LUDWIG INSTITUTE FOR CANCER RESEARCH

2010 ANNUAL RESEARCH REPORT

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2010 Annual Research Report

CORE STRUCTURE AND VALUES

The Ludwig Institute for Cancer Research is a mission-based organization dedicated to improving the understanding and control of cancer through its own research efforts. The defining characteristics of the Institute are:

- its international scope that provides the ability to reach out and engage scientists anywhere around the world.
- the excellence of its basic research maintained through rigorous and ongoing individual and institutional external review.
- its demonstrated commitment to actively translate its research into novel therapeutic approaches to control cancer.

FINANCES

Access to adequate funding is critical to any research endeavor. The Institute enjoys a distinct advantage in this regard because of its asset base. Though the Institute's asset base suffered a material setback (-24.2%) in the market meltdown of 2008, its value stands at $1.2 billion as of the end of 2010. This represents a 35 percent increase from its lowest month end value, even after withdrawals of $111.2 million to support the Institute's research during the intervening two years. Notwithstanding solid performance, the Institute's assets cannot support all of the Institute's ambitions. Accordingly, the Institute diligently seeks outside resources to supplement its own.

The Ludwig funded budget for 2010 was $67.4 million. External funding added an additional $44 million and income from royalties and other IP related payments, $5 million. Essentially all of the non-IP external funding is attributable to individual grants to Branch-based scientists. The Institute is immensely appreciative of all the efforts to garner these funds and celebrates the recipients for their success in the face of extremely stiff competition. Various of the Institute's Branches now attract as much or more funding from contributions from their host institution and grants as they receive from the Ludwig Institute's assets. This is highly desirable as it provides another external validation of scientific quality as well as enhances operational flexibility.

BRANCHES

The Institute published 368 papers in 2010, a formidable achievement. At the year end the total number of staff in the Institute stood at over 730.

Significant changes are contemplated for the future structure of the Institute. Long term, ten Branches as currently structured, each with sufficient, reliable internal funding to enable pioneering research in the 21st century, are likely not sustainable from the

resources available. Thus, the Institute is in transition to a footprint that we expect to result in a reduced number of significantly larger Branches together with a global network of satellite groups that will complement and extend our intramural program. This will be an evolutionary process and will respect obligations to current staff.

This process is already underway. For example, in the fall of 2010 the Institute signed an agreement with the University of Lausanne under which the Branch was absorbed into the University effective January 1, 2011. Many advantages accrue from this change. Member-track staff have been awarded tenured professorial positions in the University, affording them greater access to University facilities, and the University is now co-funding the unit's activities, meaningfully amplifying the impact of the Ludwig resources. Research operations in Lausanne are now known as the Ludwig Center for Cancer Research of the University of Lausanne (LICR@UNIL). As with Branches, LICR@UNIL will be externally reviewed on a quinquennial cycle to determine future Ludwig funding and support. Research operations in New York and São Paulo are also being reconfigured, converting them from full-fledged Branches to smaller efforts focused on the work of individual groups. Each of these three sites is a potential satellite of the future.

Future satellites will comprise one or two research groups, undertake very high quality and focused cancer research, will be provided longer term and highly flexible funding for defined basic and translational projects and will be reviewed with the same rigor as internal groups. These satellite foci will be distributed throughout the world and will continue to ensure that the Institute has a truly international footprint and accesses the very best scientists for the fulfillment of its mission.

The remaining large Institute Branches of the future will house a greater number of staff than any Branch has heretofore and be in localities to which it is possible to attract the best talent from anywhere in the world. The aim will be for these Branches to provide intense but collegial research environments where elite investigators can make discoveries of fundamental and far reaching impact.

COLLABORATIVE RESEARCH AND TECHNOLOGY DEVELOPMENT

The Institute seeks to enhance its core investigator-defined research by facilitating collaborations between scientists at different Branches, Centers and satellites as well as scientists at the Ludwig Trust Centers. Increased resources will be made available to these interactive groups and meetings organized to enable the identification of new potential collaborations. In 2010, meetings organized or supported with this as the objective addressed bioinformatics, genomics and TGF-beta signaling as well as melanoma and colon cancer. In each case, successful collaborations were initiated and are now ongoing. Similar meetings, involving a widening circle of scientists, are being planned for 2011.

With the appointment of Dr. Robert Strausberg as Executive Director of Collaborative Sciences in 2010, the Institute seeks to improve its access to institutional grants and increase the number of grants available to collaborative groups within the Institute. The Institute is proud and honored to have been awarded this year a grant of $2.5 million payable over five years from the Hilton Foundation that will underwrite collaborative work between the San Diego Branch and the Ludwig Center at Johns Hopkins directed at developing technology for early detection and treatment of cancer. This grant was very much made possible due to the most appreciated efforts of Mr. John Notter, Chairman of the Institute's Board of Directors.

As mentioned, a distinguishing feature of the Institute is its demonstrated commitment to extending its basic research into novel therapeutics. A recent survey revealed that over 50 Institute discoveries are either products in clinical use or are in a phase of development. In almost all cases, the cost of such development is now fully borne by external commercial entities, some in which the Institute holds equity. Over the last 15 plus years the Institute has concentrated its translational activities on the development of antibodies and cancer vaccines. For example, in 2010 the antibody known commercially as Redectane®, for which the Institute conducted extensive clinical trials and is now being developed by the biotechnology company Wilex, met the endpoints of a Phase III clinical trial. It is now expected to receive U.S. regulatory approval in 2011 for use in noninvasive detection of clear cell renal cancer.

While maintaining its interest in antibodies and vaccines, the Institute in 2010 initiated a small molecule group aimed primarily at developing inhibitors with potential applications in oncology. This small, very high quality group with biotechnology industry experience is based at the San Diego Branch. They are currently working on inhibitors of targets identified in San Diego, São Paulo, London, Brussels and Uppsala.

In 2010 clinical trials management was brought under the leadership of Dr. Ralph Venhaus. To better link clinical trials with purposeful development and to emphasize that clinical activities have the development of Institute investigational agents and science as the goal, Dr. Venhaus reports to the Executive Director of Technology Development, Dr. Jonathan Skipper.

ADMINISTRATION

An important and ongoing objective is to make the Institute's administration as cost efficient and as supportive of its scientific operations as possible. An Executive Director of Operations, Dr. Eric Hoffman, was appointed in 2010 with the task of increasingly streamlining our scientific administration in New York. This is particularly important given the introduction of formal Branch reviews by outside committees and the inclusion

of additional *ad hoc* committee members for individual reviews, both of which are under the chairmanship of the also newly appointed Head of Academic Affairs, Dr. Richard Kolodner. Coordination also is assuming an ever greater role, particularly as our senior scientists actively respond to the call to augment and reinforce the Administration.

A significant event for the New York Office in 2011 will be the move to smaller quarters at 666 Third Avenue in April. The new office will not accommodate large meetings, but will be suitable for Scientific Advisory Committee (SAC) and Board Meetings.

Dr. José Baselga has advised that he is no longer able to serve on the SAC due to increased commitments following his recent transition to Massachusetts General Hospital in Boston. The process of identifying a replacement in the clinical area is underway. As previously announced three new members have joined the SAC: Drs. Craig Thompson, Titia de Lange and Sir John Skehel. Their wisdom, experience and insight have and will add greatly to the deliberations of the SAC. A further change is that Sir David Lane has now joined the Board of Directors of the Institute. It is envisaged, nevertheless, that he will continue to attend select SAC meetings.

Consistent with the obligation of assuring that Institute science is both outstanding and well-managed, the Melbourne-Austin Branch was reviewed in 2010 and the Oxford Branch will be reviewed in 2011.

SUMMARY

The Institute is adapting to changing times and circumstances. A degree of anxiety and apprehension inevitably accompanies the process, but excitement is also palpable. As the Institute experiments with how to most effectively meet new dynamics and challenges, it remains firmly anchored in its core defining principles: a scale that is international, basic research that is world class, and an abiding commitment to improved patient outcome.

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DIRECTOR'S MESSAGE

We continued our integrated efforts to develop new cancer treatments based on therapeutic vaccination. The current focus is to identify strategies able not only to induce immune responses against the relevant tumor antigen, but also to combat the local immunosuppression that appears to develop at the tumor site and limit the capacity of the immune response to reject the tumor. We use mouse models and human tumor-infiltrating lymphocytes to uncover mechanisms responsible for this immunosuppression and devise strategies to inactivate them. We then test those approaches in preclinical models, and subsequently launch clinical trials combining peptide vaccines with the new treatment. Several approaches are currently reaching the clinical trial stage, a galectin-3 inhibitor and the local injection of cytokines and TLR ligands. Other programs are at the stage of drug discovery, such as the search for inhibitors of indoleamine 2,3-dioxygenase.

