suppressive activity. Exclusive to T cells with suppressive activity and stable expression of FOXP3 is demethylation of an intronic sequence of FOXP3. A quantitative DNA methylation analysis of FOXP3 based on RT-PCR could become a routine technique to identify the best regulatory T cell candidates.

SIGNAL TRANSDUCTION AND MOLECULAR HEMATOLOGY

Stefan Constantinescu, M.D., Ph.D.

The mechanisms by which JAK2 becomes activated by a pseudokinase domain mutation (V617F) in human myelopoliferative neoplasms

(MPNs) and the contribution of pathologic signaling by receptors for thrombopoietin (TpoR) and G-CSF (G-CSFR) in the pathogenesis of MPNs, especially Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF) are two areas of research being pursued. The role of dimerization imposed by transmembrane and juxtamembrane sequences for signaling by cytokine receptors, such as those for thrombopoietin (Tpo), erythropoietin (Epo), G-CSF and interleukins 2/9, is also being explored.

No crystal structure exists for the pseudokinase domain of JAK2, or for the entire JAK2 protein. Modeling the structure of the pseudokinase domain of JAK2 on the crystal structures of other tyrosine kinases led to identification of residues predicted to be located closest to F617: they either belonged to the kinase domain (JH1) or to the helix of the pseudokinase domain (JH2). The requirement of these residues for pathologic and physiologic activation of JAK2 was determined. Helix C of the pseudokinase domain of JAK2 was found to be essential for constitutive activity of JAK2 V617F, V617L, V617M and V617I, while mutations in the activation loop of the kinase domain had no effect. Unexpectedly, constitutive active mutants of JAK2, where mutations are located in the linker between the SH2- and the JH2 domain (K539L) or in the kinase domain itself (T875N), were dependent on JH2 helix C residues. Kinase activation originating from JAK2 absolutely requires integrity of residues in the middle of the JH2 helix C. Mutations in helix C of JH2 did not impair physiologic activation of JAK2 by Epo-activated EpoR. The same was true for Epo-activation of double, V617 and JH2, mutations. The group proposed that small molecules targeting a hydrophobic pocket in the middle of JH2 helix C might specifically inhibit oncogenic constitutive JAK2 mutants, and spare wild-type JAK2. Helix C residues might support a conformational change in mutant JAK2 that replaces receptor rotation, normally induced by cytokines, to trigger JAK2 kinase domain activation. The results are likely to be relevant for the mechanisms of activation of other JAK mutants in cancers. The homologous V617F mutation of JAK1, detected in certain T-ALL patients, was also blocked by homologous JH2 helix C mutations.

Like JAK2 V617F, TpoR W515L/K mutants are major determinants of MPNs, being detected in 2-7% of JAK2 V617F-negative ET and PMF patients. W515 belongs to a juxtamembrane amphipathic motif shown to maintain the TpoR inactive in the absence of ligand. The group demonstrated that TpoR W515A, and the Delta5TpoR active mutant, where the juxtamembrane TpoR R/KW(515)QFP motif is deleted, induced a rapid and severe MPN phenotype in mouse bone marrow reconstitution experiments. Of the three tyrosine residues of the TpoR cytosolic domain, Y112 was required for induction of MPN and myelofibrosis. Phosphotyrosine immunoprofiling detected phosphorylated cytosolic TpoR Y78 and Y112 in cells expressing TpoRW515A. Mutation of cytosolic Y112 to phenylalanine decreased constitutive active signaling by Delta5TpoR and TpoRW515A, especially via the mitogen-activated protein (MAP)-kinase pathway, without decreasing JAK2 activation. It was proposed that cytosolic phosphorylated Y112 and flanking TpoR sequences could become targets for pharmacologic inhibition in MPNs and that inhibition of MAP-kinase, in addition to inhibition of JAK2, could be useful in myelofibrosis. A more severe phenotype in bone marrow reconstitution experiments was detected when JAK2 V617F was co-expressed with TpoR; this phenotype could be prevented by the Y112F mutation of TpoR. This suggests myelofibrosis in JAK2 V617F-positive patients might be due to hyperactivation of TpoR and MAP-kinase/STAT3 pathways.

Recognizing TpoR is down-modulated in platelets from MPN patients, the group

searched for microRNAs that would bind to 3'-UTR of TpoR mRNA and inhibit translation. Three miRNAs, miR-28, miR-151 and miR-708 were identified to bind to the same sequence in the 3'-UTR of TpoR mRNA and to decrease significantly TpoR protein levels. Targets of these miRs include E2F6 and MAP-kinase Erk2. miR-28 expression inhibited megakaryocyte differentiation. One third of MPN patients overexpress miR-28 in their platelets, especially JAK2 V617F-negative ET patients with high platelet counts. miR-28 could become a marker of pathologic megakaryocyte proliferation. Expression of miR-28 requires constitutive STAT5 signaling, while transient STAT5 activation by cytokine treatment did not induce expression of miR-28, or its host gene, Lipoma Preferred Partner (LPP).

THERAPEUTIC VACCINATION AND TUMOR EXPRESSION PROFILING

Nicolas Van Baren, M.D., Ph.D.

The Therapeutic Vaccination and Tumor Expression Profiling group develops small scale clinical trials of immunotherapy, in which patients with advanced cancer, often metastatic melanoma, are treated repeatedly with a vaccine containing one or several defined tumor-specific antigens expressed by their tumor. These projects are developed in close collaboration with J.F. Baurain (Centre du Cancer, Cliniques Universitaires St Luc) and P. Coulie (Cellular Genetics Unit, de Duve Institute).

Different types of vaccines have been tested, various peptides or a recombinant protein or virus, associated or not with an immunological adjuvant. All these vaccines were well tolerated and associated with evidence of tumor regression in patients with metastatic melanoma, albeit in a minority of them, between 10 and 20%. A real clinical benefit was restricted to 5% of vaccinated patients. There is no evidence that one of the vaccine modalities tested is more effective against the tumors than the others. Weak CTL responses were detected in a minority of patients, often without obvious correlation with the clinical responses. These trials collect biological material, usually blood and tumor samples, which can be used to analyze vaccine-associated immunological events.

The most likely explanation for the lack of effectiveness of cancer vaccines is that tumors acquire the ability to resist destruction by anti-tumoral T cells, following repetitive *in vivo* challenge with spontaneously occurring immune responses. Even though molecular mechanisms of tumor resistance remain largely unknown, recent work conducted by Pierre van der Bruggen's group has shown the state of anergy that characterizes tumor-associated T cells can be reversed pharmacologically. Inhibitors of galectin-3, a protein produced by cancer cells able to interfere with effective T cell activation, reactivated anergic T cells *in vitro* and promoted tumor rejection *in vivo* in mice. A new clinical trial is being developed in which patients with advanced melanoma will receive a treatment combining a peptide vaccine and Davanat®, an experimental carbohydrate drug that inhibits galectin-3. With this combined treatment, it is expected the peptide vaccine will induce anti-tumoral CTL, which will be more effective at rejecting the tumor thanks to galectin-3 inhibition.

A series of cutaneous metastases obtained from melanoma patients is being analyzed, using an approach that combines gene expression profiling by microarray, immunohistology and immunofluorescence. These analyses focus on the inflammatory cells and pathways associated with tumors, in order to understand interactions between tumor and immune cells at the tumor site. This approach is further detailed by laser capture microdissection of small groups of cells present in the tumors, such as T lymphocytes, followed by gene expression analysis.

The group is collaborating on

a project aimed at developing innovative imaging microscopy approaches that might improve cancer diagnosis with several European groups. These approaches are based on spectroscopical analysis of tissue sections illuminated with one or more laser beams of selected frequencies, using so-called Raman and Coherent Anti-Stokes Raman Spectroscopy (CARS) microscopes. The Raman and CARS effects involve light reflection that depends on molecular bonds present in the illuminated sample. The objective is to identify spectral signatures associated with tumor cells, which would allow detecting and quantifying these cells in conventional microscope preparations without staining. The current project, which is focused on melanoma, is in early stage development.

TUMOR IMMUNOLOGY AND ANTIGEN PROCESSING

Benoît Van den Eynde, M.D., Ph.D.

The Tumor Immunology and Antigen Processing group conducts three lines of research. The first focuses on the processing of tumor antigens, studying the role of the proteasome and other proteases in the production of tumor antigenic peptides. The second studies mechanisms whereby tumors resist immune rejection and the third develops new preclinical models for cancer immunotherapy. The long term objectives are to better understand the interaction of tumors with the immune system and devise strategies to improve the efficacy of cancer vaccines.

Tumor antigens relevant for cancer immunotherapy consist of peptides presented by MHC class I molecules and are derived from intracellular tumor proteins. They result from degradation of these proteins, mainly exerted by the proteasome. The group previously described a new mode of production of antigenic peptides by the proteasome, involving the splicing of peptide fragments, either in normal or reverse order. Splicing occurs in the proteasome catalytic chamber through a reaction of transpeptidation involving an acyl-enzyme intermediate. The same mechanism accounts for splicing a third spliced peptide, derived from FGF5, despite the fact that the fragments to splice are distant from each other by 40 amino acids. The group compared the efficiency of splicing by the standard proteasome and the immunoproteasome, which is found in antigen-presenting cells and cells exposed to interferon-gamma, and contains three inducible catalytic subunits ß1i, ß2i and ß5i instead of the standard catalytic subunits ß1, ß2 and ß5. Both proteasome were able to splice peptides, but the relative efficiency was different for each peptide, depending on major cleavage sites. This is consistent with the transpeptidation model of splicing.

Using a series of novel antibodies recognizing catalytic subunits of human proteasomes in their native conformation, proteasomes that are intermediate between the standard proteasome and immunoproteasome were identified. They contain only one (ß5i) or two (ß1i and ß5i) of the three inducible catalytic subunits of the immunoproteasome. These intermediate proteasomes represent 30 to 54% of the proteasome content of human liver, colon, small intestine and kidney. They are also present in human tumor cells and dendritic cells. The group studied the processing of a series of antigenic peptides by these intermediate proteasomes, and identified two tumor antigens processed exclusively either by intermediate proteasomes ß5i or intermediate proteasomes ß1i-ß5i.

A proteasome-independent peptide derived from tumor protein MAGE-A3 was studied and insulin-degrading enzyme was identified as the protease producing both the C-terminus and the N-terminus of this peptide. Insulin-degrading enzyme is a cytosolic metallopeptidase not previously known to play a role in the class I processing pathway. The parental protein

MAGE-A3 appears to be degraded along two parallel pathways involving insulin-degrading enzyme or the proteasome, each pathway producing a distinct set of antigenic peptides presented by class I molecules.

An important factor limiting the efficacy of immunotherapy is the development of mechanisms by tumors to resist or escape immune rejection. Immune resistance mechanisms often involve modulation of the tumoral microenvironment resulting in local immunosuppression. One such mechanism is based on the expression by tumor cells of Indoleamine 2,3-dioxygenase (IDO), a tryptophan-degrading enzyme inducing a local tryptophan depletion that severely affects T lymphocyte proliferation. Data in a preclinical model indicate that the efficacy of therapeutic vaccination of cancer patients could be improved by concomitant administration of an IDO inhibitor. In collaboration with Olivier Michielin's group at the Lausanne Branch, the group described new compounds able to inhibit IDO in the micromolar range, not only in enzymatic assays but also in cellular assays. These compounds will be further optimized with the goal of developing drug candidates. An effort was launched in collaboration with academic and industrial partners to identify IDO inhibitors by high-throughput screening of a chemical library and structure-based drug design.

A monoclonal antibody against human IDO, which was used to characterize IDO expression in normal and tumoral tissues was produced. Although high expression of IDO was reported in murine dendritic cells within tumor-draining lymph nodes, results in humans indicate a subset of mature human dendritic cells express IDO but these cells are present in normal lymph nodes and not enriched in tumor-draining lymph nodes. However, an expression of IDO in a high proportion of human tumors was observed. A mouse melanoma model was devised in which the group can induce melanoma in 70% of mice injected with tamoxifen. These tumors express the tumor antigen encoded by cancer-germline gene P1A. These tumors can be either highly pigmented and indolent, or unpigmented and highly aggressive. A correlation was observed between aggressive tumor progression and occurrence of exacerbated systemic inflammation, involving disruption of secondary lymphoid organs, extramedullary hematopoiesis and accumulation of immature myeloid cells, which may contribute to tumoral immune resistance.

Cancer-germline genes are expressed at a low level in the thymus, possibly inducing some level of central immune tolerance that may explain the poor immunogenicity of many of the antigens encoded by these genes. To address this issue, mice that are knockout for cancer-germline gene *P1A* were produced. These mice are normal and fertile. Their ability to develop an immune response against the P1A-encoded antigen is slightly higher than the wild-type mice, resulting in an ability to reject P1A-expressing tumors spontaneously. Analysis of the repertoire of TCR genes revealed some differences in Vß gene usage. This result is consistent with deletion of high affinity T cells recognizing P1A-encoded antigens in wild-type mice. It was concluded that there is a limited central tolerance towards antigens encoded by cancer-germline genes.

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Director's Message

We continued to devote our efforts to combining basic and translational studies in T cell immunology to benefit cancer patients via novel approaches to immunotherapy. The Branch consists of seven groups divided between basic and translational studies. There is extensive collaboration among the groups which facilitates rapid translation of basic discoveries into the clinic. Early phase clinical trials in T cell immunotherapy are carried out by the Branch in collaboration with the Center of Pluridisciplinary Oncology (CePO) located within the University Hospital (CHUV).

Major changes in the local environment occurred last year. The Swiss Institute for Experimental Cancer Research (ISREC), which served as our partner institution throughout the history of the Branch, was incorporated into the EPFL and has left the Épalinges campus. The laboratory space in Épalinges left vacant by ISREC will be renovated and occupied by immunology groups currently working on the hospital campus. This reorganization has been formalized by the creation of a new Center for Immunity and Infection Lausanne (CIIL) in Épalinges focused on basic and translational

Aerial view of newly re-organized Center for Immunity and Infection Lausanne (CIIL) in Épalinges. The Lausanne Branch is located in the building on the lower right.

immunology research. In this new setting, the Branch is developing closer ties with the University of Lausanne and the University Hospital. We look forward to participating in this exciting new research environment in the coming years.

H. R. MacDonald

DEVELOPMENTAL IMMUNOLOGY

Hugh Robson MacDonald, Ph.D.

The development of T lymphocytes from hematopoietic precursors is the primary focus of the developmental immunology group. Significant progress was made in several areas including the role of Notch and Myc during the early stages of intrathymic development of conventional and unconventional T lymphocytes, respectively.

In the past decade, the group studied the role of Notch signaling during T cell development in collaboration with Freddy Radtke (EPFL Lausanne). This collaboration led to identification of a critical requirement for interaction between Notch-1 on lymphoid precursors and Delta-like 4 (DL4) on thymic cortical epithelial cells (cTEC) in order to specify T cell fate in the thymus. To define further the mechanism of T cell fate specification, novel monoclonal antibodies directed against Notch receptors and ligands have recently been generated in collaboration with Michel Pierres, CIML, Marseille. Using these reagents, it was established that Notch-1 is already expressed on bone marrow precursors of T lymphocytes and remains highly expressed during early (CD4- CD8-) stages of intrathymic T cell development. Notch-1 expression decreases sharply at the CD4+ CD8+ thymocyte stage and remains at low levels on mature CD4+ and CD8+ T cells. Staining of thymus sections with Notch-1 and Keratin 5, a marker for the thymic medulla, reveals Notch-1 high cells are restricted to the thymic cortex and highly enriched in the subcapsular zone. These data confirm and extend other studies indicating Notch signaling is largely confined to early T cell progenitors in the outer thymic cortex.

In related studies, the novel monoclonal antibody to the Notch ligand DL4 revealed specific staining in cTEC. Somewhat unexpectedly, it was discovered that DL4 was highly expressed in the embryonic thymus but weakly in the adult. Analysis and reconstitution of mutant mice with an early block in T cell development further revealed that lymphostromal interactions control DL4 expression on cTEC. These data suggest a model in which "crosstalk" between thymocytes and cTEC can quantitatively regulate the rate of Notch-1-dependent thymopoiesis by controlling DL4 expression levels. This model is currently being directly tested in transgenic models where DL4 expression on cTEC can be regulated *in vivo*.

In addition to conventional T cells, there is a longstanding interest in the development of natural killer T (NKT) cells. NKT cells are a subset of regulatory T cells that recognize glycolipid antigens associated with non-polymorphic CD1d molecules via a semi-invariant TCR. In contrast to conventional T cells, the developmental pathway of NKT cells involves agonist selection of CD4+ CD8+ thymic precursor cells interacting with self CD1d: glycolipid complexes via their semi-invariant TCR. Using mice with specific inactivation of the transcription factor c-Myc in CD4+ CD8+ thymocytes and their progeny, it was discovered that c-Myc plays a selective role in NKT cell development without affecting development of conventional T cells. In the absence of c-Myc, NKT cell precursors are blocked at an immature stage in a cell autonomous fashion. Thus c-Myc plays a critical role early in NKT cell development, perhaps by inhibiting proliferation of precursor cells responding to agonist signals.

INNATE IMMUNITY

Werner Held, Ph.D.

Pursuit of the identification of factors that impact elimination of diseased (stressed, infected or transformed) host cells by Natural Killer (NK) and cytotoxic T cells is the research objective of the group. Significant progress has been made in understanding how the host's Major Histocompatibility Complex class I (MHC-I) molecules improve elimination of a specific type of diseased cells by NK cells.

Similar to cytotoxic T cells, NK cell function is adapted to the

inherited MHC-I molecules of the host. This adaptation, known as NK cell education, allows efficient NK cell reactions to host cells lacking MHC-I molecules, a hallmark of many metastatic tumors. Inhibitory receptors specific for MHC-I molecules play a key role for NK cell education. It had been assumed education was mediated via an interaction of inhibitory receptors with MHC-I molecules expressed on specific other (but unidentified) host cells. However, the group found that certain MHC-I receptors are able to bind MHC-I molecules expressed in the plane of the NK cells' membrane *(in cis)*.

To determine whether cis interaction plays a role in NK cell education, a variant receptor was designed which retained the ability to engage MHC-I expressed on other cells but lacked the capacity to bind MHC-I in cis. Even though this receptor variant inhibited NK cell effector function, it was unable to mediate NK cell education. These data dissociated the classical inhibitory from an educating function of these cell surface receptors and suggested cis interaction plays an essential role for NK cell education. Consequently, reactivity of NK cells to diseased host cells, which have lost MHC-I-expression, is dependent on an unconventional interaction of certain cell surface receptors with MHC-I in the plane of the NK cell membrane.

Cis/trans interactions are reported for an increasing number of cell surface receptors. These data raise the question of how a cell surface receptor can interact with ligand expressed on an apposed or the same cell membrane. In addition, it is not clear why ligand binding in trans and cis can have distinct functional consequences. A combined structure-function analysis, performed in collaboration with Roy Mariuzza of the University of Maryland, Rockville, revealed that Ly49 family receptors bind two MHC-I molecules in trans when the two ligand-binding domains of the homodimeric receptor are back-folded onto the long stalk region of this receptor. In contrast, the dissociation of the ligand-binding domains from the stalk and their reorientation relative to the NK cell membrane allows monovalent binding of MHC-I in cis. The distinct conformations (back-folded versus extended) define the structural basis for cis versus trans binding by these MHC-I receptors. The distinct stoichiometries of these complexes provide a likely explanation for the divergent functional consequences of cis versus trans interactions. These analyses represented the first determination of the structural basis for cis/trans binding by a cell surface receptor. Overall, investigations have identified essential host factors that impact on the ability of NK cells to eliminate a specific type of diseased host cells.

MOLECULAR IMMUNOLOGY

Immanuel Luescher, Ph.D.

Three lines of research are being pursued, which better enable a molecular and mechanistic understanding of T cell immunity, in particular tumor antigen-specific CD8+ (and CD4+) T cell responses; how they can be induced and monitored by means of MHC-peptide complexes; and how they depend on the coreceptor CD8αβ.

Although tumor-associated antigen (TAA)-specific CD8+ T cells can infiltrate and eradicate tumors, current cancer vaccines rarely elicit tumoricidal T cell responses. To indentify key variables critical for efficient priming of NY-ESO-1 (ESO)-specific CD8+ T cells, HLA-A2/DR1 H-$2^{-/-}$ transgenic mice and sequential immunization with DR1 and A2-resticed ESO epitopes was used. Strong ESO+ tumor cell killing by primary and memory CD8+ T cells was induced in mice pre-immunized with DR1-restricted $ESO_{123-137}$ peptide upon immunization with DC presenting this and the A2-resticed $ESO\text{-}1_{157-165}$ epitope. Their efficient priming in draining lymph nodes required strong Th1 responses and DC co-presentation of both peptides and resulted in up-regulation of sphingosine-1-phosphate receptor 1 ($S1P_1$) on freshly primed CD8+ T cells and egress in the circulation. This well defined system allowed detailed mechanistic

analysis, which revealed that the Th1 cytokines IFNγ and IL-2 play critical roles in CD8+ T cell priming; e.g., up-regulation of CCR5 on naïve CD8+ T; and that the CCR5 binding, inflammatory chemokines CCL4 (MIP-1β) and CCL3 (MIP-1α) chemo-attract primed CD4+ T cells to mature, cognate DC and activate naïve CD8+ T cells to DC-CD4 conjugates, respectively.

Critical evaluation of cancer vaccines requires conclusive and stringent analysis of TAA-specific T cell responses. In cancer patients such analysis is considerably more difficult than in simplified animal models. Because MHC-peptide tetramers are provided for monitoring of TAA T cell responses in LICR and CVC clinical trials, the developing and testing novel reagents is ongoing.

Multimeric MHC I-peptide complexes containing phycoerythrin-streptavidin are widely used to detect and investigate antigen-specific CD8+ T cells. Since such reagents are heterogeneous, their binding characteristics were compared with those of mono-disperse dimeric, tetrameric and octameric complexes containing linkers of variable length and flexibility on Melan-A-specific CD8+ T cell clones and peripheral blood mononuclear cells (PBMC) from HLA-A*0201$^+$ melanoma patients. Striking binding differences were observed for different defined A2/Melan-A_{26-35} complexes on T cells depending on their differentiation stage. In particular, short dimeric, but not octameric A2/Melan-A_{26-35} complexes selectively and avidly stained incompletely differentiated effector-memory T cell clones and populations expressing CD27 and CD28 and low levels of cytolytic mediators (granzymes and perforin). This subpopulation was found in PBMC from six melanoma patients analyzed and proliferated upon peptide stimulation with modest phenotypic changes. In contrast, influenza $matrix_{58-66}$ -specific CD8+ PBMC from nine HLA-A*0201$^+$ healthy donors were efficiently stained by A2/Flu $matrix_{58-61}$ multimers, but not dimer and upon peptide stimulation proliferated and differentiated from memory into effector T cells. Thus, PBMC from melanoma patients contain a differentiation defective sub-population of Melan-A-specific CD8+ T cells that can be selectively and efficiently stained by short dimeric A2/Melan-A_{26-35} complexes, which makes them directly accessible for longitudinal monitoring and further investigation.

While MHC I multimers have become essential tools for analysis of CD8+ T cell analysis, applications of MHC class II multimers, especially to *ex vivo* analysis of TAA- specific CD4+ T cell responses have been far less successful. Limiting factors include high polymorphism of class II molecules, low binding capacity of the peptides and promiscuous binding to multiple alleles. In collaboration with Danila Valmori (INSERM Unité 892, CLCC René Gauducheau, Saint Herblain, France), molecularly defined tetramers using tagged ESO peptides and purification of monodisperse HLA-DR52b-ESO peptide monomers by chromatography were generated. Immunopure DR52b-ESO multimers produced from such monomers avidly and stably stained specific CD4+ T cells with negligible background on non-specific cells. Using molecularly defined DR52b-ESO multimers, it was demonstrated that in DR52b+ cancer patients immunized with a recombinant ESO vaccine, vaccine-induced multimer+ cells represent *ex vivo* on average 1:5000 circulating CD4+ T cells, include central and transitional memory polyfunctional populations and exclude CD4+CD25+CD127- regulatory T cells. This novel approach may significantly accelerate the development of reliable MHC Class II tetramers to monitor immune responses to tumor and self-antigens.

Studies of CD8αβ, in particular on CD8β are ongoing. It had previously been shown that CD8β *in vitro* increases the range and sensitivity of antigen recognition and *in vivo* plays an important role in the thymic selection of CD8+ T cells. It

was also observed that CD8+ T cells from CD8β KO P14 TCR Tg mice proliferate inefficiently *in vitro*. In contrast to these findings, the group found CD8β KO mice mount normal CD8 primary, secondary and memory responses to acute infection with lymphocytic choriomeningitis virus (LCMV). Tetramer staining and cytotoxic experiments revealed a predominance of CD8 independent CTL in CD8β KO mice. The TCR repertoire, especially the TCRα chain, was different in CD8β KO mice compared to B6 mice. The results indicate that in the absence of CD8β, CD8 independent TCR are preferentially selected, which *in vivo* effectively compensate for reduced coreceptor function of CD8αα. In order to investigate this phenomenon in depth, the group is currently establishing a powerful technique that combines quantitative real-time PCR and Illumina mass sequencing.

TRANSLATIONAL TUMOR IMMUNOLOGY

Pedro Romero, M.D.

The Translational Tumor Immunology group combines studies in human, with or without tumors, with studies in mouse models. Emphasis is on two major subjects: 1) T cell differentiation and T cell mediated immunity; and 2) identification of novel potent immunotherapy strategies. The key objectives are to understand T cell responses against tumors taking place in patients with cancer and the design of clinically effective immune intervention approaches for the treatment of cancer.

Flow cytometry based assays have been established to quantify and perform the functional characterization of T cell responses to tumor associated antigens. In collaboration with Daniel Speiser and Nathalie Rufer, the group studied the CD4 T cell responses to the melanocyte/melanoma associated antigen Melan-A and successfully combined HLA-DQ6/Melan-A26-35 multimers with anti-Foxp3 labeling to identify Melan-A antigen specific Tregs. While these cells are occasionally detectable in blood lymphocytes from healthy individuals, they could be readily identified in ten out of eleven HLA-DQ6+ metastatic melanoma patients. The proportion of Foxp3+ multimer+ CD4 T cells in these patients ranged from approximately 19 to 74% (median 33%) in blood lymphocytes taken before any immunotherapy. Interestingly, this proportion shrank to 0 – 21% (median 7%) over time during vaccination with peptide and CpG-ODN emulsified in ISA-51 adjuvant. This drop in the relative levels of Melan-A antigen specific Tregs correlated with the recovery of the proliferative response of CD4+ blood lymphocytes upon *in vitro* stimulation with the cognate peptide. Moreover, it also correlated with a sharp rise in circulating levels of HLA-A2 restricted CD8 T cells specific for a highly related Melan-A peptide.

A study of vaccination with tetanus toxoid was conducted with the aim of comparing characteristics of anti-microbial antigen specific CD4 T cell responses with those of tumor antigen specific CD4 T cell responses. To quantify the numbers of specific CD4 T cells, limiting dilution cultures were combined with fluorescent multimer labeling to determine the frequency of CD4 T cells recognizing a DP4-restricted T cell epitope represented by synthetic peptide 947 – 60. The frequency of tetanus specific CD4 T cells was in the range of 1 in 10^5 to 1 in 4 x 10^5 in the blood sample before vaccination. This is below the detection limit of *ex vivo* lymphocyte labeling with fluorescent multimers. After vaccination, there was approximately an order of magnitude increase in the frequency of specific CD4 T cells, to the range of 1 in 2 x 10^4 to 1 in 8 x 10^4 at the peak of the response, around day 12. Thus, despite the measurable expansion of specific CD4 T cells, these frequencies are still below the detection limit of direct flow cytometric analysis. These findings have important implications for the monitoring of tumor antigen specific CD4 T cell responses in clinical trials of cancer vaccines.

An analysis of the naturally acquired T cell response to MAGE-A3 and MAGE-A4 shared tumor antigens in head and neck cancer patients has been completed. All patients had detectable CD4 T cell responses directed against a multiplicity of epitopes. Specific CD8 T cells could not be detected. A panel of CD4 T cell clones from three of the patients were isolated and characterized in detail.

The studies in mouse models use transplantable tumors such as B16 F10 melanoma and an autochthonous melanoma tumor developed by scientists at the Swiss Institute for Experimental Cancer Research (ISREC). The focus is on identification of novel adjuvants for vaccination. Two candidates are double stranded RNAs, which provide ligands for TLR3 and which can activate dendritic cells, and saponins. The mechanisms of adjuvanticity of the latter are under investigation. Therapeutic vaccination using synthetic peptides or recombinant lentiviral vectors in combination with chemotherapy is also being explored. The group is using TCR transgenic mouse strains to model the interplay between effector CD8 and CD4 T cells and regulatory CD4 T cells during vaccination using different vaccine formulations.

HUMAN T LYMPHOCYTE BIOLOGY

Nathalie Rufer, Ph.D.

Although tumor-specific cytolytic T cells can be detected in cancer patients, immune responses often fail to control or eliminate the disease. Therapeutic vaccines aim at inducing and boosting T cell mediated immunity. Such approaches provide an excellent opportunity to study T cell priming and memory formation in humans. The research work has concentrated on three major aspects.

The first is devoted to identification of the type of cytolytic T cells and their T-cell receptors (TCR) that are most efficient in anti-cancer therapy, as well as on the characterization of TCR repertoire dynamics in melanoma patients following therapeutic peptide vaccination. One approach is to analyze tumor- and vaccine-specific T cell responses at the individual T cell level. A novel molecular-based strategy was developed that combines cell sorting by flow cytometry, followed by gene expression profiling and TCR clonotyping, allowing the dissection of human tumor-reactive T cell responses *ex vivo*. T cell responses are very dynamic, with the establishment of a few dominant tumor-reactive T cell clonotypes that are specific for each individual patient, with high T cell frequencies *in vivo*, and potent TCR mediated recognition of tumor cells.

These results highlight that, similarly to anti-viral T cell responses (i.e, specific for EBV and CMV), tumor-reactive effector T cells are composed of small numbers of codominant clonotypes, which are selected during T cell differentiation.