Cytokines, such as TGFß1, may contribute to tumor-related immunosuppression. We developed innovative approaches to inactivate endogenous cytokines by 'auto-vaccination'. This allows the role of individual cytokines in various *in vivo* models to be precisely defined and could lead to new therapeutic approaches.

In parallel, we pursued work on the processing of tumor antigens and discovered the new role of insulin-degrading enzyme in antigen-processing and described two new proteasome types that are intermediate between the standard proteasome and the immunoproteasome.

The roles of cytokines IL-9 and IL-22, discovered at the Branch, are extensively studied in various models of immunity and inflammation, with a focus on signal transduction by cytokine receptors. Mutations in JAK1 and JAK2 were described in human leukemias and myeloproliferative neoplasms, respectively. These mutated JAK kinases are currently characterized functionally and represent potential targets for specific inhibitors.

~ Benoît Van den Eynde

TUMOR IMMUNOLOGY AND ANTIGEN PROCESSING

Benoît Van den Eynde, M.D., Ph.D.

The group follows 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 derived from intracellular tumor proteins. They result from degradation of these proteins, mainly exerted by the proteasome. We have continued to characterize this new mode of production of antigenic peptides, which involves the splicing of peptide fragments by the proteasome. Splicing occurs in the proteasome catalytic chamber through a reaction of transpeptidation involving an acyl-enzyme intermediate. We have described four spliced peptides, two of which are spliced in the reverse order. One of these peptides also contains two additional post-translational modifications, resulting in the conversion of asparagines into aspartic acids, through a process of N-glycosylation/deglycosylation. Both the standard proteasome and the immunoproteasome have the ability to splice peptides. However, their ability to produce a given spliced peptide varies according to their ability to perform the relevant cleavages to liberate the fragments to splice.

We have completed the description of new proteasomes subtypes that are intermediate between the standard proteasome and immunoproteasome. 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 and uniquely process several tumor antigens. Other functional aspects of these intermediate proteasomes are being evaluated.

We studied a proteasome-independent peptide derived from tumor protein MAGE-A3 and identified insulin-degrading enzyme as the protease producing 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 MHC class I molecules.

We previously described an important mechanism of tumoral immune resistance 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, we identified 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. Two promising families were identified.

We made transgenic mice in which melanoma can be induced with a 70% incidence after tamoxifen injection. These tumors express the tumor antigen encoded by cancer-germline gene *P1A*. They can either be 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. Current efforts aim to devise therapeutic vaccination approaches able to induce tumor rejection despite this abnormal inflammation.

REGULATION OF T LYMPHOCYTE FUNCTION IN TUMORS

Pierre van der Bruggen, Ph.D.

The group studies the dysfunction of tumor-infiltrating lymphocytes, resulting from exposure to galectin-3 produced by the tumor, and how this anergy can be reversed by galectin competitor ligands. They also started to examine whether the spontaneous anti-tumor T cell response of non-metastatic breast carcinoma patients can be used as a clinical prognostic factor.

We observed that human tumor-infiltrating CD8 T lymphocytes (TIL), in contrast with CD8 blood cells, show impaired IFN-γ secretion upon *ex vivo* re-stimulation. This was attributed to the decreased IFN-γ secretion to a reduced mobility of T cell receptors trapped in a lattice of glycoproteins clustered by extracellular galectin-3. It was previously observed that treatment of TIL with N-acetyllactosamine (LacNAc), a galectin competitor ligand, restored this secretion. Why do galectin-3 ligands improve human TIL function? Galectin-3 is abundant in many solid tumors and carcinomatous ascites, because tumor cells and macrophages secrete it. We surmise that TIL have been chronically stimulated by antigens and, compared to resting T cells, harbor a set of glycans that are either more numerous or better ligands for galectins, in particular galectin-3. Galectin-3 would thus bind to surface glycoproteins of TIL, form glycoprotein-galectin lattices, and thereby reduce TIL function. The release of galectin-3 by soluble competitor ligands would restore TCR mobility and boost IFN-γ secretion by TIL. We recently strengthened this hypothesis by showing that CD8+ TIL treated with an anti-galectin-3 antibody produced more IFN-γ. We are trying to understand the very early activation events that are defective in TIL and will examine a possible role of galectins in the T cell dysfunction observed in different human chronic pathologies.

Galectin competitor ligands, e.g., disaccharides lactose and LacNAc, are rapidly eliminated in urine, preventing their use *in vivo*. We recently found that a plant-derived polysaccharide, currently in clinical development, detached galectin-3 from TIL and boosted their IFN-γ secretion. We observed that not only CD8+ TIL but also CD4+ TIL treated with this polysaccharide 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 of the mice, whereas all control mice died. In non-vaccinated mice, the polysaccharide had no effect by itself. These results suggest that a combination of galectin-3

ligands and therapeutic vaccination may induce more tumor regressions in cancer patients than vaccination alone. We recently identified another plant-derived polysaccharide that binds to galectins and is already used in phase II clinical trials in colorectal cancer patients in combination with chemotherapy. This compound was as effective as LacNAc in boosting the secretion of IFN-γ by TIL. A clinical trial with this new compound, in combination with anti-tumoral vaccination, is expected to be launched in 2011 in clinical centers working with the Branch.

Several retrospective studies suggest a correlation between the survival of patients with ovarian or colorectal carcinoma and the infiltration of their tumors by immune cells. Considering our experience in quantitative approaches to detect very weak T cell response in the blood of melanoma patients, Danièle Godelaine set out the prospective evaluation of the frequencies of anti-tumor CD8 T lymphocytes in the blood of patients with non-metastatic breast cancer recruited in several Belgian clinical centers. Frequencies are evaluated by mixed lymphocyte-peptide cultures, carried out with HLA-A2 and A3-restricted HER2/neu and hTERT peptides, followed by detection of specific cells with HLA-peptide tetramers. Blood samples are collected before and after surgery. Tumors removed at surgery are analyzed by immunohistology for infiltration by immune cells, and fragments are frozen for further analysis. The prospective follow-up of 172 patients will extend over a five year-period. So far, 25 patients have been included. Eight of them have a frequency against the targeted antigens ranging from 10-5 and 10-6 among blood CD8 T cells, whereas the frequency in healthy donors' blood was estimated to be lower than 5x10-7. We anticipate identifying the patients with a better prognosis in order to offer them an adapted care and thus avoiding unnecessary treatments.

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 group tests various approaches to overcome the anergy of such infiltrating T cells. These approaches involve cytokines and Toll-like receptor ligands, as well as antibodies directed against inhibitory cytokines such as TGFβ.

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. Repeated immunizations with male lymphoblasts did not induce rejection of established skin grafts. We tested local approaches to break this tolerance. Repeated local injections of a low dose of IL-12, combined with IFNα, caused graft rejection in all mice. This was also the case when IL-12 was combined with ligands of Toll-like receptors 3, 7 or 9 (TLR). Like IFNα, IL-1α, IL-18 and IL-2 were incapable of inducing rejection on their own, but synergized effectively with IL-12.

We tested combinations of agents approved for clinical use. Repeated local injections of a combination of low doses of IL-2, GM-CSF and IFNα with TLR7 ligands gardiquimod or imiquimod caused 100% rejection. The crucial components appear to be IL-2 and gardiquimod. A clinical trial has been launched in which a small number of patients with superficial lesions of metastatic melanoma will receive vaccinations with tumor antigens

combined with a local treatment composed of the three cytokines available as registered medicines and Aldara, a cream containing imiquimod.

For this treatment to be therapeutically effective, rejection of the locally treated metastasis would be necessary to result in an antitumoral T-cell expansion that would also eliminate other metastases. In CBA females carrying two male skin grafts the local treatment of one graft which caused its complete rejection induced, in a fraction of the mice, a systemic immune response sufficient to cause partial or complete rejection of a distant graft. We are presently exploring additional local and systemic treatments that may improve this outcome.

To enhance our understanding of the action of the local agents, we are tracking naïve CD8 T-lymphocytes endowed with an anti-H-Y-Kk T-cell receptor, collected from transgenic mice and transferred into the grafted mice. With a PCR specific for the α chain gene of this receptor, we observed that these lymphocytes concentrated in the graft seven days following the onset of the local treatment with IL-2, GM-CSF, IFNα and imiquimod. In the absence of treatment, these lymphocytes did not accumulate in the graft.

In collaboration with Jean-Christophe Renauld's group, we have made significant progress in our efforts to develop auto-vaccines against cytokines as a tool for studying their functions *in vivo* as well as develop a panel of monoclonal antibodies of mouse origin against mouse and human cytokines with therapeutic perspectives. Self cytokines linked chemically to a non-self protein or genetically associated to a defined foreign sequence become immunogenic. The proposed rationale underlying this process is that the self-reactive B cell that has captured the complex or fusion protein will present foreign peptides on its MHC Class II membrane proteins and thus attract help from T cells reactive with the non-self structure.

While there are gaps in our understanding of the precise mechanisms involved in this auto-vaccination, we recently noted a positive correlation between immunogenicity and immunogen size in a series of anti-IL-12 vaccines that were fractionated according to size. This raised a dilemma, since the larger the complex, also the greater the risk for structural alterations of the antigen.

To circumvent this problem, we experimented with a two-step procedure. First we made large ovalbumin (OVA) multimers by treating OVA with glutaraldehyde and, after purifying the polymerized products by size exclusion chromatography, reacted these with the target cytokine before saturating remaining glutaraldehyde sites with a pan DR epitope peptide (PADRE) to maximize immunogenicity.

With this procedure, mice were successfully immunized against the chemokine GCP-2/CXCL6, the cytokines GM-CSF, IL-17F, IL-17E/IL-25, IL-27, TGF-β1 and the matrix metalloproteinase-9 MMP-9/gelatinase B. We obtained monoclonal antibodies (mAb) from these mice, including a mAb against TFG-β1 which inhibits TGF-b1 bioactivity; an anti- IL-17F mAb, which abrogates the neutrophil chemotactic activity of IL-17F; a mAb against GCP-2, which demonstrates the essential role of GCP-2 in rapid neutrophil mobilization after Leishmania major infection; and, finally, a mAb against mouse IL-27, which potently inhibits its bioactivity.

THERAPEUTIC VACCINATION AND TUMOR EXPRESSION PROFILING

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

The group develops early phase clinical trials, in which patients with advanced cancer, often metastatic melanoma, receive an experimental immunotherapy treatment aimed at promoting cytolytic T lymphocyte (CTL)-mediated tumor rejection. These projects are developed in close collaboration with J.F. Baurain (Centre du Cancer, Cliniques Universitaires Saint-Luc) and P. Coulie (de Duve Institute). Previous studies have investigated various therapeutic vaccines containing one or several defined tumor-specific antigens expressed by the patients' tumors. All these vaccines were well tolerated. Tumor regressions were observed in a minority of patients with metastatic melanoma. However, objective tumor responses were achieved in a marginal number of patients. We are following two different approaches to try and improve these results: find more immunogenic vaccines, and combine vaccines with treatments that modify the tumor environment in favor of

effective tumor rejection. Indeed, there is increasing evidence that this environment plays a key role in the inhibition of anti-tumoral T cell activity.

In a recently started phase I trial, we are testing the safety, immunogenicity and anti-tumoral effect of a promising new vaccine called Theravac. Theravac is a recombinant chimeric protein vaccine aimed at targeting dendritic cells (DC) *in vivo*, and forcing them to express the Tyr.A2 antigen, a peptide derived from the melanocyte and melanoma-specific tyrosinase protein. Theravac is derived from a bacterial toxin that, upon binding to CD11b, is internalized and neutralizes its target DC. In the vaccine, the toxin has been inactivated by insertional mutagenesis, and coupled to the Tyr.A2 peptide. Preclinical experiments have shown that Theravac has a very potent capacity to activate Tyr.A2-specific CTL. In our trial, patients with tyrosinase-expressing metastatic melanoma are immunized with repeated injections of Theravac, at increasing doses. This project was developed in collaboration with C. Leclerc (Pasteur Institute, Paris).

In another ongoing clinical trial, melanoma patients are vaccinated with a synthetic peptide, either MAGE-3. A1 or NA17.A2, both tested in previous trials. In addition, patients receive peritumoral injections with a cocktail of pro-inflammatory cytokines, in one or two superficial metastases. The same cocktail has been tested in a mouse model of skin graft rejection and is able to induce effective tissue rejection.

A third clinical trial is in development,

whereby melanoma patients will receive the same peptide vaccine, in association with repeated infusions with an experimental drug called Davanat®, a plant-extracted oligosaccharide that binds to and inhibits galectins. Galectin-3 is a protein produced by cancer cells that is able to inhibit T cell activation. Pierre van der Bruggen's group has shown the anergy that characterizes tumor-associated T cells can be reversed with galectin-3 inhibitors including Davanat®.

Next to clinical trial development, our group analyzes a series of cutaneous metastases obtained from melanoma patients, using an approach that combines gene expression profiling by microarray, immunohistology, immunofluorescence and laser capture microdissection of small groups of cells present in the tumors, such as T lymphocytes, followed by gene expression analysis. The inflammatory cells and pathways that are associated with tumors are being studied in order to understand the interactions between tumor and immune cells at the tumor site and we are characterizing lymphoid structures present in tumors in which B cell responses occur.

We are collaborating with several other European groups on a project aimed at developing innovative imaging microscopy approaches that might improve cancer diagnosis. These approaches are based on spectroscopical analysis of tissue sections illuminated with one or several 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 dependent on the molecular bonds present in the illuminated sample. The objective is to identify spectral signatures associated with melanoma cells, which would allow detection *in vivo* and in histological preparations without staining.

CYTOKINES IN IMMUNITY AND INFLAMMATION

Jean-Christophe Renauld, M.D., Ph.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, including asthma and anaphylaxis. In addition, 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. However, alternative pathways of IL-9 production by T cells also exist, such as combinations of TGFß and IL-1 family members.

IL-22, originally identified as a gene induced by IL-9 in T lymphocytes, is produced by TH17 lymphocytes. Recent work has shown that innate lymphoid cell populations including intestinal NK-like cells and LTi-like cells also contribute to the production of IL-22 during inflammatory processes in the intestinal mucosae. In response to the TLR5 agonist flagelin or the TLR4 agonist LPS, IL-22 was produced by a new innate lymphoid spleen cell population expressing CD25, CCR6 and IL-7R representing 1% of spleen cells from recombination activating gene (Rag2)-deficient mice. The importance of LPS-triggered IL-22 production is highlighted by the fact that IL-22-deficient mice are more resistant to LPS-induced mortality, pointing to the pro-inflammatory activity of this cytokine. In the same line, we previously demonstrated that these mice are partially protected against collagen-induced arthritis. In contrast, IL-22 plays a protective role in mouse IBD models and in infectious models of the skin and mucosae.

Beside conventional gene targeting strategies, we developed novel strategies of anti-cytokine vaccination leading to the production in vaccinated mice of anti-cytokine autoantibody that block the biological activities of endogenous cytokines. Neutralizing auto-antibodies against IL-9, IL-12 and IL-17 were induced upon vaccination with the autologous cytokines coupled with OVA (IL-9, IL-17) or with the Pan DR T helper epitope PADRE (IL-12). This strategy was instrumental in demonstrating the role of IL-9 in an intestinal helminth infection, of IL-12 in atherosclerosis and of IL-17 in experimental autoimmune encephalomyelitis. We recently improved the method using OVA multimers and successfully applied it to the chemokine GCP-2/CXCL6, the cytokines GM-CSF, IL-17F, IL-17E/IL-25, IL-27, and TGF-β1, and the MMP-9/gelatinase B. We showed that functional blocking of GCP-2 inhibits tumor growth and metastases of human melanoma over-expressing muGCP-2. To further extend the scope of this strategy, a new procedure of anti-cytokine vaccination was developed based on DNA vectors encoding the targeted antigenic peptides in fusion with a human transmembrane protein to allow for their cell-surface expression. Using either transfected cells as a vaccinal vector or direct intra-muscular DNA electrotransfer, we induced blocking antibodies against IL-9 or IL-22BP. These vaccination methods represent simple and convenient approaches to knock down the *in vivo* activity of soluble regulatory proteins, including cytokines and their receptors, and are currently validated with additional targets in inflammatory models.