The second aspect of the research is to enhance understanding of the structural basis of TCR affinity/avidity for the antigen. For instance, distinct sets of alpha-beta TCRs confer similar and efficient recognition of the cancer testis antigen NY-ESO-1$_{157\text{-}165}$ by interacting with its central Met/Trp residues. Moreover, peptide-based cancer vaccines are often prepared with altered "analog" peptide antigens that have been optimized for HLA class I binding, in order to enhance their immunogenicity. An obvious but crucial point is that structural modifications of peptides should not alter TCR αβ repertoires or TCR binding properties to ensure that vaccination-primed T cell clonotypes remain highly specific for the natural antigen and efficiently recognize tumor cells. Recently, an analysis revealed that T cell repertoires generated against native or analog Melan-A/MART-1 peptides following vaccination exhibited slightly distinct but otherwise overlapping and structurally conserved αβ TCR features.

It has been proposed that CD8 T cells directed against

tumor antigens express TCRs of lower affinity/avidity for their antigenic ligands than pathogen-specific T lymphocytes. A viable approach to improving adoptive cell transfer cancer therapy utilizing TCR gene transfer is to modify TCR sequences in order to increase affinity for cognate tumor antigen epitopes. Various strategies have led to the generation of TCR variants against the NY-ESO-$1_{157-165}$ epitope with supra-physiological affinities up to the nanomolar and picomolar range. Although such variants showed enhanced T cell function, it also led to complete loss of target cell specificity. At present, the major challenge is not to increase the affinity of any given self (tumor) antigen TCRs but in fine tuning and optimizing TCR affinity step-by-step. The third aspect of the research, performed in collaboration with Olivier Michielin's laboratory, involves the generation of a panel of sequence-optimized TCR variants for the tumor antigen NY-ESO-$1_{157-165}$ with affinities lying within physiological boundaries to preserve antigenic specificity and avoid cross-reactivity. Primary human CD8 T cells transduced with these TCR variants demonstrated robust correlations between binding measurements of TCR affinity and avidity, and the biological response of the T cells, such as TCR cell surface clustering, intracellular signaling, proliferation and target cell lysis. The data point to the existence of a TCR affinity threshold above which T cell function was not enhanced. It was proposed that the affinity of newly designed self-specific TCRs may not need to be optimized beyond a given affinity threshold to achieve optimal T cell functionality.

Identification of dominant tumor specific T cell clonotypes and detailed characterization at the single cell level are key steps to understand better the relation between structural and functional features of TCRs. The approach of T cell analysis by the group is much more extensive than that usually done in humans or even in animal models, and should provide key information for the development of immunotherapy against cancer and/or infections. These scientific projects dovetail with on-going immunotherapy-oriented studies carried out by Daniel Speiser, Pedro Romero and Olivier Michielin.

MOLECULAR ONCOLOGY AND DRUG DESIGN

Olivier Michielin, M.D., Ph.D.

Rational protein design and fragment based drug design are two approaches being developed by the group as new agents for cancer therapy. New developments in free energy simulations, like the MM-GBSA method, allow the *in silico* computation of the binding contribution of the different amino acids of proteins in a complex. These methods have been applied to the TCR-p-MHC system to perform rational optimization of the CDR loops to increase affinity for the NY-ESO-1 peptide presented by HLA A2 molecules. Simulations suggested 24 single mutations subsequently introduced into soluble TCR expressed using a bacterial system. Biacore measurements were performed to confirm the impact of the mutations on both the kinetic and thermodynamic properties of the TCRs. Using this approach, a remarkable correlation was obtained between the measured affinity and predicted binding free energy change (r=0.81), validating the strategy of producing optimized TCRs.

In addition, the simulations can also determine which mutations are compatible, allowing optimal combinations to be made. Using this approach the group increased the TCR affinity by 157 fold compared to WT. The impact of these TCR modifications on T cell function was evaluated in Nathalie Rufer's group, showing that both tumor cell proliferation after antigenic challenge and tumor cell killing were significantly improved. Based on these encouraging results, clinical trials in metastatic melanoma patients are currently being designed.

Fragment based drug design represents a very attractive alternative to high throughput methods, with

a potentially higher success rate for active compound generation, allowing successful application in the academic environment. To perform fragment based drug design, the group developed its own algorithm, EADock, based on an elaborated evolutionary algorithm and accurate force field that includes rigorously solvation effects. This approach has recently been used to tackle indoleamine 2,3-dioxygenase (IDO), an important target involved in tumor immune escape. Using EADock, around 1,500 molecules were assembled *in silico* and the best 120 were synthesized in house. Fifty percent were shown to be active *in vitro* using a standard enzymatic inhibition assay. The best activities obtained so far are around 200 nM. Most of the compounds have been tested in cellular assays in collaboration with Benoît Van den Eynde from the Brussels Branch, confirming their potency in many instances. Mouse experiments are ongoing. Successful candidates could be pushed towards phase I clinical trials in the future.

CLINICAL-TRANSLATIONAL ONCOLOGY

Daniel Speiser, M.D.

Research efforts are concentrated on T cell immunity in cancer patients and performing investigator initiated clinical phase I/II trials of T cell vaccination. Biological and clinical efficacies are assessed, with the aim of identifying vaccine components that are most efficient in generating strong functional CD8 and CD4 T cell responses against cancer. The group has applied novel forms of antigen (short and long peptides, virus-like particles), with two different types of CpGs, MPL, QS21 or Klebsiella outer membrane protein A as adjuvant. This step-by-step approach has allowed development of the most powerful existing synthetic human T cell vaccine, composed of peptide antigens, CpGs and Incomplete Freund's Adjuvant. Detailed cellular and molecular studies of elicited immune responses have provided essential results promoting understanding of immune biological mechanisms, and allowing continuous further optimization of active immunotherapy.

Active immunization is particularly promising in early stages of disease to prevent tumor relapse and perhaps, primary prevention. However, progress must rely on studies with biological endpoints, because large phase III trials are rarely done considering the enormous cost, number of patients and years to complete. Active immunization may be well suited as a combination therapy with various conventional cancer treatments, or with small molecules targeting specific pathways in cancer and inflammatory cells.

A recent technical innovation made it possible to perform DNA microarray analysis of antigen specific (tetramer+) T cells sorted directly *ex vivo* from patient blood and tissue samples. Careful comparison of self- vs. non-self specific T cells identified molecular pathways responsible for the reduced protective potential of self-specific T cells. Deficient functional competence is particularly pronounced in T cells from metastases, where the group found a novel "split T cell tolerance", characterized by a marked deficiency of cytokine production but surprisingly efficient cytotoxic function. The underlying mechanisms are currently being elucidated.

A new inhibitory pathway was identified that is particularly abundant in cancer specific T cells. This occurs when CD28 family member BTLA (B and T lymphocyte attenuator) interacts with its ligand HVEM (herpes virus entry mediator) expressed by tumor cells *in situ*. Immunotherapy, with CpG based vaccines but not other vaccines, induced BTLA downregulation and made T cells partially resistant to this inhibition. Overall, CpG based vaccine induced circulating cancer/self-specific T cells were remarkably competent *in vivo*, multifunctional, and showed effector cell differentiation highly similar to virus specific T cells.

Advanced effector T cell differentiation was characterized by

downregulation of the costimulatory molecules CD28 and CD27. Working with Nathalie Rufer's group, this occurrence was discovered in conjunction with marked expansion of co-dominant T cell clonotypes, expressing T cell receptors (TCRs) with increased avidity for antigen-positive target cells. Vaccination with a "weak" natural tumor/self peptide induced T cells with stronger tumor reactivity (higher TCR avidity and stronger T cell activation) than vaccination with an analog peptide, despite weaker binding to HLA. These findings emphasize the need to generate T cell responses with high affinity TCRs to tumor/self antigens, a major challenge for the future success of T cell based therapy of cancer.

Positive conclusions can be drawn from studies of long term T cell activity over several years. Astonishingly, repetitive vaccinations induced sustained T cell responses with continuously functional effector T cells, and persistence of co-dominant clonotypes. In contrast to other studies, loss of clonotypes was rare and occurred due to passive attrition (competition) rather than active attrition (cellular senescence). Vaccination induced sizeable T cell responses in patients aged 60 to 80, with no significant difference, indicating that immunotherapy is feasible for all generations. In summary, CpG based cancer vaccines lead to sustained activity of competent T cells and are able to overcome many of the obstacles that contribute to the immuno-deficiency of tumor specific T cells.

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Director's Message

Our Branch research programs were enhanced during 2009 by a number of key appointments, discoveries and translational science achievements. The appointment of A/Prof John Mariadason as head of the Oncogenic Transcription laboratory will allow research into transcriptional regulation of cancer to complement our basic and clinical research activities. We had considerable success in peer-reviewed grants, including A/Prof Weisan Chen being part of a successful $10.4 million NHMRC Program Grant exploring vaccine immunology, which also included Nobel Laureate Prof Peter Doherty. The licensing of mAb806 to Abbott Pharmaceuticals was a major highlight of 2009, and culminated over 10 years of dedicated research, and collaboration with the LICR New York and San Diego Branches. Our publications in major basic and clinical research journals included important discoveries in T cell immunology, melanoma biology, structural studies of immunoglobulins, and lead author publications in colon cancer, urological cancer, and PET imaging clinical trials that have contributed to regulatory approvals of novel drugs and PET indications in the USA and Australia.

A number of our scientists / clinicians continue to have major roles in Australian and international research committees, clinical co-operative trial groups, and journal editorial boards. Participation in LICR global vaccine and antibody programs remains a strong focus of our activities, consistent with our commitment to translational science and extending LICR global discoveries into the clinic. During 2009 we held the inaugural LICR Translational Oncology Conference, together with the LICR Melbourne-Parkville Branch,

which was a major success, and brought together international and local speakers and attendees to address this important area of cancer research.

The partnership between LICR and Austin Health to provide routine cancer care, and innovative clinical research, for cancer patients is a unique endeavor and continues to be a major success. We are working with Austin Health on the establishment of the Olivia Newton-John Cancer Centre, which will be a comprehensive cancer center providing a full range of clinical services and research facilities, including our Branch administration and laboratories. These initiatives will enhance our research capacity, and enable our discovery and clinical programs to flourish and make substantial contributions to our understanding and control of cancer.

Andrew M. Scott

JOINT AUSTIN LUDWIG ONCOLOGY

Jonathan Cebon, M.D., Ph.D.

The unit was established in 1991 with the aim of providing the very best cancer care and conducting high quality clinical cancer research in an integrated environment. A unique partnership between Austin Health and LICR has been forged to achieve this with Austin Health providing clinical care and LICR providing clinical research opportunities. This relationship enables patient access to trial protocols sponsored by the Institute. The unit has become one of the premiere academic oncology units in Australia and supports multidisciplinary clinics for the management of patients with cancers of the lung, breast, gastrointestinal tract, and genitourinary tracts as well as specialized clinics for patients with melanoma, hepatocellular carcinoma, lymphoma and neuro-oncology. At any one time a substantial number of clinical trials are conducted with much of this effort being directed towards LICR studies, particularly in the field of cancer immunotherapy. Clinical and laboratory science are integrated in an iterative bench-to-bedside and back again program. Substantial research focus supports the careers of academic oncologists each of whom now has a high national and international profile in their respective subspecialty. Additionally, the unit trains advanced physician trainees specializing in Medical Oncology and clinical research fellows. Physicians graduating the unit often go on to undertake higher degrees and establish academic careers in Australia and abroad.

Immunotherapy studies are performed under the auspices of the Cancer Vaccine Collaborative (CVC), an international collaborative group of scientists and physicians supported by the Cancer Research Institute (New York) and LICR. During 2009 this has included an ongoing evaluation of the clinical and immunologic efficacy of the full-length recombinant NY-ESO-1 protein formulated with ISCOMATRIX® adjuvant in patients with advanced melanoma. Immune responses against NY-ESO-1 were found to be attenuated in patients with metastatic melanoma when compared to the unit's previous experience in patients with fully resected cancer. This was associated with a significantly higher proportion of circulating regulatory T cells (Tregs), thereby suggesting a mechanism for tumor-induced systemic immune suppression. This is being further studied by adding cyclophosphamide to the vaccine schedule as an approach for reducing Tregs. In a parallel study with Ipilimumab (a monoclonal antibody that blocks the immune-inhibitory interaction between CTL antigen 4 (CTLA-4) and T cells) in patients with advanced melanoma, detailed studies were undertaken to characterize the immune underpinnings of a complete clinical remission in one patient and found that the antibody expanded circulating and tumor-infiltrating Melan-A-specific T cells. The simultaneous development of an acute vitiligo-like infiltrate in skin demonstrated that anti-tumor immunity and autoimmunity were both mediated by cytotoxic lymphocytes directed against the same melanocyte differentiation antigen, MelanA.

The unit has continued to provide a national and international leadership in clinical research into urological and gastrointestinal cancers. A/Prof Ian Davis established and chairs the recently formed the Australian and New Zealand Urogenital and Prostate (ANZUP) cancer clinical trials group, a cooperative group involved in genitourinary cancer, comprising hundreds of individuals from all disciplines, including basic science. He initiated the SORCE trial (adjuvant sorafenib in renal cell carcinoma), and was study chair for Australia and New Zealand, the highest accruing group outside the UK. He was second author on two pivotal pazopanib trials published in the Journal of Clinical Oncology, one of which led to US registration of pazopanib in renal cancer. Studies in metastatic castration-resistant prostate cancer have included an evaluation of gefitinib,

an oral signal transduction inhibitor of the epidermal growth factor receptor tyrosine kinase.

Niall Tebbutt, a medical oncologist, has been a strong supporter of collaborative studies run by the Australasian GastroIntestinal Trials Group (AGITG). He has been the study chair for five national trials, including the MAX (Mitomycin C, Avastin and Xeloda) study in advanced colorectal cancer, the largest study ever undertaken by the AGITG and the first international study run by the group. In 2009, the final results were presented at the American Society of Clinical Oncology (ASCO) meeting and the European Society for Medical Oncology meeting. The related manuscript has been accepted for publication in the Journal of Clinical Oncology. He was previously a member of the Scientific Advisory Committee for the AGITG and in 2009 was also elected to the Board of Directors.

In 2009, the unit also completed accrual to the DUX study, an investigator initiated trial of combined biologic blockade of EGFR signaling by Cetuximab and Erlotinib in chemotherapy refractory colorectal cancer. Preliminary results from this study will be presented at the ASCO 2010 annual meeting by DMedSci student, Dr. Andrew Weickhardt, who will receive an ASCO Merit Award for the abstract.

CANCER VACCINE

Jonathan Cebon, M.D., Ph.D.

The laboratory integrates clinical and laboratory teams in order to better understand immune responses against cancer and to develop new cancer therapies based on this knowledge. Three research streams have been developed in order to achieve this: cancer antigens (with a focus on NY-ESO-1), melanoma stem cells and dendritic cell (DC) biology.

The Cancer-Testis antigen (CTAg) NY-ESO-1 is an attractive target for therapeutic cancer vaccination based on its highly restricted expression pattern in cancers, with very limited expression in normal tissues, and its ability to induce both cytotoxic lymphocyte and humoral immune response. The laboratory has examined the requirements for the killing of melanoma cells by cytotoxic T lymphocytes that specifically recognize a number of NY-ESO-1 epitopes. It was found that T cell recognition of NY-ESO-1 epitopes did not necessarily correlate with the level of NY-ESO-1 expression within the tumor. Furthermore, the peptide sequences of epitopes generated by vaccination can differ from those that are present as targets on tumor cells. This mismatch may explain why vaccines that generate immunity against cancer targets may not eradicate it. To investigate this further three epitopes have been studied in detail and differences in the intracellular processing of these epitopes were identified. Ongoing research will help us understand the endogenous processing pathways that lead to NY-ESO-1 epitope generation on tumor cells and antigen-presenting cells of the immune system.

Another requirement for effective vaccination is that the quality of CD4+ and CD8+ T lymphocyte responses should favor tumor elimination. Yet it is now recognized that tumor cells have the ability to recruit regulatory T lymphocytes (Tregs) which suppress immunity. In patients with advanced melanoma a significantly higher proportion of circulating Tregs was found compared to those with minimal residual disease or healthy controls, suggestive of tumor-induced systemic immune suppression. This association between the stage of melanoma progression, and the number of Tregs in the blood was further related to reduced clinical and immunological efficacy of a vaccine that targets NY-ESO-1. In ongoing studies, in conjunction with the T Cell laboratory, the group is evaluating the quality of vaccine-induced immunity against NY-ESO-1. Future vaccine trials will need to monitor such responses in order to develop strategies to counter induction of antigen-specific Tregs.

Recent work has found that the expression of CTAgs (MAGE-A1, MAGE-A4 and NY-ESO-1) in cutaneous stage II primary melanoma was associated with poorer prognosis, comparable in magnitude to Breslow thickness, ulceration and mitotic rate. Time to relapse was a median of 45 months compared with 72 months for those patients whose tumors did not express these CTags. It is not known if this difference is simply an association, or that CTAgs confer an advantage to the tumor, possibly through mechanisms that protect it from p53-mediated apoptosis. This disadvantage for melanoma-bearing patients was not seen for overall survival and the normalization of survival occurred over the time-frame that was associated with the development of spontaneous anti-cancer immune responses against NY-ESO-1. It was proposed that this is circumstantial evidence for immunity modifying the natural history of melanoma.

Stem-like cells in melanoma may be responsible for treatment failure. Tumor cells display phenotypic and functional heterogeneity, yet treatments are often directed at defined targets. Such treatments will fail if these targets are absent on cells with lethal potential. Subpopulations within melanoma cell lines have been characterized in a collaboration funded by the Melanoma Research Alliance and NHMRC.

Initial studies with the stem-cell markers have shown that clonogenicity *in vitro* can be defined by CD133 yet any effect appears to be lost when cells are transferred into an *in vivo* xenograft tumorigenicity model. This highlights that melanoma cells are very 'plastic', and the importance of environmental signaling on determining cellular behavior. In more recent studies the group has identified cellular subpopulations based on functional assays, and are using genomic approaches to identify potential targets and better understand gene regulation in these cells.

Previous work from the laboratory showed that activated human DCs produce considerable activin-A, a TGF-superfamily member, which autoregulates DC function. It is accepted that natural killer (NK) cells and DCs and play important roles in the innate and adaptive immune responses. Within the past year, the laboratory demonstrated that NK cells express type I and II activin receptors and that activin-A triggers NK-cell Smad 2/3 signaling. These findings provide evidence for an immune regulatory role of activin-A during DC-mediated NK-cell regulation, which suggests that antagonizing activin-A signaling *in vivo* may enhance NK cell–mediated immune functions and adaptive immunity.

T CELL

Weisan Chen, Ph.D.

Research within the laboratory is geared towards improving vaccine development. Activities are divided between human T Cell research and using models of influenza infection and tumor antigen systems to elucidate the mechanisms associated with immunodominance, the interaction between T cells with different antigen specificity, the interaction between T cells and antigen-presenting cells, and methods to enhance cross-presentation of tumor antigens. In addition, the laboratory continues to play an important role in the Institute's global Cancer Vaccine Program, monitoring patient T cell responses to cancer vaccines from clinical trials conducted at the LICR Melbourne-Austin Branch.

In the human system, the laboratory has been focusing on the discovery of antigenic epitopes associated with cancer testis antigen NY-ESO-1. Many novel CD4+ and CD8+ epitopes have been identified in melanoma patients either naturally occurring immune responses, or responses following vaccination with NY-ESO-1 formulated in ISCOMATRIX™ adjuvant (NY-ESO-1/ISCOMATRIX™). The laboratory has shown a clear HLA-dependent immunodominance hierarchy of T cell responses in these patients.

Such knowledge will guide the improvement of vaccine designs and implementation of more efficient T cell monitoring for future vaccinated patients.

Regulatory T cells (Tregs), a specialized T cell sub-population involved in tolerance to self antigens, are also a focus of the laboratory. Tregs have been recognized to play a major role in attenuating anti-tumor immunity and may contribute to the failure of the vaccines in advanced disease patients. Because Tregs generally do not produce measurable cytokines upon activation, the laboratory showed that down regulation of surface CD3 can be used as a novel method for identifying activated Tregs and normal effector T cells (Teffs) responding to the tumor antigen NY-ESO-1. Using this approach, it was found that vaccination of melanoma patients with NY-ESO-1/ISCOMATRIX™, which was designed to induce anti-tumor effector T cells, could actually enhance and stimulate antigen-specific Treg responses in patients with advanced malignant melanoma. Induction of tumor antigen specific Treg may be a novel mechanism to explain the lack of efficacy of immunotherapy based on whole tumor antigen vaccines. The laboratory is in the process of exploring whether these antigen-specific Tregs are able to suppress Teffs, especially antigen-specific Teffs.

Other recent findings from the T-cell laboratory include the observation that expression of FoxP3 by tumor cells and virally transformed cells may allow them to evade immune destruction through immune suppression. A novel FoxP3 splice variant in tumor cells was discovered and patented. Additional data suggest that FoxP3 expression in non-Treg cells, such as in the placenta, brain and testis may convey suppressive activity to the immune system.

Using murine model systems, the laboratory is investigating immunodominance, especially the observed immunization route-dependent immunodominance hierarchy through use of an influenza A virus (IAV) model to explore CD8+ and CD4+ T cell responses. Preliminary evidence indicates potential involvement of tissue-specific dendritic cell subsets and their role in mediating differential antigen presentation. The acquired knowledge should be beneficial to future vaccine design.

Using SV40-transformed allogeneic tumor cells, the laboratory has also shown that IAV-infection of these tumor cells enhanced cross-priming of naïve CD8+ T cells (T_{CD8+}) response to tumor antigens. This enhanced cross-priming was found in part to involve Toll-like receptor 7 (TLR7-), but not TLR3-, mediated sensing of IAV and is entirely dependent on MyD88 and IFN signaling pathways. Importantly, plasmacytoid dendritic cells (DCs) and $CD8\alpha^-$ DCs were shown to enhance cross-priming when provided in co-culture, whilst the inflammasome and IFN-γ were not involved in such enhanced priming. It was further demonstrated that $CD8\alpha^+$ DCs were the only antigen-presenting cells able to cross-prime TCR transgenic T_{CD8+}. These observations suggest that IAV-infection of tumor cells may facilitate cross-presentation of tumor antigens and may be used as clinical vaccine therapeutics.

The T-Cell laboratory also reported that the immunoproteasome, originally thought to be dedicated immune-enhancing protein degradation machinery, also plays a 'house-keeping' role. It is clear that mice with deficient immunoproteasomes show impaired B cell differentiation, reduced B cell number and defective B cell function, such as diminished antibody response to IAV and antibody class switching. Studies are following up a similar observation for T cells in these mice. It appears thus far that the nuclear factor kappa B (NFκB) function is impaired in the immunoproteasome deficient mice, which lead to impaired thymocytes differentiation, specifically the transition from DN3 to DN4 stage. Work continues to elucidate the mechanisms along the NFκB signaling pathways.

ONCOGENIC TRANSCRIPTION

John Mariadason, Ph.D.

The laboratory investigates the transcriptional changes that drive colon cancer progression, with particular focus on gene expression regulation mediated by the histone deacetylase (HDAC) family of transcriptional co-repressors. The laboratory also actively investigates the transcriptional mechanisms by which histone deacetylase inhibitors and other epigenome-modifying drugs induce apoptosis in tumor cells, with the goal of identifying biomarkers predictive of response to these agents.

HDACs alter gene expression by catalyzing the deacetylation of DNA-bound core histones, a conformational change that decreases accessibility of the basal transcriptional regulatory machinery to DNA, and in transcriptional repression. HDACs also deacetylate sequence-specific transcription factors, which can either increase or decrease their transcriptional activity. HDACs can also elicit cellular effects in a transcription-independent manner by deacetylating cytoplasmic proteins such as Hsp90, and tubulin. Eighteen mammalian HDACs have been identified to date, which are categorized into one of three classes based upon homology to a prototypical HDAC found in yeast. Previous discoveries from the laboratory include the observation that a number of class I HDACs (HDAC1, HDAC2 and HDAC3) are upregulated in colon cancers. Furthermore, the laboratory has demonstrated that these class I HDACs, as well as the class II HDAC, HDAC4, play a pro-proliferative, pro-survival function in colon cancer cells, in part through transcriptional repression of the cell cycle inhibitor, $p21^{WAF1}$. Currently, the laboratory is addressing the role of these HDACs in colon cancer progression *in vivo* through the development of mouse models in which specific HDACs are overexpressed or inactivated in the intestinal epithelium of tumor-prone *Apc* mutant mice. The role of Wnt signaling in the transcriptional regulation of specific HDACs is also being pursued.

Recently, the laboratory also gained new insight into the molecular mechanism by which commercially developed HDAC inhibitors, such as SAHA (vorinostat), induce apoptosis in colon cancer cells. Vorinostat is approved for the treatment of cutaneous T-cell lymphoma and is presently undergoing clinical trials for treatment for a variety of hematological malignancies and solid tumors, including colon cancer. Through screening a large panel of colon cancer cell lines, the group identified cell lines highly sensitive or resistant to HDAC inhibitors (HDACi). Comparison of the transcriptional changes induced by HDACi in sensitive and resistant cell lines identified a set of 48 genes, comprising multiple immediate-early and stress response genes, preferentially induced in sensitive lines. Further analysis demonstrated that this HDACi-induced transcriptional response and subsequent apoptosis is largely mediated through activation of the Sp3 transcription factor. Current work is focusing on determining whether HDACi-induction of this transcriptional response is universally observed across multiple tumor types, particularly hematological malignancies such as cutaneous T-cell lymphoma in which HDACi are clinically utilized.

A separate focus of the laboratory is the development and application of high throughput genomic technologies including gene expression profiling, methylation profiling and mutation screening, to identify novel regulators of colon cancer progression and for biomarker discovery. Using these approaches a number of observations have been made relating to the distinct molecular etiology of the two major subclasses of colon cancer, microsatellite stable (MSS) tumors and colon tumors with microsatellite instability (MSI). Specific recent findings include the identification of a mutation in a polyA(13) repeat within the 3'UTR of epidermal growth factor receptor (EGFR) in MSI colon cancers, which have been linked to increased

EGFR mRNA stability and expression.

Finally, with regards to the discovery of biomarkers predictive of drug response, the laboratory recently demonstrated that colon cancer cell lines harboring activating mutations in the *PIK3CA* gene, or lacking expression of PTEN, are inherently resistant to the EGFR inhibitor, cetuximab. These findings are presently being extended to patient samples in collaboration with members of the Joint Austin Ludwig Oncology Unit.

TUMOUR TARGETING

Andrew Scott, M.D.

The laboratory undertakes research focused on the targeting and molecular imaging of tumors, and exploring receptor based signaling pathways responsible for cancer cell growth. Immunological approaches to enhancement of cell killing mechanisms of recombinant antibodies are being pursued, and clinical trials of novel antibodies, small molecules and imaging ligands are also being undertaken.

The laboratory has a major focus on the biology and signaling pathways involved in erbB family members of receptors. The development of the monoclonal antibody 806, which targets a conformationally exposed epitope of erbB1 (EGFR), has been successfully extended to the clinic and confirmed the tumor selectivity and therapeutic potential of this antibody. The group has completed the generation of a humanized form of 806, in collaboration with the LICR New York Branch, and LICR affiliate Prof Christoph Renner in Zürich. MAb806 has been licensed to Abbott Pharmaceuticals, and over the last year the group has engaged with Abbott regarding the clinical development of 806, as well as continued research into the mechanism of action of 806. Through a collaboration with Prof Tony Burgess (LICR Melbourne) and A/Prof Tom Garrett (WEHI), the crystal structure of 806 and EGFR extracellular domain has been identified and published in *PNAS*, providing new insights into erbB receptor structural biology. The research of the laboratory has extended to identifying similar conformational epitopes in other erbB receptors, and generating and characterizing novel antibodies against these targets.

The interaction of IgG Fc with FcRn and FcγR plays a pivotal role in the pharmacokinetics and immune effector function of recombinant antibodies. Hu3S193, a humanized antibody against the Le^y antigen, is being used as a model for research into Fc function through homology modeling, crystallography and site directed mutagenesis. Key amino acids responsible for Fc:FcRn interaction have been identified, and a series of single and double amino acid mutant constructs have been demonstrated that retain antigen binding and CDC / ADCC activity, but have faster serum clearance *in vivo*. The crystal structure of the CDR region of hu3S193 Fab, and the impact of Zn ions on structure / binding, was identified and published in *PLoS One*. Molecular imaging of mutant construct biodistribution in mouse models with PET/CT has also been demonstrated. Experiments aimed at optimizing the ephA3 receptor binding antibody IIIA4 are also being performed, including the exploration of the biochemical and biologic sequelae of EphA3 function in mouse models.

Through a collaboration with LICR New York and scientists at the City of Hope, USA, the laboratory is exploring the ability of siRNA linked to hu3S193 to abrogate intracellular signaling pathways in tumor model systems. Successful knockdown of signaling pathway targets including STAT3 have been demonstrated. Nanoparticle approaches to payload delivery of toxins / drugs to cancer cells are also being studied with polymer nanocapsule systems.

The clinical trial program is conducting a protocol with anti-CAIX antibody cG250, combined with Sutent® (Sunitinib malate), in patients with advanced or metastatic

renal cell carcinoma (RCC). This trial is exploring the molecular expression of CAIX, and targeting of RCC by cG250 in these patients, and the efficacy of the combination treatment is also being evaluated.

Molecular imaging of tumor metabolism, and evaluation of new therapy pharmacodynamics, remains a major focus of the research program. Studies into hypoxia in metastatic colorectal carcinoma, utilizing ^{18}F-FMISO Positron Emission Tomography (PET), have demonstrated patterns of low oxygenation in a subset of tumors, which are being further evaluated for protein / gene expression changes. Molecular imaging of hypoxia and proliferation is also being performed in patients with head and neck, glioma, RCC, lung cancer and other tumors, as part of prospective studies exploring new treatment modalities and understanding the prognostic ability of PET in these patients. Radiotherapy treatment planning has been shown to be more accurate, and with improved outcomes, by utilizing ^{18}F-FDG PET and ^{11}C-choline in patients with mesothelioma, prostate cancer and lung cancer. The group has demonstrated significant management change (25%-50%) and improved treatment outcomes as a result of ^{18}F-FDG PET in large prospective studies of patients with colorectal, head and neck, and gastro-esophageal cancer (over 900 patients accrued), and these results have contributed to public funding approval for these PET indications through Australian Medicare during 2009. The laboratory has extended animal model PET/SPECT/CT/Doppler/2D photon microscopy platforms to the investigation of nanoparticle biodistribution, antibody pharmacokinetic and pharmacodynamic studies, and the development of novel PET hypoxic probes.

URO-ONCOLOGY

Ian Davis, M.D., Ph.D.