The role of IL-9 in tumorigenesis was previously shown in various models. However, in many cases, IL-9-dependent tumor cells eventually acquired the ability to proliferate autonomously. Using an *in vitro* transformation model, we

found that overexpression and activating mutations of the JAK1 tyrosine kinase represent efficient mechanisms leading to cytokine-independent growth with constitutive STAT activation. Twenty-five *de novo* JAK1 activating mutations were identified, including five mutations already described in human leukemias. While most JAK1 mutants were sensitive to ATP-competitive JAK inhibitors, mutations targeting Phe958 and Pro960 in the hinge region of the kinase domain rendered JAK1 constitutively active, but also resistant to all tested JAK inhibitors. Furthermore, mutation of the homologous Tyr931 in JAK2 wild-type or JAK2 V617F mutant found in patients with myeloproliferative neoplasms also conferred resistance to JAK inhibitors, such as the clinically used INCB018424. The data indicate that some activating mutations not only promote autonomous cell proliferation but also confer resistance to ATP-competitive inhibitors. *In vivo*, such a mutation can potentially occur as primary JAK-activating mutations but also as secondary mutations combining oncogenicity with drug resistance.

In collaboration with Marco Tartaglia's group, activating mutations of JAK1 in human acute lymphoblastic leukemias were previously found. 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 recapitulated this signature in the absence of IFN, but also strongly potentiated the response to IFN. Leukemia cells expressing mutants such as JAK1(A634D) are hypersensitive to the anti-proliferative and anti-tumorigenic effect of type I IFN, suggesting that type I IFNs should be considered as a potential therapy for ALL with JAK1 activating mutations.

SIGNAL TRANSDUCTION AND MOLECULAR HEMATOLOGY

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

The major lines of research in the group are: 1) the mechanisms by which the JAK2 tyrosine kinase becomes activated by pseudokinase domain mutations, such as the V617F mutation, in human myeloproliferative neoplasms (MPNs); 2) the contribution of pathologic signaling by the receptor for thrombopoietin (TpoR) in the pathogenesis of MPNs, especially Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF); and 3) the identification of other markers of MPNs, such microRNA-28.

Inhibitors of JAK2 are becoming standard therapy in advanced (myelofibrosis) forms of MPNs, but since such molecules do not discriminate between wild-type and mutated JAK2, side effects such as anemia and thrombocytopenia are common. Ideally, inhibitors specifically targeting JAK2 V617F should be used. In 2010 we alanine scanned the helix C of the kinase domain of JAK2 and that of the pseudokinase domain of JAK2, and identified a pocket located in the pseudokinase domain of JAK2 where small molecules could induce specific inhibition. Modeling the structure of the pseudokinase domain of JAK2 on the crystal structures of other tyrosine kinases led us to identify the residues predicted to be located closest to F617: the closest residue, F595, emerged in functional assays as crucial for constitutive activation of JAK2 V617F, but not for cytokine-activated JAK2. We showed that kinase activation originating from JAK2 itself due to mutations (K539L, T875N,

R683G) located in regions other than the pseudokinase domain absolutely requires integrity of residues in the middle of the JH2 helix C, especially of F595. These 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 the homologous JH2 helix C (F536A) mutation.

Using bone marrow transplantation models, we showed that an activating mutant of TpoR (TpoR W515A), identified in myelofibrosis patients, induces in mice a severe disease that is dependent upon signaling via one cytosolic TpoR tyrosine residue, Y626. Phosphotyrosine immunoprofiling and mass spectrometry detected phosphorylation of this cytosolic TpoR residue in cells expressing TpoRW515A. The same tyrosine residue was required for inducing a severe disease when TpoR and JAK2 V617F where simultaneously transduced in bone marrow stem cells. We proposed that hyperactivation of TpoR and MAP-kinase/STAT3 pathways, which are dependent on the Y626 residue, are key to myelofibrosis progression, and a small molecule binding to TpoR p-Y626 could be beneficial in the treatment of MPNs.

TpoR is down-modulated in platelets and megakaryocytes from MPN patients. We showed that co-expression of TpoR and JAK2 V617F in cell lines also leads to down-modulation of TpoR, and that this occurs via several mechanisms: i) enhanced internalization, ubiquitinylation and proteasome-mediated degradation of the receptor; and ii) selection against an antiproliferative effect of TpoR in cells that express high levels of JAK2 V617F. We discovered that, physiologically, Tpo induces proliferative and antiproliferative effects at low and high JAK2 expression levels, respectively. The antiproliferative effect of Tpo is essential for late postmitotic megakaryocytes, where Tpo prevents division allowing formation of platelets; iii) microRNA28 (miR-28) is pathologically induced in 1/3 of MPNs. miR-28 inhibits TpoR mRNA translation, and synthesis of several proteins that are involved in megakaryocyte differentiation. The host gene of miR-28, Lipoma Preferred Partner (LPP), is induced by constitutive active STAT5, but not by transient cytokine-induced STAT5 activation, suggesting that persistent STAT-induced chromatin changes are required for LPP induction. We use miR-28 as a biomarker of a subset of ET/MPN where no JAK2 or TpoR mutation can be demonstrated, and where other STAT5-activating mutations responsible for ET and myelofibrosis in humans can be searched.

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DIRECTOR'S MESSAGE

2010 marked a watershed in the history of the LICR in Lausanne. After more than 35 years as an independent entity, the Branch was formally integrated into the University of Lausanne (UNIL) on January 1, 2011 and has been renamed the Ludwig Center for Cancer Research of UNIL (LICR@UNIL). Most of the Branch staff were given UNIL contracts and several group leaders have been appointed as UNIL professors. Core funding is provided jointly by LICR and UNIL. We are grateful to both institutions for their enthusiastic support and look forward to playing a major role in the establishment of a new Lausanne Cancer Center which will focus on translational research in oncology in collaboration with the University Hospital (CHUV) and Federal Institute of Technology (EPFL).

In this new framework the LICR@UNIL will continue to combine basic and translational studies in T cell immunology to benefit cancer patients via novel approaches to immunotherapy. To this end we will adhere to our long-standing policy of encouraging

extensive interactions among research groups so that basic discoveries can be rapidly translated to early phase clinical trials which are carried out in collaboration with the Center of Pluridisciplinary Oncology (CePO) located at the CHUV.

While the creation of LICR@UNIL marked the end of my term as Branch Director on December 31, 2010, I will continue to serve as Interim Director of the new organization during the transition period. I am grateful to LICR for all the support they have provided to the Branch over the years and will continue to provide in this exciting new joint venture.

~ H. R. MacDonald

DEVELOPMENTAL IMMUNOLOGY

Hugh Robson MacDonald, Ph.D.

Work in the group continues to be focused on the development of T lymphocytes from hematopoietic precursors. Current studies highlight the role of Notch and Myc signaling pathways in the generation of conventional and unconventional T lymphocytes respectively.

Many aspects of the role of Notch signaling in T cell development have been elucidated by the group over the past decade in collaboration with Freddy Radtke (EPFL, Lausanne). These studies have established a critical and non-redundant interaction between Notch-1 on bone marrow precursors and its ligand Delta-like 4 (DL4) on thymic cortical epithelial cells (cTEC) in the specification of the T cell lineage in the adult thymus. We have extended these studies to the fetal liver and fetal thymus using novel monoclonal antibodies to Notch-1 and DL4 in addition to mice with a conditional inactivation of DL4 in cTEC. We found that Notch-1 is already highly expressed in hematopoietic T cell precursors in the fetal liver and remains highly expressed on immature T cells present in the fetal thymus. It is interesting to note that DL4 is expressed at much higher levels on fetal cTEC than on adult cTEC. Conditional ablation of DL4 in cTEC in the fetal thymus leads to an almost complete block in fetal T cell development, including absence of an unusual subset of fetal γδ T cells which are precursors of so-called dendritic epidermal T cells that ultimately home to the skin. It was concluded that the unique interaction between Notch-1 and DL4 is also critical for T cell development during embryogenesis.

In addition to conventional T cells, the group is interested in unconventional T cells such as natural killer T (NKT) cells and intestinal intraepithelial lymphocytes (IEL). Using mice with a specific inactivation of the transcription factor c-Myc in the T cell lineage, we have previously shown that the development of NKT cells (but not conventional T cells) is critically dependent upon c-Myc. In order to determine whether this selective role of c-Myc in NKT cell development can be generalized to other subsets of unconventional T cells, IEL development in the absence of c-Myc was examined.