The laboratory is studying cancers related to the urinary system: kidney, prostate and bladder cancers. These cancers are highly prevalent in Australia, but current treatments are not ideal. As the group begins to understand the basic biology of these cancers more clearly, it is possible to discover new potential targets for treatment. The laboratory has very close interactions with the surgeons and cancer specialists who care for these patients and is examining the processes underlying the causes and growth of these cancers. Research in the laboratory is specifically concentrating on the nature of interactions between the cancers and their environments, including stromal cells and the immune system, the biology of cancer stem cells, how renal cell cancers use signaling pathways and become resistant to signal inhibitors, and how these processes correlate with the clinical outcomes and responses to treatment observed in patients.

Currently, little is known about the mechanisms that cause prostate cancer to metastasize, and why bone is the preferred site. Whilst numerous studies have identified bone-derived factors (including cytokines and extracellular matrices) that influence prostate cancer cell biology, these studies have almost exclusively been performed using a limited number of cell lines. Over the past year, a major achievement of the Uro-Oncology research team has been to establish methods to isolate various populations from single cell suspensions of primary prostate cancer samples from patients that have undergone radical prostatectomy. The laboratory routinely obtains approximately 500,000 prostate cancer epithelial cells from 70-200mg tissue. These cells are now being used to study the behavior of prostate cancer in response to bone-derived stimuli in an effort to investigate the preference for metastatic disease in bone. Thus far, paracrine effects of numerous bone-derived factors have been identified and potential autocrine signaling through IL-8, BMP2, and BMP4. These freshly-isolated primary prostate cancer cells are also being processed for gene expression analysis or being assessed using a suite of functional assays that have

been developed within the laboratory in order to monitor cell proliferation, survival, motility, and adhesion in response to bone-derived factors.

The involvement of cancer-testis antigens (CTAgs) in urologic cancers is also a focus of laboratory research. CTAgs are considered targets for immunological treatment of cancer. CTAgs are often expressed heterogeneously in cancers, or at low levels, which may compromise the efficacy of therapeutic targeting. CTAg gene expression is silenced by DNA methylation. Treatment with 5-Azacytidine (Aza) demethylates DNA and has been shown to increase expression of CTAg in various cancer types, including melanoma and ovarian cancer lines. Aza may also increase CTAg expression in bladder cancer, thereby potentially increasing susceptibility to CTAg-specific or non-specific immunotherapy. These *in vitro* studies have determined CTAg upregulation appears to be both dose and schedule dependent which has implications for *in vitro* use of Aza to upregulate CTAg expression for immunotherapy. A clinical protocol is currently under development.

Clinical programs from the laboratory involve collaborators within the Centre for PET and Department Radiology and are exploring the use of ^{18}F-fluorodeoxyglucose and ^{11}C-choline (CHOL) positron emission tomography (PET) scanning in men with intermediate or high risk localized prostate cancer. This research involves two clinical trials for men with prostate cancer that is believed to be confined to the prostate. One study is for men planned for surgery (intermediate risk) and the other for men planned for radiotherapy (high risk). The projects evaluate the utility of PET scanning compared to other standard assessments, its potential contribution to making treatment decisions and whether it can be used to monitor the results of treatment. The group has confirmed the feasibility of the approach and has had excellent support from colleagues in all professional disciplines involved in the project. As of the end of 2009, 15 patients had been enrolled in the radiotherapy study and 12 patients for the surgical study. Preliminary results indicate that CHOL PET scanning identifies the dominant intraprostatic lesion, correlating well with conventional imaging and histopathology, and that data from the PET scans can be used for radiotherapy treatment planning.

The laboratory is involved more broadly in the Austin Uro-oncology Group (AUrOG), representing urology, medical oncology, radiation oncology, endocrinology, clinical pharmacology, pathology, radiology, nursing, allied health, palliative care and psychology. AUrOG is involved in numerous clinical trials and a broad range of basic and translational research linked at various levels. Examples include a renal cell carcinoma database, a population-based prostate cancer registry, a comprehensive men's health initiative, and leadership in multiple national and international clinical trials.

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Director's Message

2009 was a significant year. It marked the end of the era for Tony Burgess as the inaugural, visionary director who established the Branch in 1980 with a group of six people. Twenty nine years later, when Tony stepped down on July 14 to focus his efforts on his laboratory science, the Branch had become one of the Institute's largest sites with a proud record of achievements and one of the jewels among Australia's medical research institutes. Tony was also the inspirational leader who paved the way for our new and exciting future as part of the Australian Federal and Victorian State Governments-funded $1 billion Victorian Comprehensive Cancer Centre (Victorian CCC). With the signing of the Victorian CCC Joint Venture Agreement attended by the Institute's president, Mr. Edward McDermott on November 11, the Institute re-emphasized its commitment to world class translational cancer research as one of the six founding members of this new center due to open in 2015. Together with our present host, the Royal Melbourne Hospital, and long-time collaborative partners, the Peter MacCallum Cancer Centre, Walter and Eliza Hall Institute and Melbourne University, the Branch will be physically embedded in the Victorian CCC and able to capitalize on basic and translational opportunities with the intent of delivering the best outcomes to cancer patients.

Scientists have continued to leverage the Branch's resources with numerous competitively-awarded research grants including the National Health and Medical Research Council (NHMRC) of Australia's A$20mio Colorectal Cancer Program Grant, the Victorian Cancer Agency's A$5mio Translational Cancer Research Grant and Biomarker Pilot Project (the latter co-funded by the State Government) alongside many other project grants. Several laboratories have strengthened interaction with the biotechnology and pharmaceutical industry, signing three collaborative research agreements.

The Branch's contribution to basic and translational research in the area of colorectal cancer continues to be recognized at the highest international levels with publications in *Cancer Cell, Nature Structural Biology, PNAS* and others, alongside numerous presentations at meetings, including the American Society of Clinical Oncology (ASCO).

Several scientists at the senior and junior level have received prestigious national and international awards for the quality and innovation of their research. Jeanne Tie was the recipient of the *Bradley Stuart Meller Merit* from ASCO and Tracy Putoczki a postdoctoral award from the *International Cytokine Society.* Marc Achen and Steven Stacker were recognized by NHMRC, Australia's largest granting body for exceptional research projects.

Andrew Clayton and the biophysics group left the Branch and we have begun the consolidation phase to strengthen efforts for colorectal cancer research by building on existing expertise and research activities that extend from gene discovery, basic research, and preclinical animal models to biomarker discovery and translational research spearheaded by the Institute's Colon Cancer Initiative.

Matthias Ernst

ANGIOGENESIS

Marc Achen, Ph.D. and Steven Stacker, Ph.D.

Tumors cannot grow beyond the size of a few millimeters without blood vessels that supply nutrients and oxygen. Accordingly, tumors and their surrounding tissue — the stroma — secrete growth factors to induce angiogenesis, the formation of new blood vessels. In a similar way, tumors also induce lymphangiogenesis, formation of new lymphatic vessels. Angiogenesis and lymphangiogenesis facilitate metastasis (cancer spread) since cancer cells that detach from a tumor can be dispersed through the body via the bloodstream or lymphatic vessels. Metastasis is the ultimate cause of most cancer deaths. Substantial efforts in cancer research are aimed at developing therapeutic means to prevent tumor growth and metastasis by controlling tumor-induced angiogenesis and lymphangiogenesis.

The pro-angiogenic vascular endothelial growth factor (VEGF)-D stimulates angiogenesis and lymphangiogenesis via VEGF receptors (VEGFR)-2 and VEGFR-3, expressed by endothelial cells lining the inner surface of blood vessels and lymphatics. In order to efficiently activate the receptors, secreted VEGF-D protein must be processed into an active variant. A number of proprotein convertases (PCs) - a type of protein-cleaving enzyme - were previously identified that mediate VEGF-D processing. This study was subsequently expanded showing that the proteolytic processing of VEGF-D is essential for its effects in promoting tumor angiogenesis and lymphangiogenesis, as well as growth of the primary tumor and metastatic spread to regional lymph nodes. These findings support the hypothesis that PCs, which promote the proteolytic activation of VEGF-D, and the structurally-related growth factor VEGF-C, may be useful targets for novel therapeutics designed to restrict the growth and spread of cancer.

The studies also explored the specific roles of VEGFR-2 and VEGFR-3 in cancer by targeting these proteins in a mouse xenograft model. This revealed that VEGFR-2 signaling is very important for enhancing the rate of primary tumor growth, but does not contribute significantly to lymph node metastasis. Interestingly, VEGFR-3 signaling was a key driver of lymph node metastasis and contributed significantly to primary tumor growth. These studies indicate both VEGFR-2 and VEGFR-3 receptors play important roles in tumor biology and confirm a range of other studies which indicate these receptors are attractive therapeutic targets in cancer. Furthermore, lymphatic vessels which respond to tumor-derived lymphangiogenic growth factors are small (capillary-like) lymphatics – hence the proximity of a tumor to small lymphatic vessels is likely to be a key determinant of metastatic spread.

CELL BIOPHYSICS

Andrew Clayton, Ph.D.

Epidermal Growth Factor Receptor (EGFR) is involved in stimulating the growth of many human tumors, but the success of therapeutic agents has been limited in part by interference from EGFR on normal tissues and partly because the activation processes after ligand binding is still incomplete. The epidermal growth factor receptor (EGFR) kinase is generally considered to be activated by either ligand-induced dimerisation or a ligand-induced conformational change within pre-formed dimers. The relationship between ligand-induced higher-order EGFR oligomerization and EGFR phosphorylation on the surface of intact cells was identified. The laboratory was able to combine lifetime-detected Forster resonance energy transfer as a probe of the receptor phosphorylation state and image correlation spectroscopy to extract the relative association state of activated versus unactivated EGFR. There are at least four times as many receptors in ligand-induced active clusters than inactive clusters. Contrary to the prevailing view that the EGFR dimer is the predominant, active form, the data determine

that higher-order EGFR oligomers are the dominant species associated with ligand activated EGFR tyrosine kinase.

Previously, the group reported an antibody (mab806) against a truncated form of EGFR found commonly in gliomas. This antibody also recognizes full-length EGFR on tumor cells but not on normal cells. A mutant form of EGFR (C271A/C283A) which binds mAb806 with 1:1 stoichiometry was constructed. The binding of mAb806 to the wtEGFR requires the epitope to be exposed during ligand induced receptor activation.

In order to detect cell surface receptors such as EGFR, the group has been collaborating with Paul Mulvaney's laboratory (Melbourne University) to develop a simple, economical method for generating water-soluble, biocompatible nanocrystals that are colloidally robust and have a small hydrodynamic diameter. These nanocrystals exhibit the same optical spectra as those formed initially in organic solvents, preserving their photoluminescence intensity. These nanocrystals are being modified to track the dynamics of EGFR in living cells.

COLON MOLECULAR AND CELL BIOLOGY

Joan Heath, Ph.D. and Matthias Ernst, Ph.D.

The laboratory is focused on understanding the genetic networks that contribute to gastrointestinal cancer by using *in vivo* models largely based on zebrafish and mice.

In the Heath laboratory, considerable effort has been invested over the past few years in the positional cloning of a panel of zebrafish intestinal development mutants that were identified during an ethylnitrosourea (ENU)-mutagenesis screen designed specifically to identify mutants with defects in endoderm organ development. Seven morphologically striking intestinal mutants have now been successfully cloned and underlying mutated genes have been found to encode interesting proteins that contribute to essential cellular functions, such as transcription, ribosomal RNA processing, mRNA splicing, nuclear pore formation and protein trafficking.

The group published a discovery that the highly apoptotic intestinal epithelium in the *flotte lotte* mutant is caused by a single point mutation that generates a premature stop codon in *elys*. The *elys* gene encodes a large nuclear pore component required at the end of every cell division to provide a platform for new nuclear pore formation. The *flotte lotte* intestinal epithelium is particularly susceptible to aberrant expression of this gene after three days of development; by then stocks of maternally-deposited wildtype *elys* mRNA, which have sustained the growing embryo during early development, have diminished to zero just as the intestinal epithelium enters its most proliferative phase. Similar arguments probably explain tissue-specific phenotypes of other intestinal mutants. *Setebos* and *titania* mutants show impaired growth and differentiation after four days of development resulting in a thinner, less-polarized epithelium than observed in wildtype. The intestinal epithelium fails to elaborate folds or plicae, the functional equivalent of crypts in the mammalian intestinal epithelium. The underlying mutations in *setebos* and *titania* reside in genes that are essential at different steps during the processing of primary rRNA transcripts, resulting in reduced levels of mature 28S and 18S rRNA species, respectively. The intestinal epithelial cells in both mutants contain very large autophagosomes, probably produced in an attempt to clear defective ribosomes. In *caliban*, which harbors a similar growth-arrested phenotype, a single point mutation resides in a gene that encodes an essential component of the minor class spliceosome, a large complex of small nuclear ribonucleoproteins (snRNPs) that plays an indispensable role in splicing of pre-mRNA molecules containing one or more minor class introns. Minor class introns occur very infrequently in the human

genome; are highly conserved throughout the vertebrate and invertebrate kingdoms; and are over-represented in genes required for information processing compared to housekeeping genes. The proto-oncogene *BRAF1* and several members of the *MAPK* family of genes contain at least one minor class intron, as do a number of tumor suppressor genes relevant to colorectal cancer (CRC) and several other cancers, such as *PTEN*, *PROX1* and *LKB1*.

Several of the human homologues of cloned zebrafish genes are aberrantly expressed in colorectal cancer. The group used qRT-PCR to compare mRNA levels of *AHCTF1 (Elys)* and the minor class splicing gene in thirty pairs of human colon and colorectal cancer tissue from the same patient and found them to be consistently deregulated in cancer. This has fueled interest in the concept that essential but non-oncogenic cellular proteins, such as components of nuclear pores, spliceosomes and ribosomal RNA processing machinery may represent relevant targets for cancer therapy.

Alongside these genetic studies in zebrafish, the laboratory is part of an active program aimed at developing nano- and micro-sized capsules capable of delivering cytotoxic drugs specifically to cancer cells. The group, which includes several nanotechnologists from the University of Melbourne, characterized the biodistribution of a variety of non-targeted, non-drug loaded capsules following intravenous injection into mice using conventional biodistribution techniques and non-invasive, whole animal imaging. Phagocytic clearance of capsules from the circulation by tissue resident macrophages of the liver and spleen remains the primary limiting factor to longer capsule circulation times. Ongoing efforts are focused on development of layer-by-layer (LbL) capsules with novel polymer chemistries and structures to provide 'stealth' properties to circumvent such clearance. In parallel, a variety of covalent, reversible covalent and adsorptive drug loading methods are in development for use in a number of established syngeneic and xenograft *in vivo* tumor model systems.

The focus of the Ernst laboratory is centered on understanding the molecular control of gastrointestinal epithelial homeostasis and tumorigenesis using reverse genetics in mice. Over the years, the laboratory has established an expertise in signal transduction through the canonical Wnt/β-catenin and IL6-cytokine family/Stat3 pathways. While there is ample evidence for a causal involvement of the former in gastrointestinal cancers, the latter has been suspected for linking inflammation, obesity and aging to cancer. To investigate the role for epithelial Stat3 signaling in promoting gastro-intestinal tumorigenesis, the laboratory has taken advantage of the various (compound) mutant mouse strains previously generated in the colon molecular and cell biology laboratory, and which carry mutations in the shared receptor subunit gp130 that directly affect down-stream Stat3 signaling with gain- and loss-of-function consequences. Excessive abundance of the gp130-cytokines IL6 and IL11 within the tumor microenvironment of inflammation-associated cancers results in excessive Stat3 activation within the neoplastic epithelium of the stomach and intestine. Accordingly, mutagen-induced tumor growth and multiplicity is reduced following intestinal epithelial cell (IEC)-specific Stat3 ablation, while its hyperactivation promotes tumor incidence and growth in a colitis-associated cancer model. Conversely, IEC-specific Stat3-deficiency enhances susceptibility to chemically-induced epithelial damage and subsequent mucosal inflammation, while excessive Stat3 activation confers resistance to colitis. The laboratory identified the capacity of Stat3 to mediate IL6 and IL11-dependent survival of gastrointestinal cells and promote proliferation through G1/S and G2/M cell cycle progression as the common tumor cell-autonomous mechanisms that bridge

chronic inflammation to tumor promotion. In light of emerging findings of abundant expression of gp130 cytokines in gastrointestinal tumors alongside accumulation of excessive Stat3 activation in cells of the tumor and its microenvironment, these findings are likely to have implications on the human disease. Potential therapeutic benefits are being explored by antibody-mediated cytokine depletion and functional interference with other components of the gp130/Stat3 signaling cascade as well as by pharmacologically targeting other signaling cascades to which Stat3-dependent tumors may have become "addicted".

There is considerable interest in genetic determination of tissue-specific signaling thresholds for canonical Wnt/β-catenin signaling that convert physiological to pathological outcomes. In order to foreshadow the impact of systemically acting potential future therapeutics, the laboratory is taking advantage of genetically modified mice that harbor different germline combinations of hypomorphic alleles encoding β-catenin or the gate-keeper protein Apc, which act as a positive and negative mediator of intracellular Wnt signaling, respectively. By genetically restricting intracellular β-catenin expression in the corresponding compound mutant mice, the laboratory is able to ascribe a genotype-to-phenotype relationship which directly correlates with the strength of canonical Wnt/β-catenin signaling *in vivo* and corresponding cells *in vitro*. Different permissive Wnt signaling thresholds are required for embryonic development of head structures, adult intestinal polyposis, hepatocellular carcinomas, liver zonation and development of natural killer cells. A homozygous *Apc* allele combination with signaling capacity similar to that in the germline of the Apc^{min} mice was identified which distinguishes whether co-morbidities in Apc^{min} mice arise independently of intestinal tumorigenesis that arises from somatic *Apc* loss-of-heterozygosity (LOH). These studies were expanded by targeting overexpression of the *de novo* DNA methyltransferase Dnmt3a to the intestinal epithelium to enforce epigenetic silencing of members of the Sfrp family of soluble Wnt antagonists. The resulting elevation of canonical Wnt signaling is largely insufficient to promote polyposis as is mono-allelic absence of Apc function in Apc^{Min} mice. However, when genetically challenged with the Apc^{Min} allele, Dnmt3a transgenic mice develop intestinal tumors independently of *Apc* LOH and the resulting tumors are associated with excessive nuclear β-catenin accumulation alongside activation of the Wnt-target gene *Axin2/Conductin*. These results suggest epistatic interactions between otherwise innocuous genetic and epigenetic events enable initiation and promotion of disease as a mechanism likely to play a role in human colorectal cancer, where elevated *DNMT3a* expression coincides with repressed *SFRP5* and enhanced *AXIN2/CONDUCTIN* expression.

EPITHELIAL

Tony Burgess, Ph.D.

The laboratory aims to improve the understanding of colon cancer biology and use this knowledge to improve treatments for colorectal cancer (CRC) patients. Research involves several major themes: Wnt ligands and their receptors; expression and role in colon cancer; role of CRC stem cell-specific molecules in maintenance of the tumor phenotype; structure/function analysis of APC; investigation of phosphorylated β-catenin; Phosphatidyl Inositol phosphate (PIP) associated proteins and colon cancer.

The Wnt family of ligands, through their receptors (Frizzled and LRPs), regulate multiple intracellular pathways important in development, tissue homeostasis and cancer. As activators of the APC/ canonical pathway, Wnt ligands and receptors are postulated to play a role in intestinal cancer; however the dearth of specific reagents to these molecules has hampered understanding of

their role in the physiology and pathology of the mammalian gut. The laboratory has developed Apc, axin and iso-form specific β-catenin monoclonal antibodies, reagents and target cell lines to determine Wnt ligand affinity and specificity for their cognate receptors and measured Wnt sensitivity of colon cancer cells with Apc, β-catenin, PI3K and BRaf mutations.

Intestinal tumors arise from the stem cell compartment through a series of mutations which allow cells to escape normal physiological controls. CRC stem cells share many characteristics with normal intestinal stem cells, such as self-replication and generation of more differentiated cell types. Accordingly, many cell lines derived from human CRC maintain many stem-cell characteristics and express molecules, such as LGR5, PHLDA-1, CD133, CD44 and Msi-1, which are normally restricted to intestinal stem cells and their early progeny. These antigens have been very useful as markers for identification of normal and cancer stem cells; however very little is known on the functional role these proteins play in the maintenance of the cancer stem cell phenotype. Using well-defined human CRC cell lines, the expression of these stem-cell markers by knockdown (RNAi) or inducible overexpression has been modulated. These experiments expanded understanding of 'stemness' associated with several colon cancer cell lines and the factors that control the balance between self-renewal and differentiation.

APC is a large (2843 aa) protein with a modular structure containing a series of predicted protein-protein interaction domains. In CRC, the APC protein is truncated losing a large part of the C-terminus of the protein. Even though APC is clearly an important molecule in colon cancer, little is known about its structure due to its large size and lack of homology with other proteins. A classical structure/function analysis to characterize the shape and intramolecular interactions of the full-length and truncated APC protein was used.

The current paradigm for how truncation of APC results in initiation of tumorigenesis is through failure to regulate cellular concentration of β-catenin by proteasome-mediated degradation within a complex known as the 'destruction complex'. A critical step in β-catenin regulation is the phosphorylation of serine and threonine residues in the N-terminus of β-catenin. Dysregulation of phosphorylation of β-catenin is proposed to result in its accumulation and subsequent activation of Wnt/β-catenin-target genes, which are important in gut development and in driving colorectal tumorigenesis. It was shown that the axin destruction complex is localized to cytoplasmic puncta and that formation of axin-puncta is required for axin associated β-catenin degradation. However, the subcellular distribution of phosphorylated β-catenin is different in colon cancer cells with mutated APC. Populations of N-terminally phosphorylated β-catenin not part of the destruction complex were detected.

Phosphatidyl inositol phosphates (PIPs) are important components of the plasma membrane and associated with several signal transduction systems in cancer biology. In recent years the laboratory has used synthetic PIP analogues to identify PIP binding proteins in colon cancer cell lines. These cell lines have different combinations of tumor suppressor and oncogenic mutations, often leading to significant perturbations in the levels and distribution of PI(3,5)P2, PI(4,5)P2 and PI(3,4,5)P3. Using affinity capture and proteomics, proteins which bind to the different PIPs as a result of oncogenic or growth factor stimulation were identified. Under investigation is the role of specific PIP binding proteins in maintaining the transformed state in colon cancer and extending analyses to study the role of phosphoinositol polyphosphates in colon cancer.

JOINT PROTEIN RESEARCH

Richard Simpson, Ph.D.

Epithelial-mesenchymal transition (EMT) describes a process whereby immotile epithelial cells escape structural constraints imposed by cellular architecture and acquire a phenotype characteristic of migratory mesenchymal cells. Implicated in carcinoma progression and metastasis, EMT has been the focus of several recent proteomics-based studies aimed at identifying new molecular players. To gain insights into extracellular mediators associated with EMT, an extensive proteomic analysis of the secretome from MDCK cells was conducted following oncogenic Ras-induced EMT (21D1 cells). Using Orbitrap technology and a label-free quantitative approach, differential expression of several secreted modulators were revealed. Proteomic findings were further substantiated by mRNA transcript expression analysis with 71% concordance. MDCK cells undergoing Ras-induced EMT remodel the extracellular matrix (ECM) via diminished expression of basement membrane constituents (collagen type IV and laminin 5), up-regulation of extracellular proteases (MMP-1, kallikreins -6 and -7), and increased production and secretion of ECM constituents (SPARC, collagen type I, fibulins -1 and -3, biglycan, and decorin). Collectively, these findings suggest hierarchical regulation of a subset of extracellular effectors may coordinate a biological response during EMT that enhances cell motility. Transient silencing of MMP-1 in 21D1 cells via siRNA-mediated knockdown attenuated cell migration. Many of the secretome proteins identified broaden understanding of the EMT process.

Exosomes are 40-100-nm diameter nanovesicles of endocytic origin released from diverse cell types. To better understand the biological role of exosomes and avoid confounding data arising from proteinaceous contaminants, it is important to work with highly purified material. Here, the laboratory described an immunoaffinity capture method using the colon epithelial cell-specific A33 antibody to purify colorectal cancer cell (LIM1215)-derived exosomes. LC-MS/MS revealed 394 unique exosomal proteins of which 112 proteins (28%) contained signal peptides and a significant enrichment of proteins containing coiled coil, RAS, and MIRO domains. A comparative protein profiling analysis of LIM1215-, murine mast cell-, and human urine-derived exosomes revealed a subset of proteins common to all exosomes such as endosomal sorting complex required for transport (ESCRT) proteins, tetraspanins, signaling, trafficking, and cytoskeletal proteins. A conspicuous finding of this comparative analysis was the presence of host cell-specific (LIM1215 exosome) proteins such as A33, cadherin-17, carcinoembryonic antigen, epithelial cell surface antigen (EpCAM), proliferating cell nuclear antigen, epidermal growth factor receptor, mucin 13, misshapen-like kinase 1, keratin 18, mitogen-activated protein kinase 4, claudins (1, 3, and 7), centrosomal protein 55 kDa, and ephrin-B1 and -B2. Furthermore, presence of the enzyme phospholipid scramblase implicated in transbilayer lipid distribution membrane remodeling was reported. The LIM1215-specific exosomal proteins identified in this study may provide insights into colon cancer biology and potential diagnostic biomarkers.

Exosomes, membrane microvesicles (40-100 nm) secreted by most cell types, can be isolated in several ways while characterizing them is heavily based on electron microscopy and, most importantly, identification of exosome marker proteins. Researchers rely on identification of certain exosomal marker proteins including Alix, CD9 and CD63 to confirm the presence of exosomes in their preparations. An evolutionary-conserved set of protein molecules have been identified in most exosomes studied to date. However, with the complexity of tissue/cell type-specific

proteins being incorporated in the exosomes, some of these so-called exosomal markers are not always present in all the exosomes. The presence of tissue/cell type-specific proteins in exosomes allows researchers to isolate them using immunoaffinity capture methods. A compendium for exosomal proteomes will aid researchers in identifying proteins more commonly found in various exosomes (exosome markers) and those specific to certain tissue/cell type-derived exosomes. ExoCarta, a compendium for proteins and RNA molecules identified in exosomesis first of its kind and the resource is freely available to the scientific community through the web (http://exocarta.ludwig.edu.au).

COLON CANCER INITIATIVE

Peter Gibbs, M.D. and Oliver Sieber, Ph.D.

The laboratory was established in 2007 with the aim of 1) increasing knowledge of colorectal cancer genetics, epigenetics and biology; 2) identification of predictive and prognostic biomarkers; and 3) translation of research findings into clinical practice through biomarker-directed early phase clinical trials.

Discovery and validation of prognostic biomarkers in colorectal cancer have continued and expanded in several areas. Systematic resequencing of 1000 colorectal cancers for fourteen known cancer genes and selected pharmacogenetic variants in collaboration with the J Craig Venter Institute is nearing completion, with analysis completed for over 700 cases. Preliminary analysis of sequencing data reveals higher mutation prevalence in certain genes than previously reported and complex pathways of tumorigenesis defining molecular subtypes of cancers. Individual gene mutation data for tumors are being analyzed with longitudinal follow-up data to assess prognostic value, with novel associations apparent for *KRAS* mutation status and site of metastatic disease.

All tumor and matched normal samples included in this resequencing project are further analyzed using genome-wide SNP microarrays (Illumina 610 Quad BeadChips). Algorithms for accurate, high resolution analysis of chromosomal gains, losses and loss of heterozygosity in cancer genomes have been developed in collaboration with the Department of Statistics, University of Oxford, and developed into an analysis software package called OncoSNP. DNA copy-number data have been used to fine-map novel loci showing high-frequencies of gain or loss across cancers, and screening of candidate cancer genes encompassed in these regions is ongoing. Development of mathematical methods for identification of associations between germline variants and somatic aberrations and cancer outcome is in progress.

Using gene expression microarray data generated for 550 colorectal cancers in collaboration with the H. Lee Moffitt Cancer Center, a prognostic gene expression signature has been identified for use in stage B and C colorectal cancer. This signature adds prognostic information over and above traditional staging methods and has been identified using a novel approach, with genes showing expression differences between stage A and D tumors used to distinguish good prognosis (stage A-like) from bad prognosis (stage D-like) stage B and C cancers. This classifier is currently being adapted for use with paraffin embedded tissues prior to prospective validation studies.

Gene expression microarray data have been used to identify novel genes consistently upregulated in adenomas and carcinomas compared to normal colorectal epithelium. These candidates are currently being evaluated as markers for detection of early colorectal cancer and cancer recurrence after curative-intent surgery, and functional studies on these proteins have provided new insights into normal and cancer biology. One candidate has been shown to mark the putative normal stem cell of the human intestine, with

overexpression in tumor tissue contributing to cell growth and migration.

A study of BRAF mutations in colorectal cancer generated the first large series demonstrating clinical characteristics associations with BRAF mutations and adverse impact of these mutations on patient outcomes. This work earned Jeanne Tie the Bradley Stuart Meller award for the best abstract presented by a fellow at the American Society of Clinical Oncology annual meeting. In parallel with this study, Jayesh Desai led a phase I clinical trial at Royal Melbourne Hospital of a novel agent targeting the BRAF mutation in patients with colorectal cancer.

The clinicians have continued to collect and expand prospective, comprehensive data collection on patients treated for colorectal cancer. This resource has generated multiple publications across the spectrum of cancer care including screening initial staging, treatment, follow-up and outcomes of patients with colorectal cancer. Combined with routine collection of fresh tissue and blood, this effort continues to build a resource for translational research.

The laboratory obtained a major competitive research grant from the Victorian Cancer Agency, which will fund collection of tissue specimens and clinical data for 1600 colon cancer patients over the next four years. This collection effort will involve over ten hospitals, capturing the majority of colon cancer patients treated in Victoria. This resource will be used for prospective validation of prognostic marker candidates identified in current discovery studies.

PROTEIN BIOSENSING

Edouard Nice, Ph.D.

The major focus has been on generation and validation of reagents and assays for detecting and surveillance of colorectal cancer. The laboratory obtained RMH human ethics approval for the collection of blood and fecal samples from 1000 CRC patients, 1000 controls and 1000 patients with benign bowel disease. In blood based biomarker studies a number of ELISA assays have been successfully translated into the Luminex magnetic bead-based or 96 well plate format and have validated these assays (in collaboration with Dr. Leah Cosgrove, CSIRO, Adelaide) for matrix related effects, sample stability and assay reproducibility and sensitivity. Clinical data have been collated from Biogrid Australia and previous study 2 data have been extensively analyzed. In this study a total of 33 separate biomarkers were analyzed. Ten biomarkers were measured using different reagents or in different media (e.g., serum rather than plasma). Ultimately 38 CRC patients and 40 controls with a complete set of data on 28 different biomarkers were used in the final analysis. Statistical modeling using automated variable selection algorithms gave combinations of biomarkers that have encouraging predictive properties. This biomarker panel is now being tested using clinical samples from 100 patients and 100 age and sex matched control samples (study 3) to further refine the assay panels.

The development of fecal proteomics has been the key discovery focus of the laboratory. The hypothesis is tumor related proteins (e.g., hemoglobin peptides due to bleeding, CEA) will be relatively more abundant in stools than blood, and these proteins can be efficiently detected and quantified using mass spectrometric techniques. This has been supported by initial studies using the APC/Min mouse animal model of CRC where many potential biomarkers have been identified in stool samples, including isoforms of CEA, cathepsins, orosomucoid-1, orosomucoid-2, neutrophil defensin 1, complement factor H, fibrinogen, antithrombin-III, galectin-3 binding protein and selenium binding protein. The presence of bacterial proteins from the gut microflora did not interfere with the analyses.

The laboratory has extended murine studies to investigation of human proteins present in fecal samples from patients

with colorectal cancer (samples are obtained under full SOP post colonoscopy and prior to surgical resection) and from volunteers who have no evidence of any colon pathology. Under investigation is a new paradigm in biomarker discovery and validation: key to the discovery phase is identification of suitable ms/ms fragmentation data for proteins present in the biological sample under investigation (in this case feces). These ms/ms data can then be used to identify proteotypic peptides and their specific ms/ms transition ions which can be used in Multiple Reaction Monitoring (MRM) experiments, in a multiplexed format, using a triple-quadrupole (QQQ) ms, to confirm the presence or absence of potential biomarkers in a small set of clinical samples. Isotopically-labeled standards are then used for their absolute quantitation (limits of sensitivity of Agilent 6410B QQQ, determined using an enolase digest standard, is approximately 10amole). The subsequent validation phase, not the discovery phase, will reveal whether selected proteins are potential biomarkers with improved sensitivity and/or selectivity either individually or as a panel. The laboratory has generated a human fecal database, using samples from both CRC patients and non-diseased controls, which currently contains ms/ms data on over 485 fecal proteins yielding 2260 unique proteotypic peptides (with the associated ms/ms transition ions). Using these data to generate the appropriate MRM conditions, 97 colon cancer associated proteins (CCAP) have been identified in an unfractionated fecal sample from a patient with CRC. Nine isotopically labeled peptide standards (purchased from JBT Technologies, Germany) have been purified and absolutely quantified, three using quantitative amino acid analysis. The laboratory has performed absolute MRM quantitation on CEA5 (400-2800ng/mg stool) and hemoglobin (3-1300ng/mg stool) on eight CRC patients and compared these data with a similar number of samples from normal volunteers. These studies form part of a proof-of-concept study to establish biomarker translational development in Victoria which is jointly funded by DIIRD and VCA.

In collaboration with the epithelial laboratory and Prof Holmes (University of Melbourne), a proteomics-based approach has been developed for the comprehensive analysis of the phosphoinositide interactome using analogues of PI(3,5)P2, PI(4,5)P2 and PI(3,4,5)P3 phosphatidyl phospholipids which were immobilized onto Affi-10 beads or incorporated into liposomes for use as affinity absorbents with cytosolic extracts from colonic carcinoma cell lines. The lab has also been involved in the generation and validation of reagents and biological assays to support long term collaboration on nanoparticle drug delivery with Frank Caruso (University of Melbourne) and Joan Heath.

SIGNAL TRANSDUCTION

Margaret Hibbs, Ph.D.

The laboratory is focused on the study of signaling pathways in hematopoietic cells to understand antibody-mediated autoimmune disease and define pathogenic mechanisms in lung inflammation and lung cancer. Autoimmune diseases are characterized by self-reactive antibody and chronic inflammation, culminating in tissue damage. The two prongs act in a reinforcing manner, with immune complexes inducing inflammation, which in turn recruits and activates B and T lymphocytes. Interfering with either arm is likely to be of therapeutic benefit. One autoimmune disease, systemic lupus erythematosus (SLE), is still managed with general immuno-suppression rather than a specific therapy targeting a causative agent or cell type, making discovery of potential points of intervention critical. Lyn-deficient mice harbor hyper-responsive B cells and macrophages, and develop autoimmunity akin to SLE. They are an excellent model for exploring the intersection between inflammation and lymphocyte activation in the development of autoimmune disease, providing also an opportunity for trialing potential therapeutic agents.

In recently published studies, it was shown that perturbations in Lyn-deficient B cells precede a skewing of the T cell compartment. While young Lyn-deficient mice show no obvious T cell defects, aged mice have increased numbers of activated T cells and dramatically expanded numbers of Tregs; phenotypes that are extrinsic to the T cell compartment as Lyn is not expressed in any T cell subset. Lyn-deficient Tregs are functional and there is speculation they have expanded to control disease. The laboratory has recently generated Lyn-deficient mice expressing a soluble form of the T cell inhibitory molecule CTLA4 (CTLA4-Ig) and shown that loss of T cell co-stimulation alters the course of autoimmune disease development, changing it from IgG-mediated to one dominated by IgA immune complexes.

The laboratory recently examined the role of inflammation in the autoimmune process in Lyn-deficient mice, showing that Lyn-deficient B cells establish an inflammatory milieu that perturbs other cell compartments and drives autoimmunity. Lyn-deficient leukocytes, notably B cells, constitutively produce IL-6, which facilitates B and T cell activation, enhanced myelopoiesis and generation of pathogenic autoantibodies. Lyn-deficient DCs show increased maturation suggesting an association with disease, but this is independent of autoimmunity as it is reiterated in B cell-deficient Lyn-deficient mice, which are disease-free. IL-6 was deleted on a Lyn-deficient background and this does not alter B cell development, plasma cell accumulation or DC hyper-maturation but autoimmunity is ablated. Hyper-activation of B and T cell compartments, extramedullary hematopoiesis and expansion of the myeloid lineage is ameliorated in compound $Lyn^{-/-}IL\text{-}6^{-/-}$ mice, as is autoantibody production and associated kidney pathology. This work highlights the role of inflammation in autoimmune disease, and demonstrates that although Lyn-deficient B cells may be autoreactive, it is their inflammatory environment that dictates their disease-causing potential. The goal now is to define the relationship between inflammation and lymphocyte activation in the development and progression of autoimmune disease, which may provide an opportunity to identify points where potential therapeutic agents may act.

Chronic obstructive pulmonary disease (COPD/emphysema) and lung adenocarcinoma are major global health problems. There is strong evidence that the two diseases share common susceptibility determinants and macrophages play an important role in the pathogenesis of both conditions. Macrophages are also essential for tissue homeostasis, host defense, disease resolution, tissue repair and immune defense against cancer. These beneficial properties preclude broadly targeting macrophages as viable therapeutic strategies against lung disease. Identifying subpopulations of macrophages with destructive phenotypes might determine their signature and identify possible therapeutic targets. However, lung macrophages have been difficult to study due to their uniquely high autofluorescence. The laboratory has developed a novel flow cytometry approach which allows identification and characterization of lung macrophage subpopulations. This method is being used to define subpopulations of macrophages recruited to the lungs in response to various pathological stimuli, and in mice harboring inflammatory lung disease. The ultimate goal will be to understand how to treat or prevent inflammatory lung diseases and lung cancer by selectively targeting pathogenic macrophage subpopulations without paralyzing macrophage-dependent innate immune defenses and tissue homeostasis.

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Director's Message

Detailed serological analysis of human cancer has been a major objective of the New York Branch since its inception. Powerful techniques for analyzing the antigenic structure of the two domains of the cancer cell – the cell surface and the intracellular space – have been developed, and a broad view of the antigenic distinction between cancer cells and normal cells is beginning to emerge. With regard to cell surface antigens, the generation of thousands of mouse monoclonal antibodies provided the tools for a fine scale dissection of human cancer and the discovery of a range of surface antigens on tumor cells, stromal cells, and endothelial cells. Antibodies recognizing the most tumor-restricted antigens have been identified by extensive immunohistochemical analysis of normal and malignant tissues, and the antigenic targets have been characterized by biochemical and molecular techniques. Chimeric and humanized versions of selected antibodies were generated, purified, and developed for use in Phase I / II clinical trials to determine their ability to selectively target human cancers and for their potential as targeting agents for radioimmunotherapy. These clinical studies were carried out in close collaboration with Memorial Sloan-Kettering Cancer Center and the Melbourne Branch. Twelve of the monoclonal antibodies developed by the New York Branch have been licensed to pharmaceutical and biotechnology companies, with two of the antibodies in Phase III and eight in Phase I/II trials. The three objectives of our current cell surface serology program center on: 1) Defining the surface antigenic structure of cancer stem cells. With advances in the definition and isolation of these critical cells, we now have the tools to focus on this challenge. 2) Expanding the program on antibody - conjugates, combining the specificity and targeting capacity of antibodies with the activity of chemical toxins, siRNA, and immunomodulating agents, and 3) Integrating serological discovery with deep sequencing and bioinformatic tools to construct a comprehensive picture of the cancer cells surface (which we have termed the "cancer surfaceome"), a program being carried out with the São Paulo Branch.

The analysis of intracellular antigens of cancer cells has lagged behind the rapid growth of knowledge about cell surface antigens, because, with the available techniques at the time, intracellular antigens represented an impenetrable forest for the cancer serologist. However, this perspective has changed remarkably with the introduction of SEREX (serological analysis of recombinant expression libraries of cancer), GRAND SEROLOGY (large scale ELISA surveys of patient sera with recombinant intracellular proteins), and SEROMICS (serological screening of arrays containing up to 9,000 human proteins). A publicly available SEREX database was established based on a large LICR-initiated collaboration with a number of different laboratories around the world analyzing an array of human cancers. Combining data from SEREX with the rapidly accumulating results of GRAND SEROLOGY and SEROMIC analysis, we can expect to assess the repertoire of the humoral immune response to human cancer in the foreseeable future. Although many antigenic targets of interest for diagnosis, monitoring and vaccine development have been found, we have focused our attention on cancer/testis (CT) antigens because of their

remarkable specificity for cancer. The first CT antigen (MAGE) was discovered by T cell epitope cloning by the Brussels Branch, and the subsequent introduction of SEREX uncovered a number of other CT antigens, including NY-ESO-1 by the New York Branch. NY-ESO-1 is one of the most immunogenic human tumor antigens discovered to date, and our detailed analysis of the humoral and CD4 and CD8 T cell response to NY-ESO-1 rivals understanding of HIV and influenza immunity. Because of NY-ESO-1's strong immunogenicity, restricted normal tissue expression (only germ cells in testis), and expression in a wide range of cancers, NY-ESO-1 has been chosen as the prototype CT antigens for the Cancer Vaccine Collaborative (CVC), established as a partnership between LICR and the Cancer Research Institute and involving laboratory and clinical investigators at 17 academic centers in Japan, Australia, USA, UK, Europe and Brazil. Over 44 phase I/II clinical trials of various vaccine formulations of NY-ESO-1, e.g., peptide, protein, DNA, vaccinia constructs, combined with different delivery systems and adjuvants, have been carried out in patients with a range of tumor types. To select the most immunogenic vaccine for therapeutic trials, a key component of these trials is the detailed monitoring of the immune response elicited by NY-ESO-1 vaccines, and the New York Branch is the central NY-ESO-1 reference laboratory for the CVC. A major challenge for cancer vaccine development is the profound immunosuppressive environment of the tumor mediated by T reg cells and other immunological factors. To overcome this obstacle, modulators of immunosuppression (MOI), such as CTLA-4 and PD1 blocking antibodies, and GITR agonist antibody, are being integrated into our vaccine strategies. In addition, additional vaccine targets (CT and non-CT antigens) are being evaluated with the aim of developing polyvalent vaccines to broaden the immune response and prevent antigen escape variants.

A third therapeutic approach, enzyme-mediated amino acid depletion, has been a long-term interest of our group, starting with our contribution to the clinical use of L-asparaginase, now a part of the standard therapy for acute childhood leukemia. Asparagine is a non-essential amino acid for humans, but certain cancer cells lack the ability to synthesize asparagine due to the lack of asparagine synthetase, and die when deprived of asparagine. Arginine, like asparagine, is considered a non-essential acid, but certain cancer cells lack the enzyme argininosuccinate synthetase (ASS) necessary to convert citrulline to arginine. Normal cells express ASS and can convert citrulline into arginine. The Mycoplasma-derived enzyme arginine deiminase (ADI), which converts arginine to citrulline, has been found to have anti-tumor activity *in vitro* and *in vivo* in mouse models, and the New York Branch has played a critical role in developing ADI for the clinic. A survey of human cancers using an antibody reactive with ASS developed by our laboratory showed that a high frequency of melanoma and small cell lung cancers lack ASS and are potential targets for ADI therapy. Phase I/II trials of ADI in melanoma have shown excellent depletion of blood arginine, little or no toxicity, and some evidence of therapeutic activity. A Phase II clinical trial of ADI in patients with SCLC is now planned to begin in the near future. The three targeted approaches to cancer pursued by the New York Branch – therapeutic antibodies, antigen-specific vaccines, and enzyme depletion therapy - are connected by a common theme of high specificity for cancer, and reflect the Branch's commitment to developing a continuum between laboratory discovery and clinical application.

Lloyd J. Old and Gerd Ritter

CANCER CELL SURFACE ANTIGENS

Gerd Ritter, Ph.D., Achim Jungbluth, M.D., Otavia Caballero, M.D., Ph.D., Andrew Simpson, Ph.D., Lloyd Old, M.D.

A major goal of human cancer immunology is the identification and validation of antigenic surface targets expressed on cancer cells suitable for antibody-based immunotherapy of cancer. To pursue this goal, several different approaches have been utilized, each tailored for different classes of antigens or targets. These include, 1) immunization of mice with selected tumor cell preparations, generation and screening of hybridomas for novel monoclonal antibodies with selective cell surface binding to human cancer cells and subsequent molecular elucidation of the cell surface target antigen; 2) utilization of bioinformatic tools to identify the set of all putative genes coding for cell surface antigens from available human genome sequences and construction and definition of the human cancer cell surfaceome (SURFACEOMICS).

The laboratory has recently identified and characterized, at the molecular level, the sodium-dependent phosphate transport protein 2b (NaPi2b) as the antigen recognized by the anti-ovarian cancer antibody MX35. NaPi2b represents a new family of potential cell surface targets for immunotherapy of cancer with monoclonal antibodies. This project has been undertaken in a coordinated collaborative international effort, involving among others collaborators at the LICR Uppsala Branch, the National Academy of Science, Kiev, Ukraine and UC London. The mouse mAb MX35 was developed about 20 years ago in the laboratory as a therapeutic reagent for the treatment of ovarian cancer. In phase I clinical trials radiolabeled murine antibody MX35 targeted well to tumors in patients with ovarian cancer. Without detailed knowledge of the antigen on the molecular level it would be impossible to support the clinical development of this antibody further. With the identification of NaPi2b as the molecular target of mAb MX35 it became evident that the MX35 antigen is not only expressed on ovarian cancers but also on a series of other epithelial cancers, including lung cancer, thyroid cancer and renal cancer potentially expending the therapeutic application of the MX35 antibody to those and other types of cancer. NaPi2b is the first example of a membrane transporter that can be targeted as cancer antigen with a monoclonal antibody. Several new monoclonal antibodies against cell surface exposed NaPi2b epitopes have been generated in collaboration with Ivan Gout (London UK) and Ramziya Kiyamova (Kiev, Ukraine). Further characterization of these antibodies are under way. Furthermore, mAb MX35 and the newly generated antibodies have been sequenced and mAb MX35 has been reengineered into a humanized antibody. Two different forms have been prepared and both, a chimeric and a veneered mAb have successfully maintained their full antigen binding properties. The humanized MX35 constructs have been transferred to Recepta Pharmaceuticals (LICR start-up in Brazil) to develop a manufacturing process and to generate clinical grade human antibody to subsequently embark on clinical trials in patients with various types of NaPi2b/MX35 expressing cancers.

A major challenge in cancer research is to find and identify markers to define cancer stem cells (CSC). These cells are considered to be cancer initiating and to provide a continued source of self-renewing cancer cells. The present failure of most current therapies to cure cancers is thought to be due to the relative resistance of cancer stem cells to chemotherapy and radiation. Since CSC exist only in very small numbers in cancers, it has been extremely difficult to obtain sufficient numbers of these cells for analysis and characterization. This has been a major barrier to development of effective CSC-targeted cancer therapeutics. It was hypothesized that CSC can be distinguished from the more differentiated tumor cells and normal cells by a

different antigenic cell surface profile and that these surface antigens can be recognized by monoclonal antibodies. The group has embarked on the generation and characterization of a panel of novel monoclonal antibodies against CSC and the development of those that selectively recognize CSC to create therapeutic antibodies. For this purpose the group has utilized preparations of human cancer cells highly enriched for clonogenic cells with CSC features for immunization of mice and screening of resultant hybridomas by mixed hemadsorption assays, immunohistochemistry using cell array or tissue array technologies.

In close collaboration with Sandro de Souza and his colleagues at the São Paulo Branch the laboratory is using bioinformatics tools to define the human cell surfaceome, the set of putative cell surface proteins encoded by the human genome. A catalog of 3,702 transmembrane proteins located at the surface of human cells (human cell surfaceome) has been generated. The genetic diversity of the human cell surfaceome at different levels has been explored, including the distribution of polymorphisms, conservation among eukaryotic species, and patterns of gene expression. By integrating expression information from a variety of sources, the laboratory has been able to identify surfaceome genes with a restricted expression in normal tissues and/or differential expression in tumors, important characteristics for putative tumor targets. A high-throughput and efficient quantitative real-time PCR approach was used to validate 593 surfaceome genes selected on the basis of their expression pattern in normal and tumor samples. A number of candidates were identified as potential diagnostic and therapeutic targets for colorectal tumors and glioblastoma. Several candidate genes were also identified as coding for cell surface cancer/testis antigens of which several were previously identified using SEREX including SLCO6A1, GPA34 and NY-SAR-35. A surprising outcome of this search for cell surface antigens with CT characteristics is the paucity of candidates, despite the extensive list of highly cancer-specific intracellular CT antigens. The approach agrees with other estimates that at least 20% of all human genes code for proteins that are located at the cell surface. Because the expression of the surfaceome has been evaluated in a panel of normal tissues, the analyses are also useful to avoid possible clinical side effects based on the expression of a potential target in any given normal tissue. The human cell surfaceome will serve as a reference for further studies aimed at characterizing tumor targets at the surface of human cells.

CANCER SEROMICS

Sacha Gnjatic, Ph.D., Gerd Ritter, Ph.D., Yao Chen, M.D., Ph.D., Lloyd Old, M.D.

The laboratory embarked on a large-scale serological analysis of humoral immune responses from the serum of patients with various cancers. The purpose of assessing humoral immune responses in patients is: to evaluate the inherent immunogenicity of different cancers, to identify the most frequently immunogenic tumor antigens, to establish correlations with antigen expression as well as with cellular immunity to the antigen, to identify a potential functional role for antibody responses, to identify correlations with clinical events with diagnostic/prognostic potential, and to monitor changes in antibody responses during immunotherapy. The laboratory chose to focus on measuring defined antigen-specific responses, rather than random screening of tumor extracts. The laboratory established the frequency of spontaneous antibody responses to NY-ESO-1, a model immunogenic tumor antigen, in various cancer types, including lung cancer (n=725), melanoma (n=696), ovarian cancer (n=437), pancreatic cancer (n=160), cervical cancer (n=209), prostate cancer (n=188), breast cancer (n=143), esophageal cancer (n=138), myeloma (n=98), colorectal cancer (n=75), and healthy donors (n=205). These sera from individual patients were found

to have significant titers against NY-ESO-1 by ELISA from 0.6% to 19.7%, depending on the cancer type (low in pancreatic cancer and healthy donors, frequent in ovarian cancer and melanoma). Antibody responses were also correlated with stage of disease and other clinical parameters, and ranged from low titers (1/100-1/1000) to titers reaching more than 1/1,000,000. The group previously established that there is an association between NY-ESO-1 antibodies and naturally-occurring CD8 and CD4 T cell responses in cancer patients. It was also possible to correlate NY-ESO-1 serum antibodies with clinical events and with overall survival in non-small cell lung cancer and prostate cancer patients, in parallel with data on antigen expression in the tumor. The ELISA screenings were expanded to a series of 30+ known tumor antigens, mostly cancer/testis family members such as MAGE and SSX, as well as differentiation antigens (Melan-A), mutational antigens (P53), and stem cell/embryonic antigens (SOX). It was possible to rank and compare in large subsets of patient cohorts representing various tumor types (n>2000 individual sera). The group found that some cancers were more immunogenic than others, and that some antigens were rarely immunogenic while others were more commonly so. Possible factors to explain these differences include differential antigen expression, aggressiveness of tumor, and a local suppressive environment. Some interesting targets for vaccine development with sporadic high spontaneous immunogenicity could be identified. Nevertheless, only a minority of patients experience spontaneous humoral responses. To ascertain that potential highly immunogenic shared tumor antigen targets had not been missed, the laboratory embarked on an even greater scale of antibody profiling by using protein microarrays (SEROMICS).

The laboratory has pioneered a novel, massive high-throughput approach for dissecting in fine detail the repertoire of antigens that can be recognized by antibodies present in sera from patients with cancer. Developments in automation and miniaturization have allowed the production of large numbers of recombinant proteins in microarray format. To evaluate the feasibility of using such protein microarrays to screen human sera for antigen recognition by antibodies the laboratory compared side-by-side protein array analysis (using a small array from Procognia) and ELISA using a panel of sera from lung cancer patients with defined antibody reactivities and specificities. It was found that the data obtained by the new protein array technology correlated in full with the data obtained by ELISA. This approach was subsequently expanded and validated for larger microarrays (purchased from Invitrogen) containing over 8000 different, molecularly defined protein antigens. This approach was termed "SEROMICS" as it allows for the first time evaluation of the serological recognition of several thousand antigens ("serome") simultaneously in the same assay. Convinced that this approach will result in the discovery of new serum antibody signatures for early detection or diagnosis of cancer as well as in the discovery of new antigenic targets for immunotherapy of cancer, the laboratory embarked on large screenings of sera from patients with different cancer types.

Two human cancers were selected for the initial analysis – ovarian cancer and pancreatic cancer. Because these cancers are usually found at a late, incurable stage, there is a great need to develop early detection systems. Antibody responses to the emerging tumor could serve this purpose. In addition, identification of new antigens for immunotherapy would also be useful. A total of 164 sera were analyzed in this first SEROMICS approach including 60 highly pedigreed sera from patients with pancreatic cancer (a collaboration with Dr. Dirk Jaeger, University of Heidelberg and German Tumor Centrum) and 51 ovarian cancer (collaboration with Dr. Kunle Odunsi, Roswell Park Cancer Institute, Buffalo), and 53 age-matched of healthy individuals. In regard to pancreatic cancer, patients were subdivided

into cohorts of long-term and short-term survivors with localized disease and short-term survivors with metastatic disease. In regard to ovarian cancer, the patients were subdivided into cohorts of long-term, intermediate-term and short-term survivors. Using this approach the group identified a series of new targets that are currently undergoing further investigation for potential clinical development. By ranking antigens according to the frequency of their recognition in the cancer cohort vs. the healthy cohort, and by taking into account the signal strength in positive sera, 202 proteins with increased immunogenicity in ovarian cancer patients, 29 proteins with increased immunogenicity in pancreatic cancer patients, 6 proteins with overlapping increased immunogenicity in ovarian and pancreatic cancer were identified. It became apparent that the majority of these antigens were recognized by less than 10% of patient serum samples, and were therefore relatively rare events as expected. Correlates of autoantibody signatures with known tumor expression of corresponding antigens, functional pathways, clinical stage, and outcome were examined. It was concluded that ovarian cancer appears to induce a much broader antibody response than pancreatic cancer, an observation that may shed light on the poor prognosis and response to therapy of pancreatic cancer. Most important, the findings identify a range of cancer targets that could be the basis for new cancer diagnostic and screening tests, and for the development of cancer vaccines and other immunotherapeutic approaches to cancer. The laboratory is currently in the process of validating a series of new targets discovered by SEROMICS using recombinant proteins in non-cognate systems to confirm serological reactivity and to prepare for immunohistochemical explorations.

CANCER-TESTIS ANTIGEN GENETICS

Otavia Caballero, M.D., Ph.D., Yao Chen, M.D., Ph.D., Achim Jungbluth, M.D., Lloyd Old, M.D., Andrew Simpson, Ph.D.

Cancer/testis (CT) antigens are encoded by genes that are normally expressed only in the human germ line but which are activated in various malignancies. CT antigens are frequently immunogenic in cancer patients and their expression is highly restricted to tumors and therefore they constitute important targets for anticancer immunotherapy. There are now more than 70 CT gene families, many of them promising vaccine candidates. Due to their importance, there is a rapidly expanding body of knowledge concerning these genes widely in the literature and diverse databases. To gather and uniformly present the available information on CT antigens, and to assess the relevance of individual CT proteins, a user-friendly interface termed the Cancer-Testis database (CTdatabase, http://www.cta.lncc.br/) has been created. The database integrates heterogeneous data including basic gene, protein and expression information in normal and tumor tissues as well as immunogenicity in cancer patients. The CTdatabase contains links to external databases although a priority has been to specifically process relevant data so that it can be stored locally. The information available was expertly curated and annotated, and regular updates are done.

In addition, with the view to guiding further characterization of these potentially important diagnostic and therapeutic targets, the laboratory undertook a comprehensive survey of CT gene expression by combining information from an existing corpus of more than 8000 gene expression libraries from normal and cancer tissues. The expression of the total number of 153 putative CT and CT-like genes was directly tested in 22 normal tissues and 32 cancer cell lines by RT-PCR. It was found that although the genes are generally highly expressed in testis, they exhibit heterogeneous gene expression profiles, allowing their classification into testis-restricted (39), testis/brain-restricted (14), and a testis-selective (85) groups of genes that show additional

expression in somatic tissues. The chromosomal distribution of these genes confirmed the previously observed dominance of X chromosome location, with CT-X genes being significantly more testis-restricted than non-X CTgenes.

Several CT antigens have already proved to be useful biomarkers and are promising targets for therapeutic cancer vaccines. The aim of a new study was to investigate the expression of CT antigens in breast cancer. Using previously generated massively parallel signature sequencing (MPSS) data, together with 9 publicly available gene expression datasets, the expression pattern of CT antigens located on the X chromosome (CT-X) was interrogated. Whereas a minority of unselected breast cancers was found to contain CT-X transcripts, a significantly higher expression frequency was detected in estrogen and progesterone receptor (ER) negative breast cancer cell lines and primary breast carcinomas. A coordinated pattern of CT-X antigen expression was observed, with MAGEA and NY-ESO-1/CTAG1B being the most prevalent antigens. Immunohistochemical staining confirmed the correlation of CT-X antigen expression and ER negativity in breast tumors and demonstrated a trend for their co-expression with basal cell markers. Because of the limited therapeutic options for ER-negative breast cancers, vaccines based on CT-X antigens might prove to be useful.

In several different tumor types, the expression of CT-X genes is associated with advanced disease and poor outcome, indicating that their expression might contribute to tumorigenesis. A principal research focus of the laboratory is to investigate this possibility, and thus whether CT-X genes could also be potentially targeted by functional inhibitors in a therapeutic setting. One of the approaches used was to knock down their expression using small interfering RNA (siRNA). These agents were used in cell proliferation, migration and cell survival assays using cell lines derived from melanoma, a tumor type known to present high frequencies of expression of CT antigens. It was demonstrated that MAGEA, SSX, GAGE, XAGE1, MAGEC1, MAGEC2 and NY-ESO-1 siRNAs can specifically and effectively downregulate the respective genes at the mRNA level and also at the protein level in those cases where antibodies were available. The group found that of these, XAGE1 and GAGE knockdown mostly significantly impeded melanoma cell migration as well as their invasive capacity. In addition, XAGE1 and SSX knockdown significantly decreased the clonogenic survival of melanoma cells, suggesting that these genes may have roles in melanoma progression and may provide possible targets for the treatment of melanoma.

To elucidate further their functional roles in carcinogenesis, the yeast two-hybrid system to identify putative CT antigen-interacting proteins was used. The MAGE Homology Domain (MHD) of MAGEC1 was used as a bait to screen a human testis cDNA library. As a result of this investigation, the group identified another member of the CT antigen family, CTAG1B/NY-ESO-1 as a MAGEC1 binding partner. This was the first report of a direct interaction between two CT antigens and may be pertinent in light of the frequently coordinated expression of these proteins. To identify further the interactions of members of the CT antigen family, the group combined immunoprecipitation (IP) of endogenous protein complexes, with a more specific approach, tandem affinity purification (TAP-tagging), to identify proteins that interact with CTAG1B/NY-ESO-1, the CT antigen found interacting with MAGEC1. A total of five analyses were performed resulting in the identification of hundreds of proteins. Highly abundant proteins (e.g., keratins and ribosomal proteins and proteins frequently found in previously reported TAP tag purifications using other bait proteins) observed across samples were considered as background and excluded. A remaining set of 30 proteins putatively associated with NY-ESO-1 were selected for further analyses. To confirm further protein interactions, co-immunoprecipitation experiments were performed.

Members of the MAGEA family (MAGEA4, MAGEA10) and CTAG2/LAGE were identified as NY-ESO-1 putative interaction partners. Specific interactions of NY-ESO-1 with TRIM28/KAP1, BAT3 and DDB1 were also confirmed by co-immunoprecipitation experiments. Because these three proteins are known to interact with and modify TP53 activity, the group tested if NY-ESO-1 is also part of protein complexes involving TP53. It was possible to show that NY-ESO-1 interacts both with endogenous TP53 and with TP53 protein in pull-down assays, suggesting that there is a direct physical interaction between these two proteins. These findings suggest the possibility that the NY-ESO-1 may be part of multi-protein complexes that exert their activities through specific interactions with other CT-X antigens, TP53 and TP53 regulatory proteins.