The murine gut epithelium contains a large population of thymus-derived IEL, including both conventional CD4+ and CD8αβ+ T cells (expressing TCRαβ) and unconventional CD8αα+ T cells (expressing either TCRαβ or TCRγδ). Whereas conventional IELs are widely accepted to arise from recirculation of activated CD4+ and CD8αβ+ T cells from the secondary lymphoid organs to the gut, the origin and developmental pathway of unconventional CD8αα IELs remain controversial. We found that T-cell-specific inactivation of c-Myc selectively impairs the development of CD8αα TCRαβ IELs. In the absence of c-Myc, CD4– CD8– TCRαβ+ thymic precursors of CD8αα TCRαβ IELs are present but fail to develop on adoptive transfer in immunoincompetent hosts. Residual c-Myc–deficient CD8αα TCRαβ IEL display reduced proliferation and increased apoptosis, which correlate with significantly decreased expression of interleukin-15 receptor subunits and lower levels of the antiapoptotic protein Bcl-2. Transgenic overexpression of human BCL-2 resulted in a pronounced rescue of CD8αα TCRαβ IEL in c-Myc–deficient mice. Taken together, the data support a model in which c-Myc controls the development of CD8αα TCRαβ IELs from thymic precursors by regulating interleukin-15 receptor expression and consequently Bcl-2–dependent survival. Results point to a common requirement for c-Myc during the development of unconventional T cell subsets.

INNATE IMMUNITY

Werner Held, Ph.D.

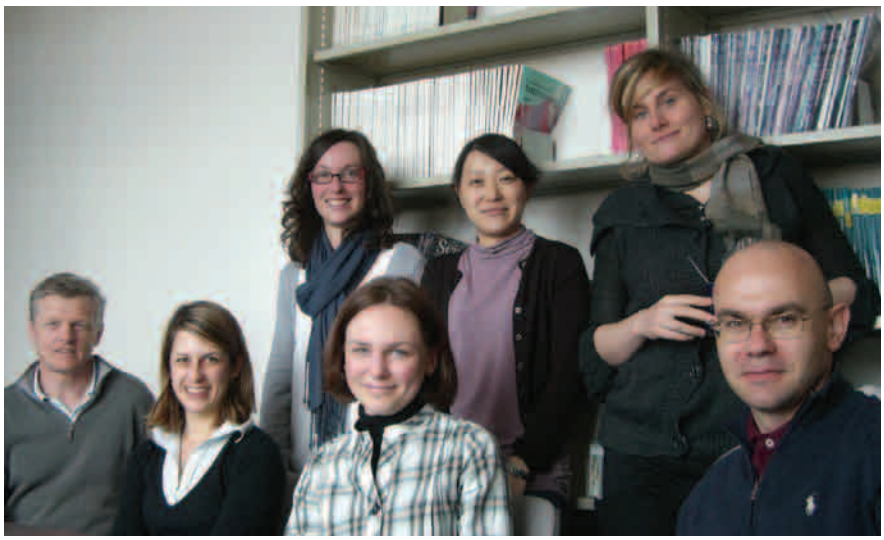
The group's research is focused on the identification of factors that improve the elimination of diseased (stressed, transformed or infected) host cells by Natural Killer (NK) and CD8+ cytotoxic T cells. This research led the laboratory to identify a signaling pathway which is essential for the development of functional CD8 T cell memory.

The first productive encounter with antigen initiates the clonal expansion and differentiation of very rare antigen-specific CD8 T cells, generating a large pool of cytokine producing and cytolytic effector T cells. Most effector CD8 T cells die once the pathogen is cleared, leaving behind a population of long-lived memory CD8 T cells. Effector memory T cells respond to antigen re-encounter with immediate effector function but limited proliferation. Long-lived central memory T cells have the unique ability to vigorously expand upon secondary antigen encounter. In addition to producing secondary effector cells, central memory precursors self-renew in order to maintain a functional memory CD8 T cell compartment. Memory CD8 T cells thus have attributes of tissue-specific stem cells.

A signaling pathway, which plays prominent roles in the maintenance and lineage specification of stem cells in a variety of tissues, is the canonical Wnt (Wingless/Integration-1) signaling pathway. A subset of extracellular Wnt proteins activates canonical Wnt signaling characterized by the intracellular stabilization of β-catenin. This allows the translocation of β-catenin to the nucleus, its association with transcription factors of the Tcf (T cell factor) family and the activation Wnt responsive target genes. In the absence of extracellular Wnt, β-catenin is recruited to a cytosolic destruction complex, phosphorylated and degraded.

To address whether the canonical Wnt pathway plays a role for CD8 T cell differentiation, we tested whether mice lacking Tcf-1, one of the nuclear effectors of the Wnt pathway, were competent to control an acute viral infection. Tcf-1-deficient mice mounted an efficient primary CD8 T cell response to viral infection. Virus-specific CD8 T cells were detected over long periods of time and these showed immediate effector functions similar to that of wild type control mice. However, CD8 T cells in immune Tcf-1-defcient mice failed to expand upon a secondary viral infection. In line with this observation, the number of central memory phenotype CD8 T cells was strongly reduced and memory CD8 precursor cells were diminished at the peak of the primary immune response. These defects were intrinsic to CD8 T cells and unrelated to possible alterations in T cell receptor usage in the absence of Tcf-1. The data suggest that central memory T cell differentiation is an early, Tcf-1-dependent event. Furthermore, CD8 T cell memory formation was dependent on the catenin-binding domain in Tcf-1 and on the intracellular Wnt signal transmitters β-catenin and γ-catenin, linking memory formation to the transduction of canonical Wnt signals. In addition to these loss-of-function data, enhanced Wnt signaling augmented the emergence of CD8 T cells with a memory phenotype using the enforced expression of Tcf-1 and β-catenin in mice.

These data demonstrate that the canonical Wnt pathway plays an essential role in the generation of central memory CD8 T cells and raise the possibility that modulation of Wnt signaling may be exploited to improve the generation of CD8 memory T cells during vaccination or for therapies designed to promote sustained cytotoxic CD8 T cell responses against tumors.



MOLECULAR IMMUNOLOGY

Immanuel Luescher, Ph.D.

The group focused on three major projects in 2010. Based on previous observations that CD8β KO mice exhibit normal CD8+ T cell responses upon acute LCMV infection, in our first project we extended these studies by comparing primary anti-LCMV CD8+ T cell responses in mice that express as transgene P14 TCR β chain (P14β tg) and CD8β or not. We found that CD8+ effector T cells from CD8β KO mice exhibited ablated granzyme/perforin-mediate cytotoxicity. Remarkably, CD8β KO mice cleared LCMV infections as well as normal mice, which was surprising in view of previous claims that for this granzyme/perforin-mediated cytotoxicity is required. The defective granzyme/perforin killing was explained by the lack of significant intracellular Ca^{2+} mobilization in CTL from CD8β KO mice, which is needed for CTL degranulation. Nevertheless, CTL from CD8β KO efficiently killed target cells as assessed by chromium release experiments, which was explained by a striking up-regulation of FasL expression on CTL and even more so on naïve CD8+ T cells and thymocytes from CD8β KO mice. These findings suggest that up-regulation of FasL might be a therapeutic modality to treat chronic viral infections.

CTL from CD8β KO are paradigm CD8 independent. Previous work advocated that CD8 dependence is essentially determined by the TCR and in our second project, we established a method to assess TCRα chain repertoires with high resolution. The TCRα chain is rearranged on CD4+, CD8+ double positive (DP) thymocytes concomitant with positive selection, and therefore CD8β KO seems more likely to impact this repertoire. Using paired-end deep sequencing on TCRα chain transcripts of naïve CD8+ T cell from C57BL/6 (B6) mice, we obtained tens of millions of complete TCRα chain sequences, analysis of which provided new insights of TCRα chain gene recombinations. The results are consistent with the previously reported coordinate TCRα chain gene rearrangements concept, according to which rearrangements are governed by the T early activation gene (TEA), which supports recombination of 5′ proximal Jα with 3′ proximal Vα genes. However, our analysis revealed that when looking at the entire repertoire, there is a much larger number of recombination that do not follow this concept and cover the entire, nearly 2 mb large Vα and Jα loci. These secondary (or later) recombinations are governed by the accessibility of Vα genes to the compact Jα locus, most likely mediated by chromatin remodeling, and determine the frequencies and the diversity of Vα-Jα recombinations. We currently apply this technique to assess the impact of CD8β on the TCRα chain repertoire formation in P14β tg mice. Preliminary results indicate that i) CD8β substantially broadens the TCR repertoire, most notably on primary D^b/gp33-specific CTL; and ii) the repertoire of CD8β KO mice is contained in the one of CD8β+ mice, with only a few (~1.3%) unique sequences.