Recently, it has begun to be possible to undertake genome-wide investigations of somatic mutations in human tumors. Within the published data, the laboratory identified reports of missense mutations in the CT-X antigen genes *MAGEA1*, *MAGEA4*, *MAGEC1*, *MAGEC2*, as well as the genes for the ubiquitously expressed *MAGEE1* (also encoded on the X chromosome) in breast and brain tumors. Although the frequency of these mutations is low, it was reasoned that their mutation might not be simply due to chance as none were observed to be mutated in colon or pancreatic tumors although the same genes were sequenced in similar numbers of tumors. The laboratory undertook a mutational analysis of the coding regions of five MAGE genes in human melanoma samples first using cell lines established from tumors and matching blood samples from 27 melanoma patients and found that 37% of the samples contained at least one mutated MAGE gene. The frequency of mutations varied from 3.7% for *MAGEA1* and *MAGEA4* to 14.8% for *MAGEC2*. One hundred and eleven melanoma samples collected from 86 patients were also examined and 44% of which exhibited mutations in one or more *MAGE* genes with the frequency of mutations in individual *MAGE* genes ranging from 6% in *MAGEA1* to 16% in *MAGEC1*, confirming the frequent mutation of *MAGE* genes in human melanoma. Mutations in melanoma are generally reflective of the mutagenic impact of ultraviolet light. This pattern can be clearly observed in that C>T - G>A alterations represent almost 87% of the *MAGE* mutations found.

The laboratory has shown previously that the cancer/testis antigen CT45 is expressed in various epithelial cancers at a frequency of <5% to ~35%. This study has now been expanded to examine the protein expression of CT45 in non-Hodgkin B-cell lymphomas and classical Hodgkin lymphoma by immunohistochemical analysis. None of 80 low-grade B-cell lymphomas, including chronic lymphocytic leukemia/small lymphocytic lymphoma, follicular lymphoma, and mantle cell lymphoma, expressed CT45. In comparison, CT45 was expressed in 28 of 126 (22%) diffuse large B-cell lymphomas (DLBCL). A remarkably high percentage (42/72, 58%) of classical Hodgkin lymphoma contained CT45-positive Reed–Sternberg cells. Nodular sclerosis and mixed-cellularity subtypes had similar frequency of CT45 expression, but most EBV-positive cases were CT45 negative. Gray-zone lymphoma (cases with features of both DLBCL and classical Hodgkin lymphoma) also showed frequent (64%) CT45 expression. Evaluation of reactive lymphoid tissues showed scattered CT45-positive lymphocytes in a single case of florid follicular hyperplasia, raising the possibility that this case was an evolving malignancy. The group measured serum antibody responses to CT45 by ELISA but despite frequent CT45 expression, only 1 of 67 Hodgkin lymphoma patients had detectable anti-CT45 antibodies in the serum, suggesting that the immune response to CT45 may be suppressed.

HUMORAL AND CELLULAR IMMUNITY

Sacha Gnjatic, Ph.D., Jedd Wolchok, M. D., Ph.D., Achim Jungbluth, M.D., Gerd Ritter, Ph.D., Lloyd Old, M.D.

SEROMICS approach and ELISA are being used to identify serum autoantibody responses during the course of treatment with immunotherapy in the hope of defining predictive markers of clinical response. Sera from melanoma patients treated with CTLA-4 blockade immunotherapy was analyzed in collaboration with the Ludwig Center for Cancer Immunotherapy at MSKCC, NY (Drs. James Allison and Jianda Yuan). When stimulated, CTLA-4, a molecule expressed by T lymphocytes, acts as a natural break to immune responses and prevents them from going into overdrive in consequence to an infection or foreign body. Monoclonal antibodies have been developed to target CTLA-4 and block this natural breaking process: as a result, immune responses are allowed to continue acting when they would otherwise be suppressed. Infusion of cancer patients with anti-CTLA-4 mAb has shown dramatic clinical benefits in a subset of patients, probably because of the unleashing of immune responses directed against tumor cells. It is not clear however what the exact targets of immune responses are, nor why only a minority of patients experience benefit from the drug. The group asked whether measuring autoantibody responses before and after treatment with CTLA-4 blockade may give clues to the mechanisms of action of this drug, as well as have potential value in predicting patients at better odds of clinically responding to treatment. To address the hypothesis that having ongoing immune responses to tumor antigens may be the underlying mechanism for the effectiveness of anti-CTLA-4 treatment, the group first looked at immune responses against NY-ESO-1. Previous work has demonstrated that immune responses to NY-ESO-1 occur spontaneously in a subset of melanoma patients expressing NY-ESO-1 in their tumors, and that these responses include serum antibody, CD8 and CD4 T cells, occurring together in an integrated manner. Antibody and T cell responses to NY-ESO-1 were measured in a series of patients treated with CTLA-4 blockade and were found to increase in frequency, breadth, and poly-functionality with treatment. Moreover, it appeared that these patients with NY-ESO-1 immune responses had a better clinical outcome to CTLA-4 blockade. To confirm this hypothesis, the group expanded its analyses through large-scale ELISA, including NY-ESO-1 and other tumor antigens, to a cohort of 117 advanced melanoma patients treated with anti-CTLA-4, in collaboration with Drs. Mario Sznol and Ruth Halaban at Yale University. It was found that 23% of patients had antibodies to NY-ESO-1, often at baseline but also in some cases developing while under CTLA-4 blockade. While one third of patients who were seronegative for NY-ESO-1 showed some clinical benefit to treatment (complete, partial responses or stabilization of disease for at least 24 weeks), close to 60% of patients seropositive for NY-ESO-1 experienced clinical benefit. This correlation of NY-ESO-1 serum antibody with clinical outcome was determined to be significant.

MONOCLONAL ANTIBODY TRIALS

Gerd Ritter, Ph.D., Achim Jungbluth, M.D., Sacha Gnjatic, Ph.D., Lloyd Old, M.D.

The laboratory has been at the forefront of discovery, analysis and validation of cell surface targets on human cancers using hybridoma technology and generation of monoclonal antibodies. Twelve monoclonal antibodies from the laboratory have been licensed to commercial partners and more than 10 have entered into clinical trials at Memorial Sloan-Kettering Cancer Center (MSKCC). Clinical trials that are currently being pursued at MSKCC in collaboration with Dr. Steve Larson and colleagues (Ludwig Center at MSKCC) investigate the ability of monoclonal antibodies to selectively target human cancers and include a study with 124-I-cG250 (phase III in

renal cancer) and 124-I huA33 in colon cancer.

To improve antibody efficacy of destroying tumor cells, the laboratory has prepared a series of antibody constructs with enhanced antibody effector functions. These new therapeutic antibody constructs utilize the targeting characteristics of an antibody to selectively deliver a highly cytotoxic or inflammatory payload to the tumor site with minimal damage to normal tissue. In collaboration with Christoph Renner, Stefan Bauer and colleagues in Zürich, Egbert Oosterwijk and colleagues in Nijmegen and Andrew Scott and colleagues in Melbourne the group prepared and studied TNF-antibody conjugates *in vitro* and *in vivo*. Biodistribution of radiolabeled G250-TNF and antitumor activity of G250-TNF, alone and in combination with IFNg, were determined using renal cell carcinoma (RCC) xenografts in BALB/c nu/nu mice. Specific G250-TNF accumulation and retention of G250-TNF at G250-positive RCC resulted in growth inhibition of RCC and improved progression free survival and overall survival. Antitumor activity induced by targeted TNF-based constructs could be enhanced by co-administration of low doses of non-targeted IFNg without significant increase in side effects. Administration of G250-TNF and IFNg resulted in significant synergistic tumoricidal activity.

Another strategy being pursued is the use of targeted monoclonal antibodies to react with cell surface targets on immune cells critically involved in controlling the immune suppressive environment in tumors. The goal is to use these antibodies as part of an integrated cancer vaccine approach to interfere with tumor mediated immune suppression. Current foci are on antibodies against CTLA4 and GITR, two key molecules in immunomodulation.
To understand better the immunomodulatory and therapeutic role of agonistic GITR antibodies or GITR ligand for therapeutic cancer vaccines, the role of agonistic GITR mAbs in two murine tumor models, a CMS5a mouse sarcoma model and the CT26 colon cancer model is being studied. Both tumor types have been transfected with NY-ESO-1, allowing the investigation of GITR mAbs and GITR-L in combination with NY-ESO-1 cancer vaccines. Both models have been initially established in collaboration with Drs. Hiroyoshi Nishikawa and Hiroshi Shiku in Mie. In these models GITR signaling was found to play a critical role *in vivo* in the induction of regulatory T cell-resistant CD8 T cells and that co-administration of GITR agonistic agents constitute a promising novel strategy for cancer vaccines. In addition, a strong synergistic anti-tumor effect with anti-CTLA4 treatment has been observed in these models.

NY-ESO-1 AND MAGE VACCINES

Sacha Gnjatic, Ph.D., Achim Jungbluth, M.D., Jedd Wolchok, M.D., Ph.D., Gerd Ritter, Ph.D., Lloyd Old, M.D.

Previous studies have correlated immune activation in ovarian cancer patients with improved clinical outcome. Over 40% of ovarian tumors express NY-ESO-1, and ovarian tumor expression of NY-ESO-1 increases with advancing clinical stage. In collaboration with Dr. Paul Sabbatini at MSKCC, the laboratory has previously completed a Phase I safety and immunogenicity study of a short NY-ESO-1 peptide and Montanide vaccination of ovarian cancer patients in high-risk first remission. The vaccine showed no evidence of toxicity with immune stimulation present in all 9 treated patients. A NY-ESO-1 overlapping peptide (OLP4) vaccine is now also being explored. This vaccine contains four 30-32mer NY-ESO-1 overlapping peptides, which cover residues 79-173 of the protein (53% of the total) that include 90% of T cell epitopes described to date for NY-ESO-1, representing the most hydrophobic and immunogenic regions of NY-ESO-1, with a high potential to generate T cell (CD4 and CD8) and B cell responses. Additional advantages of this approach include that 1) patient selection based on HLA haplotype is not required, and 2) they likely contain HLA peptide epitopes that are not yet defined.

A clinical phase I study is being conducted at MSKCC (PI: Dr. Paul Sabbatini) investigating this cancer vaccine in combination with various immunological adjuvants. The group has enrolled so far 28 women with epithelial ovarian, fallopian tube or primary peritoneal carcinoma in second or third clinical remission. The primary and secondary endpoints of the study are to determine the safety and immunogenicity of NY-ESO-1 OLP4 vaccination with or without Montanide, and with or without Poly-IC. Poly-ICLC is a synthetic compound mimicking the structure of double stranded RNA and able to bind to Toll-like receptor 3 in various cells such as dendritic cells, and thereby activate the production of an IFN type I inflammatory environment. It has been tested as a single agent in cancer, and as a dendritic cell vaccine adjuvant in recurrent glioblastoma. To establish immunogenicity, NY-ESO-1 specific humoral immunity (ELISA); NY-ESO-1 specific T-cell immunity (tetramer, ELISPOT, and intracellular cytokine staining) are assessed on weeks 0,1,4,7,10,13,16, along with delayed type hypersensitivity (DTH) before and after the vaccination regimen. Patients are monitored for progression of disease.

As a follow-up to a previous vaccine study with MAGE-A3/ Protein D/His fusion protein with or without saponin-based adjuvant AS02B in non-small cell lung cancer patients, the group explored the repertoire of CD4 T cell precursors throughout immunization using a new sensitive method based on CD40L transient upregulation and subsequent cloning and expansion. This approach allowed the characterization of CD4 T cell responses to MAGE-A3 that are usually undetectable spontaneously in healthy donors or even patients with MAGE-A3 tumors using standard assays such as ELISPOT or intracellular staining of cytokines. The low frequency of preexisting CD4 T cells to MAGE-A3 in four healthy donors, two lung cancer patients with spontaneous serum Abs to MAGE-A3, and two baseline seronegative lung cancer patients throughout vaccination with MAGE-A3 protein was compared. MAGE-A3-specific CD4 T cells were detected in all individuals tested, at low frequency in healthy donors and seronegative cancer patients and at a higher frequency in patients seropositive for MAGE-A3. By fully characterizing peptide specificity, HLA-restriction, and avidity of these CD4 T cells, it was found that MAGE-A3 protein vaccination induced oligoclonal activation of MAGE-A3-specific CD4 T cells and that the presence of adjuvant selectively expanded high avidity CD4 T cells, whereas high avidity T cells disappeared after multiple vaccinations with MAGE-A3 protein alone.

TARGETED ENZYME AMINO ACID DEPRIVATION THERAPY

Lloyd Old, M.D., Achim Jungbluth, M.D., Jedd Wolchok, M.D., Ph.D., Gerd Ritter, Ph.D.

In addition to targeted antibodies and vaccines another major area of focus of the Branch is a therapeutic approach using targeted biologics such as enzymes for depriving tumors of critical molecular building blocks (e.g., non-essential amino acids). In contrast to normal tissues some tumors lack key enzymes involved in the biosynthesis of these amino acids and the tumors depend on the uptake of these amino acids from the blood stream or surrounding tissue (auxotrophy). Deprivation of these amino acids can be achieved by treatment of cancer patients with recombinant enzymes that rapidly catabolize these amino acids in the blood. Arginine is a non-essential amino acid that can be synthesized by normal human cells but several types of tumors, especially melanoma and hepatocellular cancer, lack the key enzyme in the arginine synthesis pathway, argininosuccinate synthetase (ASS) making these tumors auxotrophic for arginine. By using arginine deiminase (ADI), a microbial enzyme that degrades the amino acid arginine into citrulline and ammonium, tumor cells can be deprived of arginine. Thus, ADI

has been developed as targeted biological therapy for these types of tumors and several clinical trials have been initiated exploring a pegylated form of ADI (ADI-PEG-20) in patients with ASS-negative melanomas and hepatocellular carcinomas. In collaboration with clinical investigators at Memorial Sloan-Kettering Cancer Center the New York Branch has initiated and conducted a phase I/II study studying the efficacy of ADI-treatment in patients with ASS-negative melanomas. Enrollment has been completed and the clinical trials data are currently being compiled and analyzed. To understand better the mechanism of how this new form of deprivation therapy may work in melanoma, microPET studies in mice were conducted in collaboration with Dr. Steve Larson and his nuclear medicine team at the Ludwig Center at MSKCC to quantitate tumor responses to arginine depletion after treatment with the enzyme ADI. It appears that the antitumor effects of ADI-PEG20 in mouse melanoma models were moderate and may be explained with adapted intrinsic resistance of melanoma cells to ADI-PEG20 treatment. Thus, there is a need to identify tumors that may be more sensitive and remain sensitive to ADI-PEG20 treatment.

The laboratory has identified by immunohistochemical screening of different tumor types that approximately 45% of small cell lung cancers (SCLC) lack expression of argininosuccinate synthase (ASS). Although SCLC is at first sensitive to chemotherapy, the majority of SCLC patients relapse, and there remain few options in the treatment of advanced disease. To investigate if SCLC is susceptible to arginine deprivation therapy a series of *in vitro* and *in vivo* experiments were conducted. The group screened a panel of SCLC cell lines for expression of ASS and found that only SCLC cell lines that lack expression of ASS at the mRNA and protein level but not ASS-expressing cell lines were sensitive to ADI-PEG20 treatment *in vitro*. Growth inhibition was caused by both, autophagy and apoptosis induced by treatment with ADI-PEG20. Based on these findings animal studies were undertaken using ASS negative cell lines in order to evaluate the anti-tumor efficacy of ADI-PEG20 against SCLC tumors. Human SCLC xenografts were established in mice, and mice were subsequently treated with ADI-PEG20 at doses equivalent to those used in the clinical exploration of ADI-PEG20. A significant and dose dependent inhibition of tumor growth was observed following repeated treatment of tumors with ADI-PEG20. Efficacy was significantly greater with SCLC tumors compared to previous studies of melanoma xenografts. These results strongly suggest that ADI-PEG20 may have a role in the clinical treatment of SCLC. Based on these *in vitro* and *in vivo* data a clinical trial exploring the use of ADI-PEG20 in patients with SCLC has been initiated at MSKCC. Additional animal studies are ongoing that assess the role of tumor burden in response to ADI-PEG20 treatment and the effect of chemotherapies and small molecule inhibitors in combination with ADI treatment of SCLC tumors.

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Director's Message

The Branch is situated on Oxford University's biomedical campus, which is home to over 1,500 scientists and researchers and affords unique opportunities for collaboration. The Old Road Campus Research Building (ORCRB), which houses the Institute, also accommodates four other major research units with strong links to cancer: the Gray Institute for Radiation Oncology and Biology; the Department of Clinical Pharmacology; the Institute of Biomedical Engineering; and the Oxford Branch of the International Structural Genomics Consortium. There are three other world-class research centers on campus: the Richard Doll Building, home to the University's cancer epidemiology and clinical trials programs; the Wellcome Trust Centre for Human Genetics, for research into the genetic basis of diseases including cancer; and the Centre for Cellular and Molecular Physiology, which has a strong focus on oxygen sensing. In addition, a 217-bed cancer hospital opened this year, across the road on the Churchill Hospital site. The new hospital includes a radiology unit, the University Department of Medical Oncology, research beds and linear accelerators for radiotherapy. The University also works closely with the Oxford Radcliffe Hospitals (ORH), one of the largest teaching trusts in the country. The Trust, jointly with the University, was recently awarded biomedical research center status by the Department of Health.

The Branch was established in July 2007 with the movement of a group from the former University College London Branch to the Weatherall Institute of Molecular Medicine (WIMM) in Oxford, which served as a temporary home. The group moved again, together with the Institute's newly established Branch infrastructure staff team, to the newly opened ORCRB in May 2008. Professor Colin Goding and Dr. Gareth Bond relocated to the new Branch in 2008 from the Marie Curie Research Institute, Oxted and the Institute for Advanced Study, Princeton, respectively, followed by Drs. John Christianson and Richard Bryant from Stanford and the University of Sheffield, respectively, in 2009. In 2010, we are welcoming Dr. Sarah De Val from the University of California

San Francisco and Dr. Skirmantas Kriaucionis from Rockefeller University. The Branch infrastructure team has also been bolstered by the arrival of an Institute Manager, Neil Carveth in 2008, along with a new laboratory technician, Janet Twitchen and Scientific Communications Manager, Dr. Claire Beveridge in 2009.

Building on the success of the London Branch's research program into tumor suppression and cancer metastasis, the focus of the Oxford Branch can be broadly divided into two areas: molecular switches in cancer therapy (Lu, Goding, Christianson, De Val and Kriaucionis); and the role of molecular switches in cancer susceptibility (Bond and Bryant). The Branch brings together synergistic, interdisciplinary teams with complementary expertise, focused on identifying and characterizing the molecular mechanisms that underpin cancer development and the responses of cancer cells to therapy. The Branch has completed its initial round of recruitment and looks forward to the future with anticipation. All research groups have successfully recruited new team members and are in the process of building new collaborative relationships, both within and outside the Branch.

Xin Lu

TUMOUR SUPPRESSION

Xin Lu, Ph.D.

Many cancer therapies, such as radio-, chemo- and targeted therapy, exploit the p53 pathway to suppress cancer cell growth by stimulating p53-mediated cell cycle arrest, apoptosis and senescence. p53 is a master regulator of the cellular stress response. In response to anti-cancer therapy or defined growth conditions, it directs the activity of specific sets of genes to instruct cells to survive, die or adopt a specific fate. Understanding how p53 switches its activity is a key issue. The Lu group was among the first to identify a key component of the switching mechanism that modulates p53's ability to activate or repress the different sets of genes implicated in cell survival or death, to suppress tumorigenesis *in vivo*. The discovery of the evolutionarily conserved ASPP family of proteins has revealed a novel mechanism by which the apoptotic function of the p53 family is regulated. The ASPP family consists of three members: ASPP1, ASPP2 and iASPP. The group previously showed that ASPP1 and ASPP2 specifically stimulate the apoptotic function of p53, while iASPP specifically inhibits it. iASPP is the most conserved member of the ASPP family, and *C. elegans* iASPP is capable of substituting for human iASPP in all assays performed to date in human cells. Detailed analysis of ASPP2-deficient mice has demonstrated that ASPP2 is a novel haploinsufficient tumor suppressor, and deficiency of p53 and ASPP2 results in synthetic lethality, suggesting that ASPP2 and p53 interact genetically. Furthermore, ASPP2 co-operates with p53 to suppress tumor growth *in vivo,* explaining why p53s ASPP2 contact residues are mutated in human cancer with a relatively high frequency.

Over the last year, research has continued to focus on the biological importance of the ASPP family, in particular on the *in vivo* functions of ASPP2 and iASPP using mouse models. It was shown that ASPP2 plays a vital role in the development of the central nervous system. Mechanistically, this role of ASPP2 is predominantly achieved by its ability to localize at cell/cell junctions, and control cell polarity and proliferation. In a different experimental system, the group further analyzed how ASPP2 is able to control cell proliferation, and made the preliminary observation that ASPP2 is an important mediator of Ras-induced senescence. This suggests a novel role for p53 via ASPP2 mediating senescence through a pathway that is independent of the p21/ARF and p53 pathway, and in agreement with its tumor suppressive function. The group will continue to investigate how ASPP2 controls cell polarity over the coming months, alongside determining the molecular details of how it regulates the cell cycle. Recently, it was also observed that ASPP2 plays a critical role in regulating autophagic activity, a research area that will continue to be explored.

In addition to work on ASPP2, mouse models have been used to study the function of iASPP. Mice in which iASPP has been knocked out show three main phenotypes: in the hair/skin, heart and eyes. As with ASPP2, preliminary evidence suggests that the heart defect observed in iASPP-deficient mice is caused, at least in part, by some sort of dysregulation of junctional components. The group will continue to investigate the molecular mechanisms underlying the phenotypes observed.

CELL FATE SWITCHING

Colin Goding, Ph.D.

The ability of cells to change or maintain their identity through epigenetic mechanisms underpins development and is misdirected in cancer. The Goding group is focusing on understanding how this is achieved at the global level via networks of transcription factors and signaling pathways using the melanocyte/melanoma system, while addressing more mechanistic gene regulation questions using *S. cerevisiae*.

In the melanocyte/melanoma system, a key transcription factor cascade was uncovered that coordinates cell survival, proliferation, differentiation, invasion and senescence, which is deregulated in melanoma.

These results suggest that, in contrast to the widely held view that metastasis arises through the step-wise accumulation of genetic lesions and subsequent clonal selection of cells with metastatic potential, melanoma metastasis is likely to be regulated by a dynamic epigenetic mechanism with major implications for current and future cancer therapy. In this model, the microenvironment of a cancer cell imposes a transcriptional program that, in some cells, would lead to cell cycle arrest and invasive potential. Once a cell has migrated to a new niche, a different microenvironment could then reverse the invasive phenotype to signal proliferation. The group has identified that a key event in the generation of invasive cells is the down-regulation of the master-regulator of melanocyte development, the Microphthalmia-associated transcription factor (Mitf).

It has also been determined that Mitf expression is repressed by the POU domain factor Brn-2, and that Brn-2 and Mitf are present in different subpopulations of melanoma cells in tumors. The question is whether up-regulation of Brn-2 is a hallmark of metastasizing cells and whether, as the model predicts, *in vivo* cells will switch phenotypes in response to microenvironmental signals. By making a melanoma cell line in which the Brn-2 promoter drives GFP expression, it has been shown, using real-time intravital imaging in collaboration with Erik Sahai, that high Brn-2 expression is required but not sufficient for invasiveness, and that high Brn-2 is inversely correlated with the pigment content of the cells that is in turn dependent on Mitf activity. Significantly, melanoma cells can switch in both directions between high- and low-Brn-2-expression, but switching from high Brn2-GFP to low is greatly favored over the reverse direction. These data validate the phenotype-switching model for melanoma and also highlight Brn-2 as a marker for a subpopulation of melanoma cells able to invade and initiate new tumors, self-renew and generate a mixed population of cells within a melanoma. In other words, the Brn2-high cells fulfill all the criteria for melanoma stem cells. The current work is, therefore, focused on understanding how Brn-2 and Mitf are regulated by different signal transduction pathways, and how post-translational modifications dictate the capacity of Brn-2 and Mitf to impose different biological outcomes on melanoma cells in culture and *in vivo*.

In the past couple of years it has become evident that senescence is a major barrier to the generation of induced pluripotent stem cells, as well as cancer. In melanoma and other cancers, two members of the T-box family of transcription factors were identified, Tbx2 and Tbx3, as key anti-senescence factors that act to prevent p53-driven activation of the p21 cyclin-dependent kinase inhibitor. In addition, it was shown that Tbx3 is a key repressor of E-cadherin, a cell-cell adhesion molecule that is lost in invasive melanoma cells. Current studies are focused on understanding what regulates Tbx2 and Tbx3 expression, and which signaling pathways determine their association with the co-factors that mediate the repression of their target genes. The results indicate that Tbx2 is regulated during the cell cycle and interacts with co-factors known to play a crucial role in epigenetic inheritance. The laboratory is now characterizing these interactions further with the aim of identifying druggable Tbx2/3-associated cofactors, since inhibition of Tbx2/3 function can lead to melanoma senescence.

While the group's work on melanoma explores the effect of signaling pathways on gene regulation, *S. cerevisiae* was used to examine the mechanistic aspects of how gene expression is regulated. Recent findings have determined that transcription activators may be dependent on the prior activity of a class of proteins called 'promoter education factors' (PEFs) that can recognize the same elements as classical activators. PEFs are strictly required for the recruitment of the basal transcription machinery, while activators are necessary for its continued promoter occupancy. The results have

major implications for work on mammalian cells, since Mitf binding sites are also recognized by a ubiquitous factor that may play a role as a mammalian PEF. Understanding how PEFs function and are regulated will be of crucial importance in deciphering the controls that operate to impose specific programs of gene expression to give a biological output.

HUMAN CANCER GENETICS

Gareth L. Bond, Ph.D.

This research program integrates computational, molecular, cellular and human genetic approaches to identify variations in the human genetic code that affect cancer risk, progression and survival and, subsequently, to describe the molecular mechanisms behind these phenotypes. The long-term goal is to gain a better understanding of the contribution of heritable genetics to both susceptibility to and progression of cancer, as well as exposing potential nodes of intervention that could prove valuable in the prevention and treatment of this disease. The task of identifying functional genetic variants is complicated by the extremely large size of the human genome and the relative abundance of variations in the genetic code found between individuals. To overcome this, the laboratory focuses on biologically relevant signaling pathways that are likely to be involved in cancer causation and progression. A large body of evidence suggests that the genes that make up the p53 tumor suppressor pathway are central in reducing cancer frequency in vertebrates and mediating response to commonly used cancer therapies. The p53 pathway is a cellular stress response pathway activated upon stresses such as DNA damage and oncogene activation. Once activated, this signaling pathway initiates cellular responses such as DNA repair, cell cycle arrest or death. One of the main functions of this signal transduction pathway is to ensure the fidelity of the genome, the result of which is the suppression of tumor formation.

Although a regulated, p53-mediated stress response is crucial in suppressing tumor formation and mediating the response to commonly used therapeutics, little is known about the human, inherited genetics of this important signaling pathway. Previous work has demonstrated that single nucleotide polymorphisms (SNPs) in the p53 tumor suppressor gene and the MDM2 oncogene can affect p53 signaling, increase cancer risk and suggest the pathway is under evolutionary selective pressure. Recently, the group attempted to accelerate the identification of functional p53 pathway SNPs by incorporating these characteristics into an analysis of 142 p53 network genes. Genomic signatures of natural selection were first utilized, and subsequently cellular chemosensitivity profiles, to identify genetic markers that affect human cancer. Scans for recent natural selection suggested the MDM4 oncogene, and the gene that encodes a regulatory subunit of the tumor suppressing protein phosphatase 2A (PPP2R5E), reside in naturally selected genomic regions. The group demonstrated that selected SNPs in these genes associate with either altered cancer risk and/or overall survival. For MDM4 SNPs, association studies in five different patient populations revealed the SNPs confer an increased risk for, or early onset of, human breast and ovarian cancers in Ashkenazi Jewish and European cohorts, respectively. For the selected SNP in PPP2R5E, association studies showed significant allelic differences in the onset (up to 19.2 years; p=0.0002) and risk (odds ratio, up to 8.1; p=0.0009) of soft tissue sarcoma development, as well as overall survival (relative risk, up to 3.04; p=0.026). This work not only identifies these genes as key regulators of these human cancers but also, for the first time, exploits evolutionary driven linkage disequilibrium as a means to identify SNPs in the p53 network that might be clinically relevant.

The group used cellular chemosensitivity profiles to identify functional cellular stress response SNPs. Using the NCI60 human tumor cell line anticancer drug screen in a scan of SNPs in the 142 p53 network genes, seven SNPs were

identified that exhibit allelic differences in cellular responses to a large panel of cytotoxic chemotherapeutic agents. The greatest differences were observed for SNPs in *14-3-3tau* (*YWHAQ*) (rs6734469, p=5.6 x 10-47) and *CD44* (rs187115, p=8.1 x 10-24). In soft tissue sarcoma patients the alleles of these SNPs, which associate with weaker growth responses to chemotherapeutics, associated with poorer overall survival (up to 2.89 relative risk, p=0.011) and an earlier age of diagnosis (up to 10.7 years earlier, p=0.002). These findings have defined genetic markers in *14-3-3tau* and *CD44* that might improve treatment and prognosis of soft tissue sarcomas.

PROTEIN QUALITY CONTROL

John Christianson, Ph.D.

This research program aims to delineate the role protein quality control in early secretory pathway plays in initiation and progression of cancer, with a specific focus on changes that occur in the cell surface proteome during metastasis. The long-term goal is to explore quality control mechanisms as a novel point of intervention for cancer therapies. The group has adopted a systems biology approach integrating cellular/molecular biological, biochemical and proteomic techniques to characterize the process of endoplasmic reticulum-associated degradation (ERAD). Genetic mutations, post-transcriptional errors, destabilized domains or missing post-translational modification/binding partners can all give rise to non-functional and orphan translation products, whose unabated accumulation or exposure to the extracellular environment can negatively impact cellular homeostasis and viability. ERAD is an essential quality control mechanism in all eukaryotic cells, which ensures fidelity and regulates the expression of proteins secreted or integral to the cell surface membrane. It has been estimated that at least a quarter of the entire human proteome transits through the secretory pathway, making ERAD an essential surveillance checkpoint for protein expression in all cells.

The complexity and adaptability of the ERAD mechanism is beginning to be appreciated. More than 40 structurally and functionally distinct proteins have been implicated in ERAD, many working in synchrony as constituents of transiently forming macromolecular complexes that span the ER membrane. The group is at the leading edge of efforts to identify novel components and map the basic functional interactions of ERAD. From these mechanism-wide systematic studies, the group decided to concentrate on several aspects. One is the regulation of the ER-resident ubiquitin ligase Hrd1 by auxiliary proteins and intracellular signaling mechanisms. Hrd1 is a core ERAD component, but how and toward which substrates its activity is directed remains unclear. Ongoing studies in the lab suggest that Hrd1 has a robust, adaptive ability to identify and ubiquitinate substrates with disparate topologies and features. The group would like to identify cell surface tumor suppressors and oncogenes, whose expression is governed by ERAD, which may represent future points of intervention for tumor progression and metastasis.