In the third project, we established an alternative procedure to prepare fluorescent MHC-peptide tetramers, reagents that are widely used to enumerate, analyze and isolate antigen-specific T cells. In conventional tetramers, monomeric MHC-peptide complexes are biotinylated at an added biotinylation sequence peptide (BSP) by means of the biotin transferase BirA and then conjugated by reaction with phycoerythrin labeled streptavidin. Tetramers avidly bind to antigen-specific T cells and induce activation dependent cell death, which seriously compromises several applications, namely cell sorting. To circumvent this, we replaced the biotin-streptavidin binding with a reversible chelate bond formed by His tag and Ni^{2+}

nitrilotriacetic acid (NTA). Commercially available mono-NTA reagents bind His_6 tagged recombinant proteins in the micromolar range, which allows their affinity purification, but not stable conjugate formation. By assessing the binding of various mono, di and tetra NTA compounds to differently His tagged MHC-peptide complexes, we found a combination consisting of double His_6 tagged ($2xHis_6$) complex and a tetra Ni^{2+} NTA compound that exhibited highly stable binding (K_D 5.6 pM); yet was rapidly and fully reversible upon addition of imidazole (100 mM). Based on this reversible binding principle MHC-peptide conjugates are currently prepared using different carriers (e.g., phycoerythrin or fluorescent labeled immunoglobulins) and evaluated for their usefulness as alternative staining reagents.

TRANSLATIONAL TUMOR IMMUNOLOGY

Pedro Romero, M.D.

The group strives at combining studies of tumor specific T cell responses in cancer patients with mouse models of immunotherapy in experimental tumors. The focus continues to be on T cell differentiation and T cell mediated immunity and the design of novel immunotherapeutic strategies that may have potent anti-tumor effects.

Adaptive immune responses to pathogens typically involve relatively large expansions, by rapid proliferation, and differentiation into effector and memory T cells. The latter will mount vigorous recall responses. Both lymphocyte differentiation and proliferation are coupled and control of these events is multifactorial. Understanding the mechanisms controlling these processes provides key insights to guide the design of effective vaccination strategies. In this regard, we wanted to identify microRNAs that may play a role in regulating T cell differentiation during specific immune responses. Low density microRNA arrays revealed a small number of miRNAs whose expression varies significantly during T cell differentiation both in human and mouse CD8 T cells. One of the prominent miRNAs in this group is miR-155. We could show that it is upregulated five to tenfold in CD8 T cells responding *in vivo* to antigen encounter in the course of an acute viral infection in mice. Likewise, differentiated CD8 T cells from human blood have three to ten times higher levels of miR-155 than their naïve counterparts. MiR-155 knockout mice mount defective CD8 T cell responses to acute viral infections and the memory phase following viral control is also abnormally low and functionally compromised, compared to the miR-155 wild type mice. Adoptive co-transfer experiments of mixtures of miR-155 wt and knockout CD8 T cells bearing transgenic TCRs, clearly indicate that the defective effector T cell phase is cell autonomous. In other words, miR-155 expression is intrinsically required for optimal effector CD8 T cell responses and miR155 deficient CD8 T cells are unable to efficiently control experimental tumor growth. Conversely, enforced overexpression of miR-155 in tumor antigen specific CD8 T cells significantly enhances their ability to control tumor growth. Further studies will aim at identifying the targets of miR-155 and its specific role in the memory phase of the CD8 T cell response, particularly in the context of therapeutic vaccination against large tumors.

We recently identified Melan-A antigen specific regulatory T cells in the majority of melanoma patients. These T cells recognize a Melan-A peptide in the context of the HLA-DQ6 molecule, which is present in approximately 20% of the

general population. Combined use of HLA-DQ6/Melan-A peptide fluorescent multimers with Foxp3 staining showed that the relative and absolute numbers of these cells were high in patients with advanced metastatic melanoma but dramatically reduced upon vaccination with Melan-A peptide in an adjuvant formulation. This, together with the strong increase in Melan-A specific CD8 effector T cells, is a desired outcome of therapeutic vaccination. To understand the mechanisms involved in this phenomenon and verify the value of Teff:Treg ratio as a biomarker of vaccination, we have begun using mouse models. Adoptive transfer of OT-1 and OT-2 x eGFP-Foxp3 TCR transgenic T cells enables testing the effect of different vaccine formulations on the ratio of effector (Teff) and regulatory (Treg) antigen specific CD8 and CD4 T cells, respectively, in the elicited response. Clearly, the use of peptide alone favors a low ratio because of selective Treg expansion.

The addition of TLR agonists, notably TLR-9 agonists, promotes high ratios because of strong Teff expansion. A saponin preparation appears to favor a low ratio. Preliminary results show that high Teff:Tregs upon vaccination are associated with better tumor growth control in the therapeutic setting.

Finally, our studies on TLR-3 expression by tumors have shown that this receptor is expressed on close to half of primary melanoma tumors and one third of primary breast carcinomas. Retrospective analysis of a clinical trial performed in the 80s using poly(A:U), a double stranded RNA analogue which engages specifically TLR-3, strongly suggests the value of TLR-3 expression in the resected primary tumors as a biomarker of the clinical efficacy of poly(A:U). These results encourage considering the use of TLR-3 agonists as anti-tumor agents in melanoma and breast cancer expressing TLR-3.

CLINICAL-TRANSLATIONAL ONCOLOGY

Daniel Speiser, M.D.

In the framework of our clinical melanoma program, we are treating patients with state-of-the-art innovative therapies, including tyrosine kinase inhibitors and immunotherapy. We achieved relatively frequent regression of metastases and/or stabilization of disease induced by a BRAF inhibitor (PLX4032, Roche) or the recently approved anti-CTLA-4 antibody (Ipilimumab, BMS). We also continued the development of cancer vaccine formulations based on antigenic peptides and CpG or Lag-3.

The daily interactions between clinic and laboratory allowed us to obtain blood samples and unusually large amounts of metastatic tissue. We analyzed antigen-specific T-cell effector functions directly *ex vivo*, combined with extended molecular characterization. The results are of unprecedented high resolution, providing novel insights into the biology of cancer specific immune mechanisms.

It was determined the gene expression profiles of antigen-specific CD8 T-cells, for both tumor- and virus-specific T-cells, the latter as reference populations of protective T-cells against acute viral diseases. In peripheral blood, the tumor-specific T-cells showed multiple features of functional effector T-cells, with only small differences to virus-specific T-cells, indicating that both are competent effector cells. In contrast, the gene expression profile of tumor-specific T-cells isolated from metastases was very different and showed extended molecular alterations. The data demonstrate an impressive co-existence of functionally competent T-cells in circulation, and dysfunctional

T-cells in metastases of the same host. The expression profile of the latter cells correlated with the profile of so-called exhausted T-cells from mice infected with *LCMVirus clone-13* causing protracted/chronic infection. In contrast, the gene profile of T-cells from melanoma metastases was significantly different from murine "anergic" T-cells. Thus, mechanisms of exhaustion rather than anergy appear responsible for the known functional T-cell deficiencies in melanoma metastases. These results represent a breakthrough because T-cell exhaustion has been postulated for cancer-associated immune deficiency not demonstrated in any species to date. Scientists can now build on the new molecular evidence and pinpoint mechanisms that can be targeted by novel therapeutic approaches with the goal of improving the functional competence of exhausted T-cells in metastases of cancer patients.

For these studies, we used sophisticated technologies to isolate the cells directly *ex vivo*; purify them by FACS sorting, and determine their molecular profiles. In addition, protein expression was analyzed and functional analysis of cytokine production and cytotoxicity was performed. Technically, this work represents a new generation-approach with systems biology, whereby blood cells and tumor tissue were analyzed at the level of highly specific cell populations, rather than whole tissue or whole blood cells/whole lymphocytes. For the first time in the field of human and animal bio-medical research, it has become possible to characterize small numbers of cells directly *ex vivo* for molecular and functional properties.

One example of our findings was that T-cell exhaustion was associated with simultaneous expression of multiple inhibitory lymphocyte receptors. Thus, there are more than the two receptors (CTLA-4 and PD-1) that are usually described in the literature. We found frequent co-expression with Tim-3, Lag-3, BTLA, 2B4, and KRLG-1 (but not CD160) even by individual T-cells. We analyzed expression and function of BTLA (B and T lymphocyte attenuator) in greater detail, and made three key observations. First, BTLA negative T-cells showed enhanced effector functions. Second, blocking of BTLA-HVEM interactions of BTLA positive T-cells increased their functional competence. And third, strong vaccines adjuvanted by CpG oligonucleotides induced BTLA downregulation on tumor-specific effector T-lymphocytes, and enhanced their functional competence. Together these findings open new treatment options for patients with cancer and chronic infectious diseases.