A handful of ERAD genes known to be regulated by ER stress are an integral facet of the unfolded protein response (UPR). Multiple studies have also linked ERAD genes to various forms of cancer. However, the impact of a coordinated response of ERAD genes to stress has not yet been fully ascertained. In a fledgling collaboration with Dr. Sandro de Souza at the São Paulo Branch, the group has embarked on a project to uncover the coordinated transcriptional regulation of ERAD genes across a range of tissue and tumor types, as well as when challenged with environmental stresses and changes in growth conditions.

PROSTATE CANCER RESEARCH

Richard Bryant, Ph.D.

The research of the laboratory is focused on the molecular mechanism of prostate cancer

progression and the goal is to investigate the hypothesis that aberrant ASPP protein function contributes towards the progression of prostate cancer. *p53* is the most frequently mutated gene in human cancer and all tumor-derived *p53* mutants have lost their ability to induce apoptosis. Around half of all human cancers contain mutant *p53* but most prostate cancers express wild-type *p53*. The acquisition of mutant p53 occurs in around 10-20% of prostate cancers and is a particular characteristic of advanced disease. The tumor suppressor function of p53 can, however, be lost through mechanisms other than gene mutation in cancers expressing wild-type p53. Interactions between p53 and cofactors such as the ASPP proteins regulate the promotion of apoptosis, but not cell cycle arrest, functions of p53.

ASPP1 and ASPP2 are p53 co-activators, whereas iASPP competes against ASPP1 and ASPP2 and directly inhibits p53. Breast cancer studies suggest roles for ASPP proteins in carcinogenesis. ASPP1 and ASPP2 are frequently down-regulated in cancers expressing wild-type p53, whilst iASPP is over-expressed in cancers expressing wild-type p53 and normal levels of ASPP1 and ASPP2. It was hypothesized that down-regulation of ASPP1 and/or ASPP2, or up-regulation of iASPP, might be mechanisms whereby p53 function is inhibited in prostate cancer cells that continue to express wild-type p53.

The p53 protein family contains p53, p63 and p73. p63 is expressed by basal cells of normal prostate epithelium and is lost in the development of prostate cancer. Its function is required for the embryonic development of the prostate gland. ASPP proteins are known to regulate p63, and the group hypothesized that normal ASPP protein function is required in order for the prostate to develop normally. It is possible that abnormal ASPP function may contribute to the development of prostate cancer through aberrant p63 function.

Through collaboration with Professor George Thalmann in Switzerland, the group is investigating the expression of ASPP proteins in tissue microarrays containing prostate cancers from radical prostatectomy samples with associated follow-up data. Having optimized conditions for iASPP, ASPP1, ASPP2 and p63 antibodies, the group is now scoring the staining pattern of these arrays in order to investigate potential correlations with clinical parameters. The identification of associations between ASPP expression and prostate cancer progression will form the basis of further translational studies.

The role of ASPP proteins in the development of the mouse prostate gland is also under investigation. The wild-type mouse does not spontaneously develop prostate cancer, but genetically modified mice develop prostate cancer precursor lesions and invasive prostate cancer. Given that p63 function is necessary for mouse prostate gland development, it is hypothesized that normal ASPP protein function is also required for normal prostate development. The initial approach has been to dissect the normal mouse prostate and process this tissue in a standardized manner in order to accurately map out the eight individual lobes of the mouse prostate gland. The peripheral zone of the human prostate represents the site of most prostate cancers, and the transgenic mice that develop prostate cancer tend to do so in the dorsolateral lobe of the mouse prostate gland. It is believed that the dorsolateral lobe of the mouse prostate is analogous to the peripheral zone of the human prostate. It is important to carefully assess the individual lobes of the mouse prostate in order to investigate any phenotype in the knock-out mouse. Having ensured that the normal mouse prostate can be examined accurately, the expression pattern of ASPP proteins in this gland is being studied. The macroscopic and microscopic phenotype of the prostate gland of $ASPP2^{+/-}$, $ASPP2^{-/-}$, $iASPP^{+/-}$ and $iASPP^{-/-}$ mice is also under investigation.

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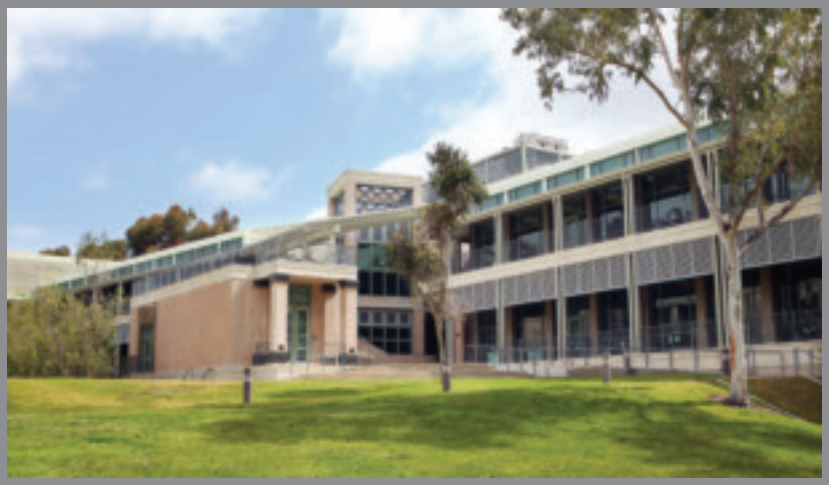
Director's Message

During 2009, we continued to make discoveries of importance; Branch staff received international awards and honors; and we grew in roles of significance in scientific societies as well as journal editorial and program review responsibilities. The seven groups at the San Diego Branch focus mainly on cancer genetics, cell signaling, gene regulation and mechanisms of cell division. The Branch has made important achievements investigating the processes that cells use to maintain the integrity of their genome, and how failure in these processes can lead to cancer.

Dr. Bing Ren was promoted to Member and earned an accelerated promotion to Professor in the University. He is only the first of the outstanding scientists we recruited a few years ago to achieve this and we expect similar accomplishments by others in the next year or two. Dr. Don Cleveland became Chair of the Cellular and Molecular Medicine Department of the UCSD Medical School while remaining a Member of the Institute, thereby establishing a new degree of integration and partnership with the University. Many postdoctoral fellows and graduate students moved on to independent positions in academia or industry after finishing their training with us.

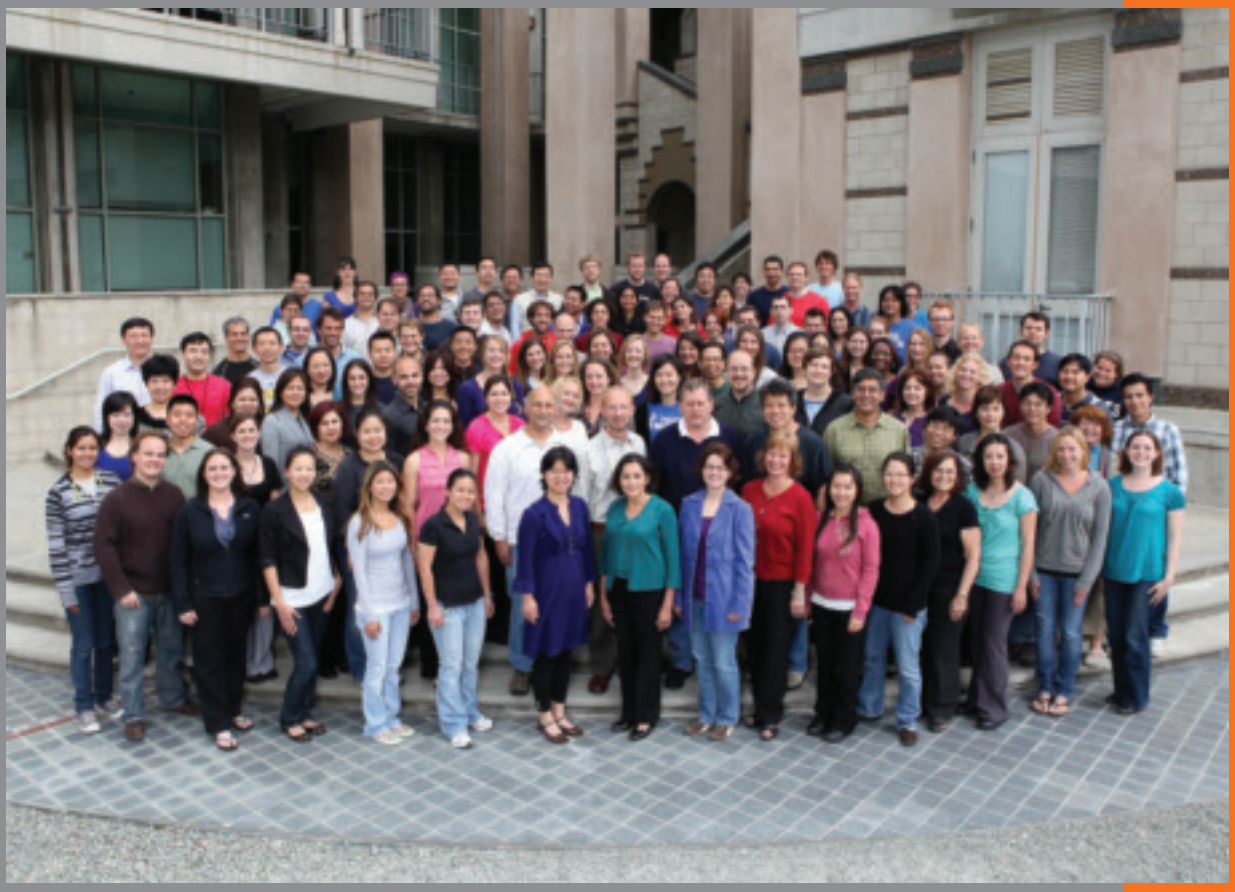
Moreover, the number of collaborative interactions between and among our research groups in the Branch and with other national and international investigators has grown significantly. Another measure of our success is the large and steadily increasing level



of competitive grant support won by Branch staff. In the past year, we developed a collaborative effort with the New York office and established a Small Molecule Group that is tasked with the development of chemical inhibitors of cancer-related enzymes and receptors, both for enhancing research and as a prelude to clinical drug development.

The quality, accomplishment and breadth of expertise of Branch staff has been praised and welcomed by the UCSD Chancellor, Dr. Marye Anne Fox, and the Dean of UCSD Medical School, Dr. David Brenner. Our partnership with the University has never been stronger and we look forward to our future with great optimism and gratitude to the Institute for its support and faith in us.

Web Cavenee



TUMOR BIOLOGY

Webster K. Cavenee, Ph.D.

The laboratory comprises two sections with complementary interests.

MOLECULAR CYTOGENETICS

Karen Arden, Ph.D.

Understanding the consequences of genetic aberrations associated with cancer is the primary focus of this section of the laboratory, concentrating on the characterization of the function of the PAX3-FOXO1 fusion gene formed by the t(2;13) translocation found in the alveolar subtype of the skeletal muscle tumor rhabdomyosarcoma. Experiments designed to determine how PAX3-FOXO1 expression contributes to the development of muscle cell-derived tumors resulted in the discovery that the fusion protein misregulates gene expression and interrupts myogenic differentiation through a unique gain of function mechanism. PAX3-FOXO1 contributes to tumorigenesis by binding to and destabilizing a transcription factor, EGR1, required for upregulation of the cell cycle regulator p57KIP2.

The quality control ubiquitin ligase, CHIP1 ubiquitinates and targets PAX3-FOXO1 for destruction. EGR1 is promoting unregulated cell cycle progression also degraded as a consequence of its association with PAX3-FOXO1. This is a novel mechanism of action for a chromosomal translocation-derived transcription factor and suggests that proteasome inhibitors might be a new and effective approach for treating ARMS. Experiments are currently underway to determine whether this mechanism is a general feature of fusion genes associated with other pediatric solid tumors. A second focus is the normal function of the forkhead FOXO sub-family of transcription factors. Disruption of these genes, as in alveolar rhabdomyosarcoma, has been associated with cancer. FOXOs have been implicated in a variety of important cellular processes, such as insulin metabolism, response to oxidative stress and apoptosis. However, the role of FOXO factors during the cell cycle has not been fully investigated. Recent experimental results have determined that changes in FOXO1 mobility are associated with different phases of the cell cycle in multiple cell types with the most significant change occurring during M phase.

In collaboration with Dr. Huilin Zhou, it was determined that the decrease in FOXO1 mobility observed at M phase is due to phosphorylation at multiple sites by CDK1/CyclinB1. This multisite phosphorylation of FOXO1 reduces its transcriptional activity and promotes cell proliferation. Thus, CDK1/CyclinB1 phosphorylation of FOXO1 provides a mechanism for assuring proper cell cycle progression and supports the role of FOXO1 as a tumor suppressor. A third focus centers on discovering the genes directly targeted by the FOXO transcription factors. Genome-wide mapping and analysis of FOXO target genes are underway in collaboration with Dr. Bing Ren. All FOX genes contain a very similar 100 amino acid DNA binding domain and differ significantly outside this domain with respect to amino acid sequence. The FOXO subfamily of transcription factors shares a 6 amino acid sequence within the DNA binding domain not seen in any other family members. Determining the specific gene targets of the FOXO family members will contribute to better understanding their normal role in gene regulation and how their normal function is perturbed in cancer.

HUMAN CARCINOGENESIS

Frank Furnari, Ph.D.

Genetic alterations that drive the genesis of high-grade gliomas is the main focus, notably the commonly amplified and truncated epidermal growth factor receptor gene (ΔEGFR or EGFRvIII) and mutation of the PTEN gene, which antagonizes PI3K/Akt signaling. Current projects include: 1) how expression

of ΔEGFR potentiates tumor heterogeneity and hence aggressiveness; 2) the role of this receptor in driving tumor maintenance; and 3) how modulators of the PTEN/PI3K signaling axis influence the effectiveness of receptor-directed therapeutics. For the tumor heterogeneity initiative, using a combination of human glioma tissues, cell lines and stem cells, and mouse astrocytes, it has been determined that a paracrine mechanism prompted by secretion of IL-6 and/or LIF cytokines from ΔEGFR-expressing cells can recruit wild type EGFR-expressing cells into accelerated proliferation *in vivo*.

Ablating these cytokines or their receptor, gp130 uncouples this cellular cross-talk and potently attenuates tumor growth enhancement. These findings demonstrate that a minor tumor cell population, through a paracrine mechanism, can drive accelerated growth of the entire tumor mass and therefore such heterotypic cancer cell interactions could be of potential therapeutic significance. In the case of tumor maintenance, the laboratory has demonstrated that *in vivo* silencing of regulatable ΔEGFR attenuates glioma growth. However, similar to the clinical experience, after a period of stasis, tumors eventually regained aggressive growth (escapers). To determine how tumors acquired this ability, comparative gene expression analysis was performed and a novel gene identified, KLHDC8A that was highly expressed in tumors that had escaped ΔEGFR dependence. KLHDC8A was also expressed in human glioma tissue. Knockdown of KLHDC8A expression in escaper tumors suppressed tumor growth. These results indicate that ΔEGFR is required for both glioma establishment and maintenance, and gliomas undergo selective pressure *in vivo* to employ alternative compensatory pathways to maintain their aggressiveness when confronted with ΔEGFR activity cessation.

Lastly, although PTEN mutation can also lead to EGFR-directed therapy resistance, some gliomas retaining wild type PTEN also demonstrate resistance, suggesting mechanisms exist that mimic PTEN mutation. Investigating modulators of the PTEN/PI3K signaling axis, the laboratory has identified such a mimic in the form of PTEN tyrosine phosphorylation in resistant gliomas. This resistance can be recapitulated in mouse astrocytes and human glioma cells that express tyrosine phosphorylated wild type PTEN and ΔEGFR. It is reversed upon mutation of identified tyrosine phosphorylation sites to phenylalanine. These findings provide a mechanistic link between PTEN regulation and drug resistance and suggest that drugs targeting the kinases that mediate PTEN tyrosine phosphorylation in combination with EGFR inhibitors could circumvent some cases of EGFR kinase inhibitor resistance.

CANCER GENETICS

Richard D. Kolodner, Ph.D.

The major research focus of the laboratory is on two broad projects using the yeast *Saccharomyces cerevisiae* and other model organisms. The first is to elucidate the genetic and biochemical mechanisms of the DNA mismatch repair (MMR) pathways that prevent cells from accumulating mutations due to errors in DNA replication. The second is to define the pathways that cells use to prevent the accumulation of genome rearrangements, such as translocations, that accumulate in many types of cancer cells. The long-term goal of these projects is to understand how mutations and genome rearrangements arise in cancer cells at increased rates and whether the underlying aberrant mechanisms can be exploited for therapeutic means. In the area of MMR, the work has primarily focused on understanding the biochemical mechanisms of MMR. Deuterium exchange mass spectrometry (DXMS) has been exploited as well as partial proteolysis to examine the early steps of MMR.

Critical to these studies has been the analysis of dominant

mutations in the *MSH2* and *MSH6* genes that allow trapping of early intermediates in MMR. Using DXMS, the Msh2-Msh6 heterodimeric mismatch recognition protein binds to base paired DNA by forming a static ring around the DNA. When a mispaired base is present in the DNA, two regions of the Msh6 subunit contact the mispair, and this appears to license two different ATP binding-induced conformational changes of the Msh2-Msh6 complex required for subsequent steps in MMR. One of these steps is conversion of the bound Msh2-Msh6 to a clamp form that slides along the DNA and the other is conversion to a form that binds a key MMR factor, the Mlh1-Pms2 complex. In addition, using genetic and proteolysis approaches, ATP binding and hydrolysis drives cyclical remodeling of the nucleotide-binding region of the protein required for both sliding clamp formation and binding of the Mlh1-Pms1 complex.

Furthermore, using a combination of genetics and analysis together with DXMS, a small surface on the Msh2 subunit responsible for binding the Mlh1-Pms1 complex has been identified. With this improved understanding of the critical mispair recognition step in MMR in hand, future efforts will be to understand how other proteins that function in MMR are engaged and activated to ultimately result in a repair reaction.

S. cerevisiae has also been used to further explore the functional consequences of mutations in human MMR genes associated with inherited cancer susceptibility. By using *S. cerevisiae* to model a series of missense mutations in human MMR genes, a number of cancer-associated mutations in different MMR genes that cause little or no disruption of MMR were identified. Using the ability to readily perform enhancer analysis in *S. cerevisiae*, it was demonstrated that many of the weak human MMR gene alleles are capable of interacting with other weak alleles in different MMR genes resulting in complete MMR defects. These studies have, for the first time, provided the basis for identifying and studying polygenic MMR gene defects and their association with cancer susceptibility.

S. cerevisiae genetics coupled with systems biology inspired approaches is also used to identify the genes and pathways cells use to prevent genome instability. This is a critical area of investigation as most cancers are associated with increased genome instability and the genome rearrangements that arise are thought to play a role in driving the development and progression of cancer. In the last year, a new assay was developed that allows measurement on the accumulating genome rearrangements mediated by both single copy and repeated DNA sequences. Using these assays, an unexpected finding indicated there are a series of pathways exclusively devoted to suppressing repeat sequence-mediated genome rearrangements and similarly there are separate pathways that suppress single copy sequence-mediated rearrangements as well as pathways that suppress both types of genome rearrangements. Current efforts are directed at experimentally verifying these genes and pathways and using genomic methods to directly explore whether defects in the human homologs of these genes are present in different cancers.

CELL BIOLOGY

Don W. Cleveland, Ph.D.

Research is focused in three directions: 1) mechanisms of mammalian chromosome movement and spindle assembly during mitosis and how errors in these processes contribute to tumorigenesis; 2) the basis for epigenetic specification of centromeres; and 3) mechanisms of neuronal growth and death, especially related to treatment of the neurodegenerative diseases Amyotrophic Lateral Sclerosis and Huntington's disease. In the past year, the major cell cycle control mechanism in mitosis, the mitotic checkpoint, was reconstructed using all purified components. With this

approach, each centromere unattached to spindle microtubules was shown to serve as a catalytic surface for generation of an inhibitor that arrests cycle advance to anaphase. Using gene replacement in mammalian cells, NuMA, a protein previously identified by the laboratory, was shown to be crucial for establishment and maintenance of mammalian spindle poles. Also identified was the chaperone (HJURP) for the centromeric chromatin and demonstrated that it is required at exit from mitosis for replication of centromeric chromatin through assembly of a centromere specific nucleosome constructed of the histone H3 variant CENP-A. A highly unexpected finding was chromatin remodeling at sites of DNA damage, including double strand DNA breaks, utilizes CENP-A assembly at DNA damage sites as a means to promote DNA repair.

It has been long known that determination of the correct axonal size of large myelinated axons, including those of motor and sensory neurons, is mediated by a three dimensional crosslinked array of neurofilaments as a means to support axonal diameter. It was demonstrated that this requires an outside-in signal from the myelinating cell to the underlying axon which targets neurofilament phosphorylation as a means to establish a volume-supporting filamentous superstructure. In the past year, using gene replacement in mice, it was shown that the target for this signal is not the repetitive lysine-serine-proline motifs in the larger two neurofilament subunits as had been predicted. The laboratory identified the mechanism through which gigaxonin, whose mutation underlies the sensory neuronal disease giant axonal neuropathy, controls intermediate filament organization. Lastly, exploiting its previous discovery of damage to the vasculature within the central nervous system as an early event in inherited ALS, it was successfully demonstrated that peripheral treatment with the serum protease Activated Protein C (APC) can slow progression of inherited ALS in rodents. This was shown to be mediated by APCs transcytosis across the endothelia of the capillaries and subsequent cleavage of surface receptors on motor neurons and microglia within the central nervous system. Receptor cleavage and activation in turn inhibits a key transcription factor (Sp1), thereby transcriptionally suppressing synthesis of an ALS causing mutation in superoxide dismutase. Since APC is already an FDA approved drug, this discovery has enabled a clinical trial for treatment of inherited ALS with APC.

CHROMOSOME BIOLOGY

Arshad Desai, Ph.D.

Understanding the mechanisms that distribute the genome during cell division is the main focus; specifically investigating kinetochores, macromolecular assemblies that form on chromosomes to connect them to spindle microtubules during cell division. The kinetochore-spindle microtubule connection is central to accurate segregation of chromosomes and prevention of aneuploidy—it achieves this goal through both mechanical and checkpoint signaling activities. This interface is also the target of anti-mitotic chemotherapeutic drugs, such as taxol. One project area is focused on the conserved KNL-1/Mis12 complex/Ndc80 complex (KMN) protein network, comprised of nine interacting proteins, provides the core microtubule-binding activity at the kinetochore. In the past year, the laboratory has extended analysis of this network to the checkpoint signaling function of the kinetochore and established that the KMN network provides the kinetochore scaffold for generating the checkpoint signal.

In addition, efforts revealed that the checkpoint pathway is bifurcated, one operates at the kinetochore and the other in the cytoplasm. In a collaborative effort, the distribution of the components of the KMN network at

nanometer resolution within the structure of the kinetochore in human cells was mapped. These efforts are a guiding principle in the investigation to understanding the mechanical and checkpoint signaling activities integrated at the kinetochore. The analysis of the KMN network to meiosis has also been extended, where homologous chromosomes, rather than sister chromatids, must be segregated away from each other. Efforts in this area have revealed a new kinetochore-independent mechanism operating during anaphase of oocyte meiosis to separate chromosomes.

In a second project area, the mechanisms that specify kinetochore formation at a localized site on chromosomes are being studied. The specification event involves formation of specialized chromatin containing a histone H3 variant called CENP-A/CenH3. The specialized chromatin domain containing CENP-A is proposed to be propagated by DNA replication and replenished during early G1 to epigenetically maintain the identity of this specialized chromosomal region. The deposition of CENP-A occurs *de novo* following fertilization in *C. elegans* and, using an unbiased functional genomic strategy, a conserved protein family with a predicted conserved DNA binding domain involved in this deposition has been identified. The human member of this protein family is required for centromeric chromatin formation in human cells, indicating conservation of function. In addition, the laboratory has analyzed the distribution of the centromeric histone variant genome-wide in *C. elegans*, which revealed a surprising connection to gene expression.

GENE REGULATION

Bing Ren, Ph.D.

The research focus of the laboratory is understanding the gene regulatory mechanisms that determine cell fate and differentiation in human cells. Two related problems under investigation are: 1) the transcriptional regulatory sequences that control cell-specific gene expression programs; and 2) the epigenetic processes that regulate cell fate in each human cell type.

To achieve these goals, high-throughput approaches have been developed for systematic identification of promoters, enhancers, and insulator elements throughout the human genome. Comprehensive maps of enhancers for undifferentiated human ES cells and various differentiated cell types were generated. The results revealed that the activities of transcriptional enhancers are highly specific to each cell type and likely play a critical role in regulating cell-specific gene expression. High throughput approaches to characterize genome-wide chromatin modification states in human embryonic stem cells and fibroblasts were also utilized. Analysis of these epigenomic profiles revealed dramatic differences in the chromatin landscapes between the pluripotent and lineage-committed cell types, which suggest potential mechanisms for cellular differentiation and reprogramming. In the past year, two large-scale collaborative projects were undertaken:

- *San Diego Epigenome Center* Comprises five participating laboratories funded by the NIH Roadmap Epigenome Project. In 2009, the Center produced the first comprehensive map of epigenome in human cells, including nucleotide resolution of DNA methylation throughout the human genome. This map reveals widespread differences in DNA methylation between human embryonic stem cells and differentiated cell types, and was selected as one of the top ten scientific discoveries in 2009 by *Time* magazine.

- *ENCODE and modENCODE consortium* Consists of hundreds of researchers worldwide aimed at identifying functional elements in the human genome and genomes of several model organisms.

MITOTIC MECHANISMS

Karen Oegema, Ph.D.

Using the nematode *C. elegans* as a model system to study cell division, three major areas of research are being pursued: 1) molecular mechanics of cytokinesis; 2) centriole duplication and function; and 3) functional genomics of cell division.

Cytokinesis completes cell division by physically remodeling the mother cell to partition a single copy of the replicated genome into each daughter cell. In animal cells, cytokinesis is accomplished by constriction of a contractile ring that forms a belt around the cell middle to pinch the mother cell into two daughter cells. Understanding how the contractile ring divides the cell is expected to facilitate efforts to prevent uncontrolled cell division in cancer. Through experiments designed to determine how cytokinesis scales with cell size during embryogenesis, the laboratory recently uncovered a remarkable property of the contractile ring. All animals begin life as a fertilized single-celled embryo. During embryogenesis, embryos are progressively partitioned into smaller and smaller cells by successive rounds of cell division. Cell volume and the length of the contractile ring around the cell middle are reduced at each successive round of cell division.

By contrast, the dimensions of the chromosomes carrying the genetic material segregated to the daughter cells remain constant. In recent work, the lab demonstrated that despite the decrease in cell size during embryogenesis, the duration of cell division remains the same. This constant timing likely facilitates the coordination of chromosome segregation with cell division. The constant duration of cytokinesis results from the fact that contractile rings close at a constant rate that is proportional to the initial size of the cell; rings in larger cells constrict at a proportionally higher rate than rings in smaller cells. Analysis of the fate of three contractile ring components revealed that the amount of ring components decreases in direct proportion to ring perimeter as the ring closes, without turning over. Cumulatively, this work suggests that the contractile ring initially forms and is progressively disassembled as the ring constricts. This work led to a new "contractile unit" model that proposes contractile rings assemble from units of fixed size that shorten at a constant speed during constriction without being lost. Bigger rings contain more units and therefore constrict at a proportionally higher rate.

To identify new genes involved in cell division the lab has taken an RNAi-based "high-content" screening approach. High-content screens utilize a complex biological assay that samples a wide variety of cellular functions in a relatively unbiased fashion. In a high content screen, each feature associated with a gene knockdown is assessed independently according to a controlled vocabulary and the compilation of features defines a phenotypic profile. In principle, high-content screening is the method of choice for generating a functional map for a collection of genes of unknown function. In practice, the difficulties of developing and executing a suitable assay, scoring the phenotypes, and analyzing the data have limited its use.

C. elegans early embryogenesis has been a prototype system for large-scale functional genomic analysis of essential genes. Of the 20,000 *C. elegans* genes, ~10% are required for embryo production or viability. This set of 2,000 genes was previously screened by filming the first two divisions of embryos individually depleted of each gene product. This screen provided high quality data that allowed for the functional classification of ~400 genes. However it did not provide high quality functional information on the ~560 genes whose inhibition blocks embryo production (the sterile collection). In recent work, the lab functionally characterized the sterile collection by performing a

high-content screen using two-color fluorescence confocal microscopy to examine gonad structure in anesthetized worms after individual depletion of each gene product. The gonad-morphology data was analyzed by binary scoring for 94 potential defects. Hierarchical clustering and extensive manual refinement was used to partition the genes, including the 175 uncharacterized genes in this collection, into 102 phenotypic classes. In addition to identifying new genes involved in cell division, embryo production and development, this work also catalyzed the development of new data analysis methods. To generate a map of the functional connections uncovered by this analysis, plans to develop a means of assessing the specificity of functional connections are underway in collaboration with Fabio Piano's lab. This method should allow automation of the labor intensive process of generating functional maps from high-content screening data.

PROTEOMIC BIOLOGY

Huilin Zhou, Ph.D.

The research of the laboratory is concentrated in three areas: 1) enzymology of the regulation of DNA damage checkpoint using the yeast *Saccharomyces cerevisiae* as a model organism; 2) identification and characterization of pathways controlled by the DNA damage checkpoint that suppress genome instabilities; and 3) development of quantitative proteomics technology for a global and quantitative analysis of protein phosphorylation and protein-protein associations.

Genetic studies have shown that mutations in genes encoding DNA damage checkpoint kinases cause the elevated genome instabilities frequently observed in cancer cells. In particular, mutation of the ATM kinase causes *ataxia telangiectasia*, a cancer prone syndrome. Using the yeast *S. cerevisiae* as a model system, the laboratory overproduced and purified most of the proteins in the DNA damage checkpoint including the Tel1 kinase, the ATM homolog in yeast, and others. The laboratory also developed a biochemical assay to study how these proteins work together to mount a robust checkpoint response. A recent study established the first biochemical reconstitution of the DNA damage checkpoint kinase cascade and demonstrated the role of DNA replication fork associated protein Mrc1 in mediating activation of this kinase cascade. This research is being extended to study regulation of the Tel1 kinase and other components on DNA damage checkpoint activation.