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DIRECTOR'S MESSAGE

Since 1990 Institute activities at the Austin Hospital have grown from an initial clinically focused research site, to one which encompasses basic, translational and clinical cancer research in an integrated Branch structure. In contrast to other LICR Branches and medical research institutes in Australia, there is an integral engagement in innovative clinical research providing routine cancer care through the unique joint arrangements with Austin Health in running the Centre for Positron Emission Tomography (PET) and Medical Oncology. This arrangement was strengthened in 2010 with the appointment of Professor Jonathan Cebon as Director, Cancer Services Unit of Austin Health. The LICR-Austin Health partnership has had a major impact on patient health delivery, as well as contributing at a national and international level to evidence based clinical care of cancer patients.

The basic science laboratory programs in the Branch comprise themes in cancer immunology, antibody-based therapeutics, signaling pathways in cancer, epigenetics of colon cancer, and tumor biology. During 2010 we successfully completed a five year review by the Scientific Advisory Committee. Our research programs are enhanced by our extensive laboratory infrastructure, cGMP facility, and in-house key technology expertise in cell biology and imaging as well as a $12M animal model Bioresources Facility that opened in February 2010.

Translational activities for cancer vaccines and antibodies, as well as clinical trials for proof-in-concept and Phase II trials continue to be highly successful. Notably, the Phase 1 bioimaging study with ABT-806, an Institute monoclonal antibody against the EGF receptor

and licensed to Abbott, is being developed through the Tumour Targeting Laboratory, with Austin Health as the lead site for 2011 study commencement.

Our 2010 publications in major basic and clinical research journals included important discoveries in antigen cross presentation and processing, novel cancer targeting immunoconjugates 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 US and Australia. During 2010 we also published a number of invited reviews in *Nature Reviews Cancer*, *Nature Reviews Urology*, and *Radiology*. Several awards were received including Andrew Weickhardt's 2010 ASCO Cancer Foundation Merit Award and the Royal Australasian College of Radiology Elekta Prize to Joe Chang for a paper on Carbon-11 PET in Prostate Cancer arising from collaborative research led by Ian Davis and Andrew Scott.

During 2010 we were successful with peer reviewed research grants, clinical fellowships, $100,00 equipment grant from the Prostate Cancer Foundation of Australia, $1.5 Million funding from Australian Nuclear Science and Technology Organisation (ANSTO) for the establishment of the ANSTO-Austin-LICR Cyclotron Partnership for Solid PET Targetry Facility; and key involvement with the $2 Million awarded to Victorian Cancer Biologics Consortium from the State Government's Victoria Science Agenda Investment Fund for the clinical development of Avibody™ products.

In May 2010 construction commenced on the Olivia Newton-John Cancer and Wellness Centre. The building site is visible in our 2010 Branch staff photo. This comprehensive cancer center will provide a full range of clinical services and research facilities, including almost 6,000 sqm for Branch administration and laboratories. Completion of stage 1 is scheduled for June 2012 and funding for stage 2 has been secured with anticipated 2013 completion. This development will considerably enhance our research capacity and enable our discovery and clinical programs to flourish and significantly contribute to the mission of the Institute.

~ Andrew M. Scott

JOINT AUSTIN LUDWIG ONCOLOGY UNIT/ CANCER VACCINE

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

The Joint Austin Medical Oncology unit was established to enable translational clinical research to be undertaken by Institute clinical investigators. The unit was joined by Dr. Hui Gan late 2009 and Dr. Tom John in 2010, with a focus on Phase I drug development and lung cancer respectively. Ian Davis and Niall Tebbutt continue to make major contributions in the clinical evaluations of new treatments for urological and gastrointestinal cancers, and the immunotherapy of melanoma also continues as a major focus.

Dr. Gan's Phase 1 program has continued to grow strongly, having now expanded into a regional program spanning four hospitals with the aid of a $400,000 Victorian Cancer Agency grant. He has also established several new research programs. One, in collaboration with Glaxo Smith Kline Ltd, is examining the role of focal adhesion kinase in cancer. The other, a translational research program in head and neck cancer in conjunction with São Paolo Branch, is projected to generate new therapies for pre-clinical testing within 12-18 months.

Dr. John has studied the role of cancer testis antigen in melanoma, lung and breast cancer. He received a two-year $400,000 fellowship from the Victorian Cancer Agency to further investigate these antigens in patients undertaking chemotherapy for lung cancer and also received competitive grants from Pfizer and Austin Health Medical Research Foundation to investigate these antigens in breast cancer. This research has been selected for presentation at the World Lung Cancer Congress annual scientific meeting of the American Society for Clinical Oncology.

A/Prof Davis has continued to develop new drug treatments for urological cancers; notably several new trials including MDV3100, a novel androgen receptor antagonist in patients with metastatic castration-resistant prostate cancer, for which he is the national principal investigator and two trials with the immunotherapeutic antibody ipilimumab against CTLA4 in prostate cancer. He is also the lead investigator in the Australian & New Zealand Urogenital and Prostate Cancer Trials Group (ANZUP) trial for renal cancer which evaluates the safety and feasibility of alternating sunitinib and everolimus. His international standing is highlighted by two pivotal publications on pazopanib published in the *Journal of Clinical Oncology* and two papers in *Nature Reviews* journals. He was co-convenor of an international conference on renal cancer in Istanbul.

The final results of the MAX study, the largest investigator driven study in gastro-intestinal cancer undertaken in Australia, were published by A/Prof Tebbutt in the *Journal of Clinical Oncology*. Several translational studies involving tumor tissue from this study are currently underway in collaboration with the Mariadason Oncogenic Transcription Laboratory and Scott Tumour Targeting Laboratory. The Phase II DUX study, a multi center study coordinated from the Joint Unit at Austin Health, involving dual targeting of EGFR in advanced colorectal cancer was presented at the annual scientific meeting of the American Society for Clinical Oncology. Recruitment to the Novartis sponsored Granite study in gastric cancer and the Amgen sponsored 447 study in colorectal cancer was completed, with Austin Health leading recruitment in Australasia.

CANCER VACCINE

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

The Cancer Vaccine Laboratory program seeks to better understand immune responses against cancer and to develop immune-based treatments based on this understanding. Clinical application has focused on the cancer testis antigens, most notably NY-ESO-1 which has been targeted in a series of clinical trials. The trials based around the full-length NY-ESO-1 antigen characterized immune responses and explored potential clinical impact in melanoma. These trials have been undertaken using ISCOMATIX® adjuvant and with protein-loaded Fl3L-mobilized dendritic cells, in patients with minimal residual disease and in those with evaluable metastatic disease. Cyclophosphamide had been incorporated to gauge its impact on immune regulation. Ongoing studies will be undertaken in conjunction with the Cancer Vaccine Collaborative, and it is anticipated the new generation on checkpoint inhibitors will become available for combination immunotherapeutic trials. Additionally our relationship with GSK Ltd is providing new clinical research opportunities. These include two international trials in which we play a leading role; phase I development of a new vaccine against NY-ESO-1 and an international trial which targets MAGE A3 in patients with resected hepatocellular carcinoma. This will be performed throughout Asia and Australia by the Asia-Pacific Hepatocellular Carcinoma Trials Group.

Translational laboratory research undertaken in conjunction with our trials has extended previous observations assessing antigen loss. These include down-regulation of class I, and the quality and specificity of the immune response including the fine specificity of epitopes generated through vaccination versus those presented by the cancer. We have also studied the presence of regulatory or immunosuppressive mechanisms in the tumor micro-environment, including the induction of Tregs, some of which may be antigen-specific. We have shown that although vaccination can generate robust immune responses, clinical impact remains elusive. Investigation of this has led to studies of cellular heterogeneity within the tumor population. This includes subpopulations in melanoma that behave like stem cells and plasticity among populations that can switch from one state to another. These may explain how tumor cell populations can evade therapy. In a collaboration funded by the Melanoma Research Alliance and Australian NHMRC, we found that CD133, which marked clonogenic cells *in vitro* did not define stem-like cells upon transfer into an *in vivo* xenograft tumorigenicity model. Thus different environmental signaling appeared to affect cellular behavior. Consequently we have defined subpopulations based on functional assays rather than phenotype and then used genomic approaches to identify potential targets to better understand gene regulation in these cells. Initial studies point to a role for epithelial-to-mesenchymal transition as a determinant of some key biological features, chiefly heterogeneity, chemo-resistance, motility and invasiveness. The molecules that mediate this process may serve as new targets for the clinic. We are developing a melanoma cell-line resource to support ongoing studies using well characterized human melanoma populations. This involves characterizing a large panel of cell lines that were derived in-house, for genetic mutations, differences in gene-expression and epigenetic profiles based on miRNAs. These studies, initially supported by the Austin Health Medical Research Foundation and a grant from the Victorian Cancer Agency, the Melbourne Melanoma Project, will be extended in partnership with the Oxford, Brussels and Lausanne Branches of the Institute as part of a Ludwig melanoma initiative.