In a second area of investigation, the lab is interested in understanding the molecular basis of the roles of DNA damage checkpoint kinases in genome maintenance. To this end, it is necessary to identify their *in vivo* substrates; to determine when, where and how they are phosphorylated; and to understand their functions.

A new quantitative mass spectrometry technology was recently applied to identify most of their known substrates, identified by many over the past decade, and almost three times as many novel substrates of these kinases. It was further demonstrated that Replication Protein-A and its associated chromatin structures help to mediate the *in vivo* action of the checkpoint kinases and identified chromatin. The laboratory will continue to study how specific substrates are recruited to the site of DNA damage and phosphorylated by the DNA damage checkpoint kinases using a combination of quantitative mass spectrometry, genetic, cell biological and biochemical analyses. In the third area, the lab has been developing mass spectrometry based technologies for high-throughput and global analysis of protein phosphorylation. This involves the development, optimization and integration of several analytical and computational tools. These tools study the molecular basis of the functions of DNA damage checkpoint and how they are related to elevated genome instabilities observed in a number of checkpoint mutants.

SMALL MOLECULE DISCOVERY

Andrew K. Shiau, Ph.D. and Timothy C. Gahman, Ph.D.

Established in the last quarter of 2009 as a collaborative venture between the San Diego Branch and the technology development effort led by Dr. Jonathan Skipper in the New York office, the group focuses on the identification and synthesis of novel small molecules that regulate the activity of enzymes and receptors important in cancer biology. The group uses a combination of high-throughput biochemical and cellular assays, robotic screening technologies, and structure-based drug design to identify lead compounds. These small molecules are iteratively optimized using synthetic chemistry for potency, selectivity and pharmacokinetic properties, and evaluated in proof-of-concept studies in human tumor xenograft and other animal models of disease.

Currently, the group is engaged in efforts to generate potent and selective inhibitors of polo-like kinase 4 (PLK4), a master regulator of centriole biogenesis. Centrioles are large, intricately structured macromolecular assemblies, which recruit pericentriolar material to form mature centrosomes, the microtubule organizing centers of the cell. Hence, small molecules that block PLK4 activity should suppress the centrosomal amplification that may underlie the genetic instability of certain cancers. In the past year, the laboratory has screened multiple small molecules against PLK4 and has identified one with significant inhibitory properties in both biochemical and cellular assays. Computational modeling of PLK4 bound to this inhibitor indicated regions of the core scaffold that would be amenable to chemical modification. Synthesis of chemical analogs has yielded a compound with nanomolar potency and at least ten-fold selectivity for the enzyme relative to a highly structurally-related kinase.

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Director's Message

The Branch is now in its fourth year at the Hospital Alemão Oswaldo Cruz where it enjoys a state of the art environment. 2009 was a most successful year evidenced for example by a significant increase in the number of scientific publications: 38 versus 23 in 2008. I was proud to be the recipient of the Ordem Nacional do Mérito Científico (National Order of Scientific Merit) and Dr. Sandro de Souza was named one of the World Economic Forum's Young Global Leaders for 2009.

The Branch was privileged to host a Scientific Advisory Committee meeting which convened in São Paulo the first week of March. It was a unique opportunity for us to discuss proposals and programs and to obtain valuable feedback on our work. It was also helpful in our preparation to convert the Branch to a more focused and locally funded research effort in 2016 when Mr. Ludwig's support in Brazil is exhausted.

Going forward, the Molecular Biology and Genomics of Cancer group headed by Dr. Ana Maria Camargo and the Computational Biology of Cancer group led by Dr. Sandro de Souza will comprise the major efforts of the Branch. The two remaining groups, the Cell and Molecular Biology of Cellular Prions and the Molecular Epidemiology and Immunology of HPV, led by Dr. Vilma Martins and Dr. Luisa Villa, respectively, will move to new host institutions. It is anticipated that Dr. Martins and her group will be relocated by the end of 2010. The Virology group is reorganizing and exploring new opportunities that will enhance its work toward new discoveries in the field of HPV infections and related tumors.

This was the beginning of the end of an extended era of success for the Branch in São Paulo as an independent, fully-funded entity spanning more than 25 years. We have

much to be proud of, not the least of which was the move to the wonderful new environment that I was able to orchestrate. I felt privileged to be the Branch Director until mid-2009.

New challenges and opportunities abound. We hope to have the wisdom to make the most appropriate choices both individually and as a group. Therefore, with a sense of mission accomplished, I'm looking forward to a bright and promising future.

Luisa Villa

VIROLOGY

Luisa L. Villa, Ph.D.

Efforts are devoted to two lines of research: epidemiology of HPV infections and associated diseases on the one hand, mechanism of carcinogenesis of the main HPV types, and variants; as well as characterization of immune responses driven by HPV on the other.

An international *HPV Infection in Men Study (HIM)* is being conducted tracking approximately 4,000 men aged 18–44 and 450 men aged 45–70 every six months for four years. The goal is to understand the natural history of HPV infection in order to develop effective programs to reduce HPV. After obtaining informed consent, subjects are submitted to visual inspection of the skin and external genitalia, in addition collection of urine, blood, oral cells, penile skin and anal samples are collected and a structured epidemiologic questionnaire administered. If anogenital lesions are present at any of the clinic visits, biopsies are taken for histopathologic examination.

Recruitment of eligible men was concluded in September 2009, with a total of 4,292 men (1,443 – Brazil; 1,426 – US; 1,423 – Mexico). The Brazilian cohort was composed of men from a public health clinic and the general population. To date, retention rates are 98% at the end of the second visit (six months from study enrollment). That same month, initial participants performed their tenth and last study visit. Results of biopsies, HPV and tested STDs results were made available to physicians and participants. Treatment was given whenever necessary. This study is generating several publications, including those related to prevalence and incidence of HPV infection in men which are high and stable along the different age ranges studied. The first results of anal HPV infections are being analyzed and submitted for publication. This cohort will continue through 2014.

The group analyzed the effect of tumor necrosis factor (TNF) on migration of primary human keratinocytes (PHK) expressing HPV16 oncogene using the "wound healing" assay. Results show that cells expressing the E6 oncogene exhibit enhanced mobility in the presence of this cytokine. In order to study the potential effect of cells expressing HPV oncogenes on normal cells, the group performed "wound healing" assays using normal PHK as targets and conditioned medium derived from cultures of PHK expressing E6E7 oncogenes. Medium conditioned by HPV16 oncogenes expressing cells inhibited PHK migration compared with control cultures incubated with medium conditioned by normal PHKs. These preliminary results indicate that PHK expressing HPV16 oncogenes exhibit a higher migratory capacity in the presence of a pro-inflammatory stimulus. Moreover, they suggest that PHK expressing the viral oncoproteins can inhibit normal cell motility by the secretion of soluble factors. A supernatant analysis is being conducted in order to identify the molecules involved in this effect.

Organotypic cultures of PHK expressing HPV16 E6, E7 or E6E7 treated with TNF and TNF-related apoptosis-inducing ligand (TRAIL) have been obtained. The effect of TNF on cell proliferation was analyzed by BrdU incorporation. RNA samples obtained from these cultures were used to analyze the effect of TNF on global gene expression of E7 expressing cells and study the effect of HPV16 oncogenes on TNF and TRAIL-regulated pathways using specific RT-array platforms for TNF, NFκB and apoptosis related genes.

Another study relates to the effect of HPV oncogenes on the expression and activity of different matrix metalloproteinases (MMPs) and their inhibitors both in monolayer and organotypic cultures. In these experiments, viral oncoproteins collaborate to inhibit the expression of the MMP regulator reversion-inducing cysteine rich protein with Kazal motifs (RECK) without affecting the expression or activity of MMP2, -9 or 14.

The group is continuing to pursue characterization of differential oncogenic potential of natural molecular variants of high-risk HPVs, particularly types 16 and 18. Nucleotide variability among HPV molecular variants can be as high as 5% in the regulatory region (LCR). In order to identify novel transcription factors (TFs) that might be involved in the regulation of E6/E7 expression, a high-throughput assay based on reverse transfection of 704 transfection ready cDNA plasmids covering all commonly known transcription factors was used. The *Transcription Factors GFC-Transfection Array* (Origene, Madison, EUA) was tested for the regulation of a reporter plasmid containing the complete HPV-18 LCR driving the expression of luciferase. Experiments were conducted in C33 cells. Of the 704 genes tested, twenty eight activated and thirty six inhibited HPV-18 LCR more than twofold. TFs of each group were chosen for further validation. For this purpose, C33 cells were co-transfected with expression plasmids of the TFs under study in addition to recombinant vectors LCR-Luciferase of different variants of HPVs 16 and 18. Some differences were observed in activation or repression between prototypes and molecular variants of HPVs 16 and 18. Among the TFs identified which could potentially interfere with the regulation of HPV expression, only GATA3, p53 and myb have putative binding sites within the LCR sequence as indicated using the TRANSFAC database. Several TFs not previously implicated in the regulation of HPV gene expression were identified. Many of these factors have also been described to be mutated in cancer or are putative cancer biomarkers, and should be targets for further studies. *In vivo* interactions of some of these factors with LCR of HPVs type 16 or 18 are being studied using CHIP and EMSA assays.

Differences in the immortalization potential between molecular variants of HPVs 16 and 18 are being investigated. Primary human foreskin keratinocytes were infected by recombinant retroviral vectors expressing the complete E6 and E7 genes. Data obtained indicated differences in a colony formation assays. Some colonies were isolated and are being cultivated after 30 passages or eight months in culture; these experiments are ongoing. Some of the colonies have reached passage 30 and studied to evaluate potential differences in the expression profile of these immortalized cells using the Human Cancer Pathway Finder PCR array.

One potential marker is the protease kallikrein-7 (hK7). Analysis of hK7 expression was performed in 367 histological samples of cervical lesions of different grades. A significant trend between hK7 positivity and severity of cervical disease was observed, suggesting that hK7 might be a useful biomarker for high-grade cervical dysplasia and cancer. Similar analyses are being performed using other biomarker candidates. Preliminary data suggest superoxide dismutase-2 (SOD-2) as a potential marker for invasive cervical disease.

The innate immune system is the first line of defense against invading pathogens. Toll like receptors (TLRs) recognize pathogen associated molecular patterns (PAMPs) and have a very important role in the immune response. TLR-mediated signaling leads to the activation of different transcription factors, such as NFκB. TLRs are linked to different pathologies, including cancer, and both plasma membrane and endosome associated TLRs recognize a wide variety of viruses leading to the production of different cytokines and chemokines. Little is known about the interactions between TLRs and HPV. The role of E6 and E7 of high risk HPV16 in TLR-mediated signaling is under investigation and 16E6 potentiates MyD88- and TRAF6-dependent NFκB activation. Moreover, 16E6 interacts directly with both molecules, suggesting a novel MyD88- and TRAF6- dependent mechanism of regulation of NFκB function by E6 oncoproteins from high-risk HPVs. The role of 16E6 in TLR signaling in primary human keratinocytes is being studied.

The characterization of cellular immune responses against HPV associated tumors in mice is ongoing. Hematopoietic chimeras are being used, where lymphoid populations from wild type mice are adoptively transferred to RAG1-/- immunodeficiency mice. Using this model, isolated T cells from mice bearing HPV associated tumors are able to respond against HPV antigens and protect against tumor growth better than T cells transplanted together with B cells. The group is investigating the cross-talk between these populations to understand better the mechanism by which B cells may influence in anti-tumor responses. IL-10 is an important molecule, among others secreted by tumor associated macrophages, because it induces a regulatory phenotype on T cells and facilitates tumor growth. The group published a paper demonstrating macrophages that infiltrate HPV associated tumors in mice are M2 and inhibit anti-tumor T cells responses through the induction of regulatory T cell phenotype.

The project on the effect of the HPV associated tumor microenvironment on myeloid populations has *in vivo* and *in vitro* goals. *In vivo*, mice with tumors have increased myeloid cell proliferation in the spleen and bone marrow and the group is investigating whether molecules secreted by the tumor stimulate this effect. *In vitro*, using tumor cell and myeloid cell co-cultures, macrophages incubated with E6/E7 expressing keratinocytes produce significantly higher levels of IL-10 than those incubated with control keratinocytes. Ongoing studies intend to explain how E6/E7 expressing cells control gene expression and the phenotype of myeloid cells.

MOLECULAR BIOLOGY AND GENOMICS

Anamaria Camargo, Ph.D.

Two complementary programs have been undertaken by the molecular biology and genomics group: functional genomics and clinical genomics. The functional genomics program explores the role of two genes (ADAM23 and SIGIRR) differentially expressed in breast tumors identified as part of the effort to characterize new cancer-related genes using both experimental and computational approaches. The ADAM23 was identified as a gene silenced by DNA methylation in breast tumors. ADAM23 silencing is strongly associated with poor disease outcome and confers enhanced invasion, migration and adhesion to the tumor cells by negatively modulating $\alpha v\beta 3$ integrin activation during metastasis. The results suggest that ADAM23 acts as a metastasis suppressor gene and ADAM23 promoter methylation could be used as a complementary marker for metastasis risk assessment in breast cancer. SIGIRR belongs to the IL-1R superfamily and acts as a decoy receptor that negatively modulates IL-1R and TLR4 signaling. SIGIRR was identified as an overexpressed gene in c-erbB2 positive tumors and demonstrated that SIGIRR overexpression downregulates lymphocyte recruitment and secretion of pro-inflammatory cytokines such as IL-8, TNFα and CXCL2. The results support a role for SIGIRR overexpression in immune suppression and immune evasion.

The clinical genomics program is focused on colon and breast cancer. The colon cancer initiative benefits from a strong collaboration with the computational biology group, led by Dr. Sandro de Souza, and with a renowned group of surgeons led by Drs. Angelita and Joaquim Gama from the Hospital Alemão Oswaldo Cruz. As part of this initiative, a tumor bank for collection of biological samples and clinical records from colorectal cancer patients was established. Five hundred patients were enrolled over the past two years and samples used in two projects: prediction of response to chemoradiation in rectal cancer patients using mRNA-seq and identification of novel cell surface targets overexpressed or mutated in colorectal tumors.

The group sequenced the genome and transcriptome of a breast tumor cell line (HCC1954) and a lymphoblastoid cell line (HCC1954BL) derived from the same patient. It was a major collaborative project involving

sequencing and bioinformatics groups both in Brazil (São Paulo Branch, National Laboratory for Computational Science and University of São Paulo) and the United States (San Diego and New York Branches and the J. Craig Venter Institute) carried out under the auspices of the Brazilian Network for Cancer Research. The normal genome presents very few somatically acquired chromosomal rearrangements compared with the tumor genome, but accumulates a similar number of somatic point mutations. Results are consistent with a crucial role for chromosomal rearrangements in tumorigenesis and suggest it is permutation rather than frequency of point mutations in the tumor genome that is crucial to complete tumorigenic transformation.

COMPUTATIONAL BIOLOGY

Sandro José de Souza, Ph.D.

The potential of cancer research computational biology can be achieved if integrated into a program involving experimental and clinical expertise and resources. This will enhance opportunities for basic scientific discoveries and translational research. An ongoing objective has been to strengthen interactions with groups focused on experimental and clinical approaches and this has been accomplished within the Branch with Anamaria Camargo's group and outside with several groups in Brazil and worldwide. A group led by Dr. Angelita Gama from the host institution, Hospital Alemão Oswaldo Cruz, is a valuable collaboration. Three groups (de Souza, Camargo and Gama) run the colorectal clinical genomics initiative.

In the last couple of years, next-generation sequencing technologies have been used to study genetic alteration in cancer. Together with Drs. Andrew Simpson and Robert Strausberg, transcriptome data generated by the 454 technology to map chromosomal rearrangements in a breast tumor cell line has been used. In collaboration with other groups in Brazil and abroad, genomes of a breast tumor cell line and lymphoblastoid cell line are being sequenced from the same patient. Exploration of these next-generation sequencing technologies will be a major focus over the next few years, especially in colorectal cancer.

A second major project involves identification of all human genes coding for cell surface proteins. Cell surface proteins are excellent targets for diagnostic and therapeutic interventions and have proved to be relevant in areas of medicine. A number of monoclonal antibodies against them are approved by the FDA for therapeutic applications, particularly in cancer therapy. Based on that finding and expertise in genomics and bioinformatics, it was decided to explore the human cell "surfaceome" in an attempt to identify potential diagnostic and therapeutic targets.

A catalog of 3,702 human proteins was identified as potential cell surface proteins by searching the human genome for genes encoding trans-membrane proteins. After a series of filters to eliminate proteins known to be part of other sub-cellular compartments, a knowledge base was generated.

The group capitalized on the availability of expression data from a panel of normal tissues to define the expression profile of the human cell surfaceome in normal cells/tissues. This large-scale profiling of the surfaceome in normal tissues has potential value in facilitating further development of therapeutic and diagnostic protocols. Almost half the surfaceome set (42%) present a very broad expression pattern being expressed in more than twenty normal tissues. A smaller fraction (~85 genes) is expressed in all normal MPSS libraries, a typical feature of house-keeping genes. However, a significant number of surfaceome genes (13%) have a restricted expression pattern among normal tissues probably reflecting significant differences in the plasma membranes of cells in different tissues. These genes are excellent candidates for tumor targets due to this restricted expression pattern.

In exploring the human cell surfaceome with respect to differential expression in tumors, emphasis was placed on finding genes exhibiting restricted expression in normal tissues and differential expression in tumor samples, as well as identifying genes showing differential expression in tumors irrespective of expression in normal tissues. The analysis was restricted to two types of tumors – GBM and colorectal tumors, based on the abundance of publicly available gene expression analyses as well as access to samples and clinical information. Genes were selected for further experimental validation based on their expression in 305 SAGE libraries (97 and 208 derived from normal and tumors samples, respectively). This analysis generated a subset of 902 genes submitted to experimental validation using a platform for large-scale measurement of cDNA levels (Biomark – Fluidigm, Inc.). Sixty-five RNA samples were used: twenty-one normal tissues (available commercially), fifteen tumor cell lines of diverse origins, eleven colorectal tumors with two pools of normal colon and eleven GBMs with five pools of normal brain. After one qPCR measurement, all 593 genes expressed in at least one sample were selected for subsequent experiments. This analysis expanded the number of cell surface targets for GBM and colorectal tumors and new cell surface cancer-testis antigens were identified as well as significantly altered pathways in those types of tumors.

MOLECULAR AND CELLULAR BIOLOGY

Vilma Martins, Ph.D.

The research effort is focused on deciphering physiological functions of the cellular prion protein (PrP^C) and in particular its association with extracellular matrix proteins vitronectin (VN) and laminin and co-chaperone stress inducible protein 1 (STI1). PrP^C modulates neuronal survival and plasticity as well as memory formation. Recent data suggest participation of PrP^C in tumoral processes. The key objective is to understand how interaction between these proteins can be explored as a therapeutic target in neurodegenerative diseases such as prion diseases and Alzheimer´s Disease as well as in tumors.

Data strongly support that PrP^C interacts with multiple extracellular ligands recruiting a multi-component cell surface complex and promoting intracellular signals that contribute to diversity of functions mediated by PrP^C. Expression of PrP^C and its ligands VN and STI1 during mouse development was investigated and distribution of the three proteins was spatiotemporally related. STI1 and VN expression became evident at an earlier time than PrP^C, in the nervous system and heart. Later a gradient of expression of the three proteins was observed in the spinal cord, being more abundant in the notochord and floor plate. These data are consistent with previous results showing the role of PrP^C-VN and PrP^C-STI1 in axonal growth. As development proceeded, the three proteins were detected in other organs, suggesting they may play a role in development of non neural tissues as well.

The function of PrP^C-STI1 binding in astrocytes has been addressed and, analogous to what has been demonstrated in neurons, STI1 increases survival and differentiation upon binding to PrP^C through PKA and ERK1/2 activation. In addition, STI1 inhibited astrocyte proliferation in a PKC-dependent manner. STI1 is secreted by astrocytes and considered a trophic factor due to its properties on neuronal and astrocyte survival and differentiation upon interaction with PrP^C. The mechanisms associated with non-classical STI1 secretion are under evaluation. An STI1-null mouse was generated and will permit the laboratory to explore the functions of PrP^C-ligand *in vivo*.

In 2007, the group was nominated by the Brazilian Ministry of Health as a reference center for the diagnosis of genetic prion diseases. Last year, 34 possible cases of Creutzfeldt-Jakob disease were notified and the most prevalent mutation in PRNP (the gene

that codifies PrP^C), E200K, was identified in one patient. Besides the epidemiological aspects, these analyses would allow identification of new mutations in PrP^C. In addition, experimental approaches using PrP^C mutants are important to delineate possible loss-of-function that can contribute to neuronal loss in these diseases. An interim plan is to explore neurotrophic properties of the PrP^C–STI1 complex to rescue the impairment in neuronal function in prion as well as in Alzheimer's diseases.

PrP^C modulates cell survival, differentiation and proliferation and is affected in cancer cells. The role of PrP^C in the aggressiveness of transformed cells was investigated. Mesenchymal embryonic cells (MEC) from wild-type and PrP^C-null mice were transformed by co-expression of ras and myc oncogenes. These cells presented similar growth rates and tumor formation *in vivo*. However, PrP^C-null transformed cells formed more aggregates with blood components and exhibited increased lung colonization compared with wild-type transformed cells. These effects were mediated by the integrin αvβ3 which is overexpressed and activated in PrP^C-null cells. PrP^C expression may have significant implications in modulating metastasis formation. A new ligand for PrP^C, ADAM23 which also associates integrin αvβ3, has been identified and its role investigated. Recent data illustrate PrP^C-STI1/HOP modulates proliferation of human glioblastoma cell lines. A major goal is to understand the role of PrP^C-STI1 complex in tumors but in particular GMB proliferation and develop strategies to impair this interaction and block tumor proliferation.

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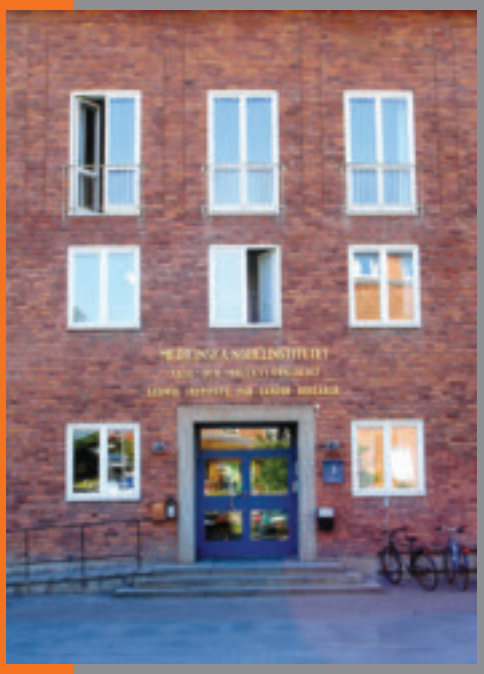
Director's Message

Since my appointment to Director in 2007, the Branch has undergone several major changes. Ulf Eriksson, a senior group leader and LICR Member, accepted a new position at the Karolinska Institutet Department of Medical Biochemistry and Biophysics. Ulf was an extremely successful group leader and we wish him and his team continued success. Two junior group leaders, Jan Stenman and Susanne Schlisio, joined the Branch in late 2008 and are establishing exciting research programs in the areas of vascular biology in the central nervous system (CNS) and gliomas, and oxygen sensing in cancer, respectively.

Jonas Muhr has continued to pursue an exciting research program focusing on Sox proteins in CNS development and gliomas and my own research on transcription factor function in neuron development is progressing in exciting new directions. We have also taken steps to recruit additional staff to further strengthen our research program in 2010. Jonas Muhr was promoted to Associate Member, effective April 1, 2010. He was also a recipient of the Sven and Ebba-Christina Hagberg Prize.



Our group leaders were very successful in competing for external funds. Susanne Schlisio attracted grants from the Swedish Cancer Foundation and the

Swedish Child Cancer Fund; Jonas Muhr received a large grant from the Swedish Research Council; and I received a major grant from the Swedish Foundation for Strategic Research. A growing number of students and postdocs have been welcomed to the Branch and everyone is invigorated by the energy and talent contributing to our research activities. We are most grateful for the strong support from the LICR and we are continuing our work with renewed determination and enthusiasm.

Thomas Perlmann

GENE EXPRESSION

Thomas Perlmann, Ph.D.

Neuronal differentiation is used as a model system to investigate how a transcription factor network is established in early differentiation and how it is organized in differentiated cells. Under the cell fate decision process, a regulatory network must be sufficiently flexible to allow for cell type specification events while it becomes increasingly robust to support the maintenance of the terminally differentiated state, which under physiological conditions is very stable. The goal is to understand how a transcription factor network is influenced by signaling and how it operates during the more plastic early differentiation steps; how it controls the maintenance of differentiated neurons; how pathological changes of a network can lead to neuron dysfunction; and how plasticity of stem cells or differentiated cells may allow for programming or trans-differentiation under experimental conditions. These studies have implications for steering differentiation to cell fates of medical interest and elucidating mechanisms that are central for pathological states such as neurodegeneration and cancer.

In studies of how dopamine (DA) neurons are generated in development, the laboratory has identified Lmx1a as a key transcription factor determinant. Lmx1a is characterized by loss-of-function in newly generated knock-out and knock-in mouse strains. Moreover, regulatory relationships to signaling pathways and other factors have been identified. This new knowledge has been used in novel methods for generating DA neurons from stem cells. This is of considerable interest since these cells are lost in Parkinson's disease (PD) and engineered DA neurons may become useful in cell therapy. During the previous year, published results have shown that forced expression of Lmx1a in mouse embryonic stem (ES) cells under the appropriate culturing conditions can generate DA neurons with remarkable efficiency (>80%). Partnering with Anders Björklund of Lund University, the laboratory is using this methodology to develop safe and reliable protocols for grafting cells in PD rodent models. Moreover, a definitive study showing that forced transcription factor expression in stem cells is an extremely powerful method to generate additional therapeutically interesting neuronal cell types with high efficiency. It is now feasible to generate DA-, serotonin-, visceral motor- and somatic motor neurons with >80% enrichment. This strategy provides a robust system for studying neuron differentiation *in vitro* and has offered the possibility of determining the global transcriptome of specific neuron types at different stages of differentiation.

Although many developmental transcription factors are known to be expressed in mature neurons, conditional gene ablation in the adult mouse brain has not previously been reported for any such factors. Mutations in Nurr1, Lmx1a, Lmx1b and Engrailed 1 genes have been associated with rare cases of PD. In addition, Nurr1 is known to be down-regulated in surviving but pathologically affected neurons in PD. The laboratory has generated conditional knockout mouse strains for Nurr1 and Lmx1a. Conditional knockout strains for Lmx1b have been provided by other labs. Nurr1 has been ablated in adult DA neurons and it was discovered that Nurr1 deficiency results in a slowly developing DA neuron dysfunction that mimics key aspects of PD disease progression.

A separate project, headed by Dr. Johan Holmberg, is independently focusing on the roles of Oct4 and Nanog expression in high grade glioma. It will explore the possibility that the presence of these ES cell-specific transcription factors in glioblastoma is a feature of self-renewing tumor cells that distinguishes them from normal neural stem cells. To explore a causative role for Oct4 and Nanog in the generation of glioblastoma, an inducible lentiviral expression vector has been generated wherein the

expression of shRNA targeting the gene of interest can be controlled through the addition of doxycyclin. Primary tumor cells derived from several human glioblastoma patients will be transduced with the inducible vector. This project is performed in collaboration with Prof. Nistér at CCK, KI and Prof. Marie at the University of São Paulo. In collaboration with Dr. Bracken at Trinity College, Dublin, the role of the putative tumor suppressor Chd5 in promoting and maintaining neuronal identity is being investigated. The data indicate that Chd5 may be involved in Polycomb-mediated epigenetic silencing of non neuronal genes. Through a series of gain- and loss-of-function experiments investigations are underway regarding the role for Chd5 in both the generation and the subsequent maintenance of mature neurons.

STEM CELL BIOLOGY

Jonas Muhr, Ph.D.

Addressing general principles that underlie regulation of stem and progenitor cells in the central nervous system (CNS) is one research goal of the laboratory. Critical goals are to characterize molecular pathways which direct neural stem cells towards neuronal differentiation, and examine how this process is antagonized by mechanisms that maintain neural stem cells in an undifferentiated and proliferative state. A second research goal is to reveal molecular similarities and differences between neural stem and progenitor cells (NSPCs) and stem cells contributing to the establishment and growth of gliomas. To understand better how neurogenesis is regulated, the group has focused on High-Mobility-Group (HMG) proteins of the Sox transcription factor family. This is a class of architectural non-histone proteins that are involved in gene regulation and maintenance of chromatin structure. Numerous examples show their importance in the regulation of different stem- and progenitor cell populations. For instance, Sox2 is expressed in the early embryo which contributes to the core transcriptional network that maintains cells of the inner cell mass in a multipotent stem cell state.

The group has previously demonstrated that Sox1-3 function to actively maintain neural cells in a self-renewing progenitor state, whereas two other Sox proteins, Sox4 and Sox11, are necessary for the establishment of neuronal gene expression in differentiating neurons. These findings raised the question which genes are directly regulated by Sox proteins. In the past year, the laboratory has used chromatin immune precipitation combined with high throughput sequencing (ChIP-Seq) to compare the genome-wide target genes of Sox3 and Sox11 in NSPCs and differentiating neurons, respectively. These analyses have demonstrated that approximately 4,000 genes are bound by Sox3 in NSPCs, whereas 3,500 genes are bound by Sox11 in neurons. Most of the genes activated and bound by Sox11 in neurons have been occupied by Sox3 in the preceding cellular stages. In NSPCs, Sox3 binds a large set of genes defining neural stem cells as well as many silent genes that first will be activated in differentiating neurons.

Similar to Sox2 in ES-cells, the binding of Sox3 is to silent genes poised for activation as indicated by trimethylated H3K4 and H3K27; a bivalent chromatin profile associated with an active state in differentiating neurons upon binding of Sox11. These findings suggest Sox transcription factors control the transition of neural stem cells into differentiated neuronal progeny by activating gene transcription in a controlled developmental time course, and by maintaining genes that will be activated at later differentiation stages in a repressed but epigenetically poised state. The group also analyzed the expression of stem cell markers in human gliomas and demonstrated that high grade gliomas, not low grade, co-express transcription factors normally engaged in regulating the maintenance of NSCs or pluripotent ES-cell in a

sub-population of tumor cells. Cells in high grade gliomas acquire traits characteristic of mesodermal and endodermal cells in addition to neural cell fates underscoring the pluripotent potential of these cells. Finally, the combined expression of neural and pluripotent stem cell markers can only be found in solid tumors. These data emphasize the importance of studying molecular characteristics in tumor cells exposed to a pathological micro environment *in vivo*.

DEVELOPMENT AND DISEASE

Jan Stenman, Ph.D.

The overall goal of the laboratory is to unravel general mechanisms regulating normal and pathological development of the highly specialized vascular endothelium in the brain and spinal cord, which together make up the central nervous system (CNS). To reach these goals, the group is concentrating on two key questions: 1) how a functional CNS vascular network forms and differentiates; and 2) how normal CNS vasculature is different from the dysfunctional vasculature formed in brain tumors, and how the brain tumor vasculature can be manipulated to the benefit of those affected by this disease.

A detailed description of the structure of a system and its components is a prerequisite for understanding the mechanisms directing its development. The CNS vasculature is quite distinct from the more porous vasculatures permeating other organs. CNS endothelial cells begin acquiring these organ-specific properties already at the earliest stages of CNS vascularization but this process is not complete until adulthood. It was previously demonstrated that two neural Wnt ligands, *Wnt7a* and *Wnt7b (Wnt7a/7b)*, act directly on the CNS endothelium via the canonical Wnt signaling pathway to regulate CNS-specific angiogenesis and early blood-brain barrier differentiation *in vivo*. However, the cellular and molecular mechanisms by which Wnt signaling regulates these processes are not clear. In addition, a comprehensive description of the changes in the global gene expression profile of the endothelium that occurs during embryogenesis is lacking. Adding to the complexity, differences exist between endothelial cells within the developing CNS vasculature with regards to morphology, function and expression of specific homeobox transcription factors. To understand better the cellular and molecular mechanisms regulating the formation and differentiation of a functional CNS vascular network, the group aims to describe the gene expression profiles of CNS endothelial cells with single cell resolution at several developmental stages; and identify the downstream targets of *Wnt7a/b* and the role(s) these play in CNS vascular formation and differentiation.

The group performed an initial screen for Wnt downstream targets and developed several reagents necessary for more advanced screens that aim at identifying direct Wnt targets in the CNS endothelium. In brain tumors, the blood-brain barrier is disrupted resulting in vasogenic brain oedema, a major cause of morbidity, abnormal blood flow causing hypoxia, and production of proangiogenic factors that keep the blood vessels dysfunctional. It has been suggested that preventing tumor vasculature development using anti-angiogenic strategies, or normalizing the vasculature and thus the tumor microenvironment could lead to improved cancer treatment. Previous studies observed that beta-catenin accumulates in the vasculature associated with certain brain tumors. This suggests a role for canonical Wnt signaling in neovascularization. The group will explore whether it can prevent neovascularization or normalize the dysfunctional tumor vasculature by modulating canonical Wnt activity in the tumor endothelium.

The group has been working on developing mouse glioma tumor models, now being tested *in vivo*. The tumor models will be characterized carefully (e.g., time until tumor

formation, tumor histology, vasculature formation, etc.). After characterizing the tumor models, a study of the consequences of manipulating canonical Wnt signaling in brain tumor endothelial cells will be undertaken as well as work on new genetic mouse models.

OXYGEN SENSING AND CANCER

Susanne Schlisio, Ph.D.

Understanding the mechanisms of how disruption of oxygen-sensing pathways can lead to cancer is the primary research focus of the laboratory. Oxygen-sensing is mediated partly via prolyl hydroxylases that require molecular oxygen for enzymatic activity. The EglN prolyl hydroxylases are well characterized in regulating the HIFα hypoxic response, but are also implicated in HIFα-independent processes. During development, EglN3 executes apoptosis in neural precursors independently of HIFα. Failure of EglN3 neuronal apoptosis is involved in certain forms of sympathetic nervous system tumors.

Research is concentrated on three areas: 1) direct impact of cancer metabolism in EglN hydroxylase functioning; 2) molecular mechanism of how prolyl hydroxylase EglN3 executes apoptosis in neural precursors during development; and 3) how failure of developmental apoptosis mediated by EglN3 predisposes to certain forms of nervous system tumors.

The long-term goal is to understand how mutations in metabolic enzymes contribute to the pathogenesis of cancer. EglN hydroxylase activity is dependent upon metabolites that take part in the Krebs cycle. Inactivation of the Krebs cycle enzyme succinate dehydrogenase, a bona fide tumor suppressor, leads to enzymatic inactivation of the EglN prolyl hydroxylase through accumulation of succinate. In the last year, the focus has been on newly-discovered metabolic cancer genes, IDH1/2. Hydroxyglutarate is a product of the gain-of-function mutation in IDH1/2. The group developed assays to measure the impact of hydroxyglutarate on EglN hydroxylase activity. A collaboration with Tim Gahman at the San Diego Branch is underway to study the effects of hydroxyglutarate *in vivo* and its impact on EglN hydroxylation substrates.

To understand the mechanism of how the prolyl hydroxylase EglN3 executes neuronal apoptosis, the group is trying to identify novel EglN3 hydroxylation substrates. Last year, a substrate trap that allows stabilization of the transient enzyme substrate interaction was established. Using this substrate trap, affinity purification coupled with mass spectrometry will help to reveal novel EglN3 substrates. This work is performed in collaboration with Ulf Hellman at the Uppsala Branch for mass spectrometry and peptide sequencing. Further, loss of function screens using lenti viral (sh)RNA libraries are used to identify genes required for EglN3 mediated apoptosis. Recently, such a screen identified the kinesin KIF1Bβ. KIF1Bβ is necessary and sufficient for neuronal apoptosis when neurotrophin factors such as NGF becomes limiting. KIF1Bβ maps to 1p36.2, a region of the genome frequently deleted in neural crest derived tumors including neuroblastomas. The aim is to understand how, mechanistically, EglN3 regulates KIF1Bβ and how this translates into cell death. The group has started to inactivate this gene in model organisms to investigate if loss of KIF1Bβ by itself or in collaboration with other oncogenes promotes neuroblastoma development.

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Director's Message

The aim of the Uppsala Branch is to elucidate signaling pathways that control cell growth, survival and migration. As malignant cells are characterized by perturbations in such pathways, we anticipate our work will unravel suitable target molecules for development of signal transduction modulators, which can be used to treat cancer patients.

We have a long-standing interest in two growth regulatory molecules, i.e., platelet-derived growth factor (PDGF) and transforming growth factor-β (TGFβ). PDGF is a family of dimeric isoforms of A-, B-, C- and D-polypeptide chains, which exert their cellular effects by binding to α- and β-tyrosine kinase receptors. PDGF isoforms are major mitogens for connective tissue cells and some other cell types, and are implicated in autocrine as well as paracrine stimulation in tumors. Our objective is to elucidate the molecular mechanisms of signal transduction via PDGF receptors and explore the clinical utility of PDGF antagonists.

The TGFβ family consists of 33 members, including TGFβ isoforms, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs) and activins. They all act via heteromeric complexes of type I and type II serine/threonine kinase receptors and have important roles during embryonal development. TGFβ most often inhibits cell growth and promotes apoptosis, matrix production and cell differentiation in normal cells. TGFβ has a complicated role in cancer. Initially, it is a tumor suppressor through its ability to inhibit cell growth and promote apoptosis. At later stages of tumor progression, however, TGFβ becomes a tumor promoter by induction of epithelial-to-mesenchymal transition, which makes cancer cells invasive, and by stimulating angiogenesis and

The picture shows the east wing of the Biomedical Center at Uppsala University where the Uppsala Branch is located on the third floor.

inhibiting the immune system. Our aim is to elucidate the molecular mechanism whereby TGFβ acts and explore whether selective TGFβ antagonists can be developed, which would inhibit the tumor promoting effects of TGFβ while leaving the tumor suppressive effects unperturbed. Our ultimate goal is to investigate the clinical usefulness of such antagonists.

Another line of research is to elucidate the role of matrix molecules, and in particular the polysaccharide hyaluronan and its receptor CD44, in tumor progression. As before, the groups are supported by expertise in proteomics and mass spectrometry.

During the year two former group leaders, Kohei Miyazono, Tokyo University, and Peter ten Dijke, Leiden University, were appointed guest professors with a twenty percent commitment at Uppsala University. Kohei and Peter will lead small groups at the Branch, and we expect this arrangement to encourage and promote additional long-standing interactions in TGFβ research.

The Branch is located at the Biomedical Center in Uppsala using lab space provided by Uppsala University. During the year, we moved into newly renovated laboratories specially designed for our needs.

C.-H. Heldin

PDGF TRANSLATIONAL RESEARCH

Carina Hellberg, Ph.D.

The elucidation of PDGF receptors as cancer drug targets and modulation of PDGF β-receptor signal transduction are the primary focus of the translational research group. Increased interstitial fluid pressure (IFP) in tumors constitutes a barrier to drug uptake. In experimental models, inhibition of PDGF receptors on stromal cells, as well as inhibition of the VEGF receptor on endothelial cells, lowers tumor IFP and increases tumor uptake of cytotoxic drugs. The group investigated whether the combination of PDGF and VEGF receptor inhibitors could synergize to lower tumor IFP. Both agents lowered Kat4 tumor IFP when given as monotherapies over four days, but the efficacy of the combination therapy was directly dependent on the treatment schedule. The decrease in IFP induced by PDGF receptor inhibition for four days was significantly greater when VEGF receptor inhibitor was given during the last two days. However, when both drugs were combined over the four day period, no reduction in tumor IFP was observed.

When anti-angiogenic drugs were given in combination with the chemotherapeutic agent taxol, anti-PDGF receptor inhibition and continuous combination therapy potentiated the effect of taxol to the same extent. To explore further the discrepancy observed between the effects of anti-angiogenic drugs on IFP and taxol treatment, the group investigated the effects on the tumor water content. The therapies that provided the greatest effect on tumor growth in combination with taxol increased the extracellular water content in the tumor. This indicates that the increase in convection may accompany IFP reduction to improve drug uptake.

Following ligand stimulation, the PDGF β-receptor signaling is terminated by dephosphorylation of the receptor autophosphorylation sites in parallel with receptor internalization and subsequent degradation. Receptor signaling can be modulated by altering the rate of receptor dephosphorylation or trafficking. It was previously found that increased PDGF β-receptor phosphorylation observed in T-cell phosphatase -/- fibroblasts is paralleled by induction of PDGF β-receptor recycling resulting in decreased rate of receptor degradation. Protein kinase C alpha has been identified as a critical component in the sorting of PDGF β-receptor into Rab 4 positive recycling endosomes. PKC alpha activation by the G-protein coupled LPA receptor also induced PDGF β-receptor recycling, paralleled by increased PDGF β-receptor phosphorylation in response to low concentrations of PDGF-BB. This increased the physiological response of PDGF-BB, as LPA potentiated the chemotactic response of wt fibroblasts in a PKC-dependent manner. These findings suggest that activation of PKC on early endosomes might constitute a point of crosstalk between receptor subclasses that modulate the amplitude and duration of PDGF β-receptor signal transduction. This is an important finding and may explain how cells respond to low concentrations of PDGF *in vivo*. The relevant substrate for PKC is currently being identified.

PDGF SIGNAL TRANSDUCTION

Johan Lennartsson, Ph.D.

The molecular mechanisms by which PDGF induces cell proliferation, migration and survival is the focus of the signal transduction group. Mitogen activated protein (MAP) kinases have important roles in the intracellular signal transduction from plasma membrane receptor to the nucleus. There are at least four major groups of MAP kinases in mammalian cells, i.e., Erk1/2, Erk5, p38 and Jnk. The activity of MAP kinases is negatively regulated by dephosphorylation of key residues in their activation loop, which can be performed by MAP kinase phosphatases (MKPs). The group demonstrated that MKP3, which is considered to be selective for

Erk1/2, is regulated by PDGF in porcine aortic endothelial cells both at the protein and gene expression level. Upon PDGF stimulation, MKP3 is rapidly degraded by proteasomes. For efficient MKP3 degradation to occur, phosphorylation of Ser174 was necessary; this occurred in a manner sensitive to Mek1/2 inhibition. This feed-forward loop was critical for PDGF-induced Erk1/2 phosphorylation. In addition, prolonged exposure of cells to PDGF resulted in increased expression of the *mkp3* gene followed by restoration of MKP3 protein level after 1-2 h. This feedback loop restricts duration of Erk1/2 activity. Consistent with a role of MKP3 in the regulation of Erk1/2, depleting cells of MKP3 using siRNA led to an increased mitogenic response to PDGF. In conclusion, the involvement of MKP3 in the regulation of Erk1/2 activation is complex and consists of an initial feed-forward phase and subsequent feed-back phase.

Erk5 is a member of the MAP kinase family involved in the regulation of cell survival and proliferation. PDGF stimulation of NIH3T3 cells results in rapid and transient activation of Erk5. The mechanism by which the PDGF receptor activates Erk5 is not clear, but depends on normal Src kinase activity. To elucidate the function of Erk5 in the context of PDGF signaling, the Erk5 expression was downregulated using siRNA; under conditions of reduced Erk5 expression Akt was activated in a transient manner, whereas under control conditions PDGF provoked a sustained Akt activation. Since Akt is an established survival kinase, the consequence of Erk5 silencing on cell survival was investigated. Consistent with the diminished Akt activation, PDGF was not able to suppress caspase 3 cleavage and suppress nuclear morphological changes under serum-free conditions in Erk5 silenced cells.

The dynamics of Erk1/2 activation are important for the cell biological outcome of signaling through this pathway. In addition to studies of MKPs, the group also investigated how pathways activated in parallel to the Erk1/2 cascade influence Erk1/2 activation kinetics. To date, results indicate that RasGAP, Src and PI3-kinase, all major pathways activated downstream of PDGFRβ, modulate the kinetics of Erk1/2 pathway activation in both time (RasGAP and Src) and strength (PI3-kinase). In contrast, mutations of either the PLCγ binding sites (PDGFRβ$^{YY1009/1021FF}$) or Shp2 binding sites (PDGFRβ$^{YY763/1009FF}$) did not significantly influence Erk1/2 signaling.

Fer is a cytoplasmic tyrosine kinase activated in response to PDGF stimulation and associates with the activated PDGF β receptor (PDGFRβ) through multiple autophosphorylation sites, i.e., Tyr579, Tyr581, Tyr740 and Tyr1021. Using low molecular weight inhibitors the group found that PDGF-BB-induced Fer activation is dependent on PDGFRβ kinase activity, but not on the enzymatic activity of Src or Jak kinases. To elucidate the function of Fer downstream of PDGFRβ, the expression using siRNA was downregulated; under conditions with reduced Fer expression, PDGF-BB was unable to induce phosphorylation of Stat3, whereas the phosphorylation of Stat5, Erk1/2 and Akt was unaffected. In addition, PDGFRβ autophosphorylation was partially dependent on Fer expression. On a functional level, it could be demonstrated that expression of Fer is dispensable for PDGF-BB-induced proliferation and migration. However, Fer was found to be essential for colony formation in soft agar, consistent with its critical role in Stat3 activation, frequently implicated in cell transformation.

TGFβ SIGNALING

Aristidis Moustakas, Ph.D.

The TGFβ signaling group focuses on signaling pathways of transforming growth factor β (TGFβ) isoforms and the related bone morphogenetic proteins (BMPs). Uncovering new mechanisms of regulation of these pathways is a key objective. Efforts have expanded to encompass the AMP-regulated kinase family and transcription factors

involved in processes such as epithelial-mesenchymal transition, tumor cell invasiveness, metastasis and cancer stem cell self-renewal.

TGFβs, BMPs and other family members signal via receptor serine/threonine kinases on the cell surface and intracellular Smad and other signaling proteins such as Erk and p38 MAP kinases and Rho GTPases. Nuclear Smad complexes regulate expression of several genes in a cell type-dependent manner by binding to DNA and several transcription factors. The TGFβ signaling pathway is negatively regulated by a growing set of molecular mechanisms involving the inhibitory Smad7, receptor and Smad de-phosphorylation and ubiquitination, leading to fine tuning of the signaling flow.

The group has identified novel regulators of Smad function in the nucleus that operate at the chromatin level. PARP-1 (poly(ADP-ribose) polymerase 1) is a nuclear enzyme that regulates chromatin remodeling during gene transcription and also ADP-ribosylates several transcription factors, regulating their function. PARP-1 associates with nuclear Smad complexes in response to TGFβ. PARP-1 ADP-ribosylates Smad3 and Smad4 and promotes dissociation from DNA. In this manner, PARP-1 controls the length of time that Smad complexes reside on DNA and protects gene expression from spurious regulation by TGFβ.

In addition, nuclear Smad complexes associate with the chromatin insulator protein CTCF. Smad-CTCF protein complexes were identified on the *H19/Igf2* imprinting control region known to undergo genomic imprinting. This research opens new avenues into the role TGFβ can play in regulating epigenetic events.

Progress has been made on new kinases that act as effectors of TGFβ signaling and mediate various physiological responses. A new line of research in collaboration with Peter ten Dijke's laboratory has been completed, focusing on the mitogen and stress-activated kinase Msk1, which provides a survival signal downstream of TGFβ/Smad signaling and counteracts the apoptotic response certain epithelial cell types exhibit upon stimulation by TGFβ. This new work explains why TGFβ causes apoptosis in a cell type-dependent and growth condition-dependent manner.

TGFβ regulates diverse physiological processes including cell proliferation and death. In addition, TGFβ positively contributes to cancer progression. One of the mechanisms by which TGFβ promotes tumor cell invasiveness and metastasis is the process of epithelial-mesenchymal transition (EMT). EMT, in the context of growing tumors, has been proposed to empower tumor cells with stem-like capacities.

The group previously established a molecular pathway downstream of TGFβ, which involves the nuclear factor HMGA2 and its downstream targets Snail and Twist. Deeper molecular analysis of the EMT process in collaboration with the Stockholm Branch determined that nuclear Smad complex associates with transcription factor Snail, leading to transcriptional repression of several epithelial-specific target genes. These findings have direct relevance to mechanisms by which TGFβ acts as a pro-metastatic factor and direct the research towards novel therapeutic approaches.

Using multi-species phylogenetic analysis, the evolutionary origins of the TGFβ pathways were re-evaluated and it was found that all metazoans have a complete TGFβ ligand, receptor and Smad complement. This demonstrates the importance of TGFβ pathways in the establishment of multi-cellular organisms, during early metazoan life.

APOPTOTIC SIGNALING

Maréne Landström, M.D., Ph.D.

The goal of the group is to elucidate the molecular mechanisms whereby TGFβ induces apoptosis. Smad7 is a negative modulator of TGFβ signaling. The group demonstrated that Smad7 is also required as an adaptor, which assembles

a TGFβ-induced signaling complex, leading to apoptosis in prostate cancer cells. Smad7 is important for activation of the TGFβ activating kinase 1 (TAK1), mitogen activated protein kinase kinase 3 (MKK3) and p38 MAP kinase pathway, presumably by bringing the kinases close to each other.

The group also demonstrated that TGFβ receptor interacts with TAK1 and that activation of TAK1 requires Lys63-dependent ubiquitination by the E3-ligase TRAF6. This post-translational modification of TAK1 determines its biological responses downstream of the active receptor complex. This finding provides a molecular mechanism by which TGFβ receptors initiate a non-Smad signaling pathway. TGFβ-induced activation of the p38 MAPK pathway is not dependent on kinase activity in type I TGFβ receptor. A consensus binding site for TRAF6 in type I TGFβ receptor was identified, which explains how TRAF6 associates with type I TGFβ receptor. Ligand-induced oligomerization of the receptor complex causes auto-ubiquitination and activation of TRAF6, followed by Lys63-dependent poly-ubiquitination and activation of the TAK1–p38 MAPK pathway. The group is now elucidating further the molecular mechanisms whereby TRAF6 determines specificity of cellular responses induced by TGFβ.

There is a close link between chronic inflammation and tumor promotion. In patients with prostate cancer, a high level of TGFβ has been associated with poor prognosis. The group is investigating detailed molecular mechanisms for activation of the TAK1–p38 MAPK pathway by inflammatory cytokines such as TGFβ, TNF-α and IL-1 and studying molecular mechanisms for activation of TAK1 and its biological responses evoked by TNF-α and IL-1β in prostate cancer cells.

For patients with advanced and metastatic prostate cancer, there is an urgent need to develop improved therapeutic strategies. The small gold compound aurothiomalate (ATM) is currently used for treatment of rheumatoid arthritis and causes growth inhibition of ovarian and non-small cell lung cancer. ATM specifically induces apoptosis of prostate cancer cells while normal primary prostate epithelial cells are not affected. The group demonstrated that ATM disrupts the association between proteins in the cell polarity complex (Par6 and aPKC) which maintain pro-survival signals initiated by the Akt kinase. Treatment with ATM induces apoptosis of tumor cells via its inhibitory effects on Akt and activation of the MAPK-family members, p38 and Erk. The putative antitumoral effects of ATM on additional prostate cancer cell lines are currently under investigation. It is anticipated that these studies will provide a basis to add ATM to future therapeutic options to treat advanced prostate cancer.

Signaling molecules downstream of TGFβ and Wnt receptors regulate cell fate during development and tissue homeostasis. Smad7 interacts with components in the Wnt signaling pathway, i.e., β-catenin and lymphoid enhancer binding factor 1/T-cell-specific factor (LEF1/TCF). By knocking down endogenous Smad7 expression, it was shown that Smad7 is required for TGFβ-induced stabilization of β-catenin in human prostate cancer cells. Moreover, Smad7 and p38 regulate the activity of glycogen syntetase kinase-3β (GSK-3β) leading to stabilization of β-catenin. This event is crucial for TGFβ-induced directed cell migration of prostate cancer cells.

MATRIX BIOLOGY

Paraskevi Heldin, Ph.D.

The matrix biology group is focused on how stromal microenvironment influences proliferative and invasive behavior of malignant cells. The aim is to dissect signaling pathways downstream of the hyaluronan receptor CD44, and mechanisms whereby CD44 assists and fine-tunes signaling by growth factor receptors, in particular the receptors for PDGF and TGFβ.

Hyaluronan is an extracellular and cell-associated polysaccharide which has a

key role in tissue homeostasis. The levels of hyaluronan are balanced through its biosynthesis by hyaluronan synthases (HASs) and catabolism by hyaluronidases (HYALs). These enzymes are differentially regulated by growth factors, including PDGF-BB and TGFβ, under pathological conditions. The general concept emerging from studies is that HAS2 overexpression leads to faster development of transplantable tumors in animal models, compared to mock transfectants. In contrast, HAS2 suppression or HYAL1 overexpression suppressed growth rate of tumor cells both *in vitro* and *in vivo*.

Additionally, the effects of peritumoral hyaluronan in breast cancer cell invasion and microvascular endothelium adherence are being investigated. The analysis revealed that breast tumor cell-retained hyaluronan is important for adhesion to microvascular but not lymphatic endothelium.

In order to elucidate the mechanism of aberrant accumulation of hyaluronan during tumor progression and persistent inflammation and unravel its roles in tissue homeostasis and cellular functions, the regulatory mechanisms of HAS activities were investigated and it was demonstrated that HAS2 forms a homo-dimer as well as a hetero-dimer with HAS3. The dimeric configuration appears to be critical for the activation of HAS2. HAS2 activity is regulated by mono-ubiquitination.

Several groups have demonstrated cross-talk between CD44 and growth factor receptors including receptors for PDGF-BB, TGFβ, hepatocyte growth factor and epidermal growth factor. Recent studies revealed that PDGFRβ and CD44 form a complex resulting in hyaluronan-dependent inhibition of PDGFRβ phosphorylation and fibroblast migration. The effect most likely involves a CD44-mediated docking of a tyrosine phosphatase (PTP) to the PDGFRβ. In an attempt to identify proteins that interact with CD44 and explain the effect of CD44 on growth factor receptor signaling, the group utilized pull-down approaches using synthetic peptides from the cytoplasmic domain of CD44 baits. Proteins from cell lysates that bind to CD44 sequences were separated by SDS-PAGE and identified by Maldi-TOF-MS. The analysis revealed several interesting candidate proteins of potential functional significance for CD44-mediated cellular functions currently under investigation.

PROTEIN STRUCTURE

Ulf Hellman, Ph.D.

With solid experience in peptide synthesis, radio-labeled amino acid sequencing and MALDI TOF mass spectrometry, the group's function is to support activities within the Branch with specialized techniques and interact with other Branches and groups. Today almost all activities are concentrated towards mass spectrometry and the group uses a top of the line matrix-assisted-laser-desorption-ionization-time-of-flight-mass-spectrometer (MALDI-TOF-MS). The present instrument (Bruker Ultraflex III TOF/TOF) offers excellent sensitivity and accuracy for both MS and especially for MS/MS, and allows for peptide sequencing possibilities.

Over 95% of the samples for analysis by MALDI-TOF-MS are delivered as bands or spots from one- or two-dimensional SDS-PAGE gels. The gel separated proteins are prepared for MS analysis by in-gel tryptic digestion. With Coomassie-visible material only a fraction of the digest is needed for analysis; with silver-stained material, often the entire sample must be applied after concentration and desalting on micro RPC columns (i.e., C18 µZipTip). A method for recovery of a sample deposited on the target plate, after which useful modifications can be made for facilitating certain measurements, is under development. A recently introduced novel approach to enrich for phosphorylated peptides, which are notorious for low sensitivity by MALDI TOF MS using home-made micro-columns comprising

TiO2 particles, increases the sensitivity significantly for this group of highly relevant peptides.

Determining protein identity by PMF is a routine procedure for known proteins. After generation of a proteolytic digest and determination of peptide masses by MALDI-TOF/TOF-MS, a search engine (ProFound or MASCOT are preferred) to uncover a match with a protein in sequence databases was employed. Should it fail to identify the target protein, the identity may be established by obtaining the amino acid sequence of one or more tryptic peptides. A sequence homology search by BLAST is, in contrast to PMF, tolerant to amino acid substitutions.

A technique of Lys-modification by an imidazol derivative is employed which renders lysine-containing peptides more basic and increases sensitivity resulting in higher sequence coverage. As a bonus, such peptides are easy to fragment for sequence analysis. Furthermore, the Lys-Tag reagent may be used to label a set of proteins with deuterium for relative quantifications. Over the last year, interesting proteomics projects were carried out in collaboration with various groups, including the Karolinska Hospital, looking for proteins that are significantly changed in various tumors. A fair amount of time is spent studying PTMs which are of crucial importance in signal transduction. The nature of the modification determines the difficulty involved and the group has been successful with e.g., methylation, acetylation, ubiquitinylation and phosphorylation.

Fragment analysis of peptides by MALDI-TOF/TOF-MS using post-source-decay (PSD) is a straightforward technique. The resulting fragment spectra are often successfully used for protein identification, but are difficult to interpret for *de novo* sequencing. The TOF/TOF technology allows complete PSD spectra to be generated in seconds, so it is possible to quickly scan through several peptides from one digest. This easy and quick PSD combines extremely well with the Chemically Assisted Fragmentation (CAF) derivatization protocol, leading to easily interpreted spectra, as they comprise a unique series of y-ions. Hence, an amino acid sequence can unambiguously (with the single exception of the isobaric Leu/Ile) be determined faster, cheaper and with more sensitivity than was possible using chemical Edman degradation. CAF-PSD is utilized for identification of uncharacterized species, as well as for analysis of modified peptides. As the CAF reagent was discontinued in early 2008, it was replaced by SPITC (4-sulfophenyl-isothiocyanate), which is a much less expensive compound with similar positive effects on peptide fragmentation by MALDI-TOF/TOF.

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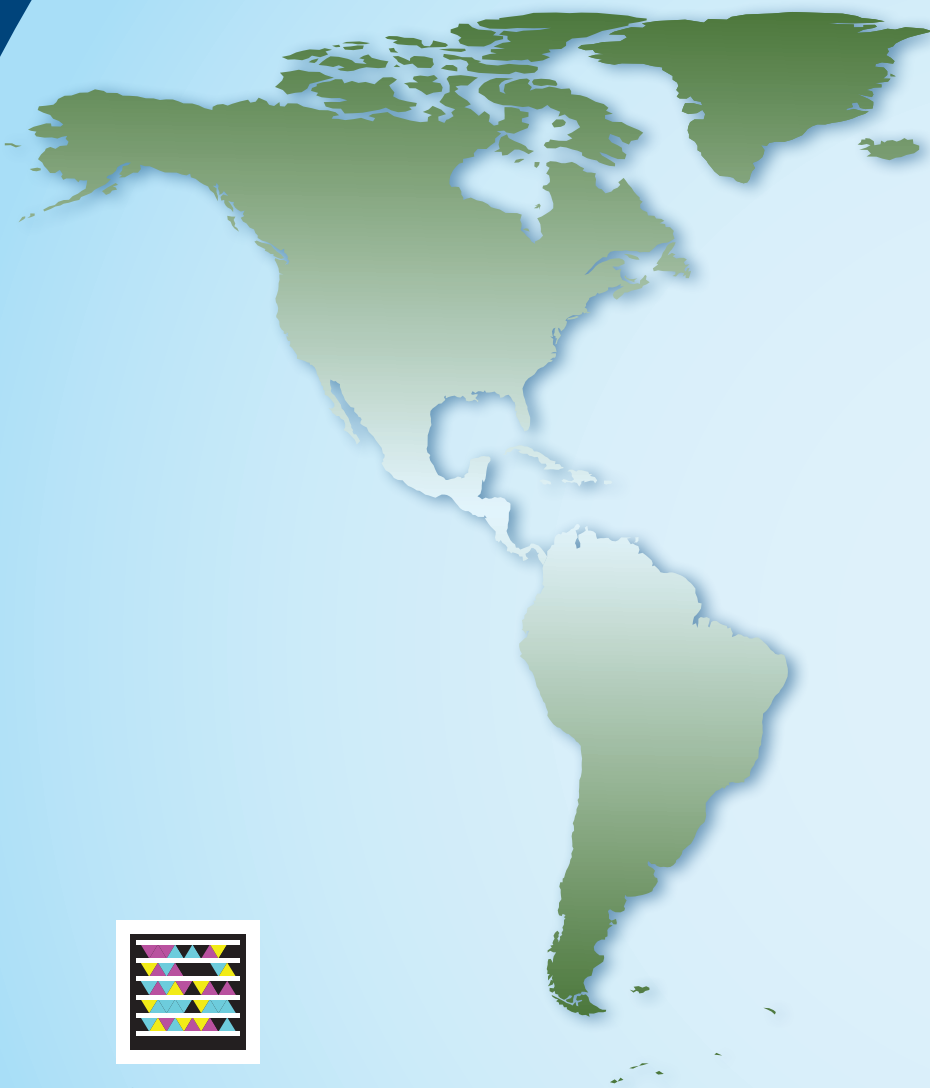

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