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. Our development of the monoclonal antibody 806, which targets a conformationally exposed epitope of erbB1 (EGFR), was successfully extended to the clinic and licensed to Abbott Pharmaceuticals. During 2010 we engaged with Abbott regarding the clinical development of

806 and a Phase I study of ABT-806 commenced at clinical sites in the US in Q4 2010. Further clinical studies are planned in Australia and the USA in 2011. Our research has extended to identifying similar conformational epitopes in other erbB receptors, and generating and characterizing novel antibodies against these targets together with continued research into the mechanism of action of 806, combination therapy of 806 with radiation and immunoPET imaging with ^{124}I-806.

Our collaborative research into the ephA3 receptor binding antibody IIIA4 with Dr. Martin Lackmann (Monash University) and licensee KaloBios continues with exploration of the biochemical and biologic sequelae of EphA3 function in mouse models, development of a humaneered form of the antibody for first in human studies, and the filing of a provisional patent during 2010.

The interaction of IgG Fc with FcRn, and FcγR plays a pivotal role in the pharmacokinetics and immune effector function of recombinant antibodies. We have previously demonstrated the long serum half-life of anti-Le^{y} humanized antibody hu3S193 in Phase I trials. Hu3S193 is now being used as a model for research into Fc function through homology modeling, crystallography and site directed mutagenesis. We have identified key amino acids responsible for Fc:FcRn interaction, and generated a series of single and double amino acid mutant constructs that retain antigen binding, but have faster serum clearance *in vivo*. Impact of Fc mutations on CDC and ADCC activity is being correlated with C1q and FcγRIII binding properties, respectively. Molecular imaging of zirconium-89 radiolabeled mutant construct biodistribution in mouse models with PET/CT has also been demonstrated.

Through continued collaboration with LICR New York and scientists at the City of Hope, USA, we are 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.

During 2010 our clinical trial program commenced a Phase I imaging trial of CS-1008 in patients with metastatic colorectal cancer in collaboration with Daiichi Sankyo Pharma Development. Our clinical trial program also continued a protocol with anti-CAIX antibody cG250, combined with Sutent® (Sunitinib malate), in patients with advanced or metastatic renal cell carcinoma (RCC). This trial explored the molecular expression of CAIX, targeting of RCC by cG250 in these patients, and also evaluated the efficacy of the combination treatment.

CENTRE FOR POSITRON EMISSION TOMOGRAPHY

Andrew Scott, M.D.

The Centre for Positron Emission Tomography (PET) at the Austin Hospital, under the direction of Prof Andrew Scott, has developed an integrated clinical and research PET program and is the largest academic PET Centre in Australia. The Centre for PET provides clinical PET studies for oncology and neurology patients, undertakes teaching and training of clinicians, technologists and scientists, and performs cutting edge research in close affiliation with the Branch leading to the development of innovative new therapies. Many of the research studies combine PET quantitative measurements of tumor metabolism with detailed protein profiles and gene arrays, in order to compare *in vivo* analyses with proteomic and genetic characteristics of tumors.

A new 18MeV (proton/deuteron) IBA cyclotron was commissioned in 2009, and a new dedicated small animal imaging suite was opened in February 2010 for preclinical investigations. Our oncology clinical PET program continued with the provision of ^{18}F-FDG PET studies for oncology patients referred from within Austin Health, and more broadly across the State of Victoria through a federal government funded access scheme. Through engagement with other hospitals within Melbourne, and Cancer Trials Australia, we have participated in a number of clinical trials exploring changes in tumor metabolism and proliferation in response to novel therapeutics with ^{18}F-FDG PET and ^{18}F-FLT PET studies. The quantitative assessment of pharmacokinetics and pharmacodynamics (metabolism, hypoxia, blood flow) of novel monoclonal antibodies, utilizing ^{124}I-antibody, ^{18}F-FDG, ^{18}F-FMISO and $H_2{}^{15}O$ PET, has also been undertaken in cancer patients during the last 12 months.

Molecular imaging of tumor metabolism, and evaluation of new therapy pharmacodynamics, remains a major focus of our research program. Our studies into hypoxia in metastatic colorectal carcinoma, utilizing ^{18}F-FMISO 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. We have 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, melanoma 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 2009-2010. We have also extended our animal model PET/SPECT/CT/Doppler microscopy platforms to the investigation of nanoparticle biodistribution, antibody pharmacokinetic and pharmacodynamic studies, and the development of novel PET hypoxic probes.

Our basic research studies have developed novel PET tracers for nanoparticles, hypoxia detection, proliferation (^{18}F-FLETT), signaling pathways (EGFR), and apoptosis over the last 12 months. Plans are underway to install solid targetry equipment for the new cyclotron, thus enabling in-house production of long lived PET isotopes (^{89}Zr, ^{64}Cu and ^{124}I) in the near future. The integration of multiple molecular imaging technologies, including PET, CT/MRI and optical imaging, remains a focus of our ongoing imaging research program.

T CELL

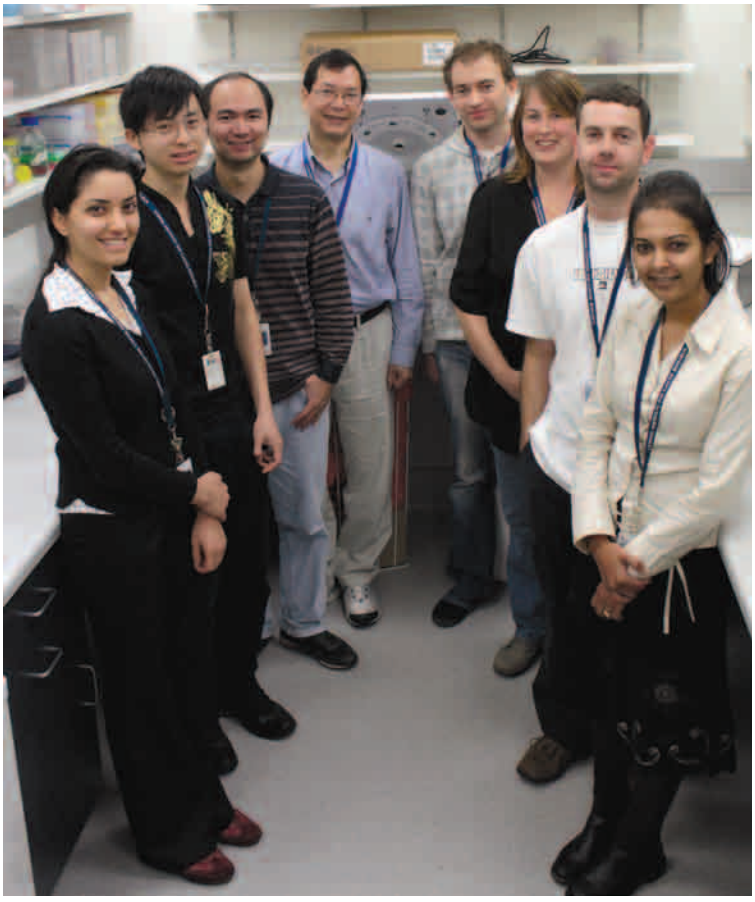
Weisan Chen, Ph.D,

Research within the T Cell Laboratory focuses on T cell biology and vaccine development. The laboratory activities are divided between human T cell research and murine models of influenza infection and tumor antigens 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 anti-tumor immunity in the presence and absence of innate signals. In addition, the laboratory continues to play an important role in the LICR 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 continued the study of antigen (NY-ESO-1) specific regulatory T cell (Treg) epitopes using the novel CD3-downregulation method recently developed in our lab. We have shown that many late stage melanoma patients who received NY-ESO-1/ISCOMATRIX™ vaccine exhibited induction of both NY-ESO-1-specific effector T cells (Teff) and Treg. We also showed that $CD4^+$ Teff and Treg can recognize the same T cell epitopes. Such knowledge assists in improving vaccine designs and T cell monitoring.

We have recently generated an orthotopic murine cutaneous melanoma model to explore the tumor-immune cell interaction, particularly the role of Langerhans cells and skin derived $CD103^+$ dendritic cells (DCs), and the biology of melanoma metastasis. B16 melanoma cells are inoculated in the skin dermal layer ensuring their interaction with dermal DCs and Langerhans cells. The cutaneous melanoma forms early metastasis in the draining lymph nodes. This is in contrast to the widely utilized subcutaneous tumor model which continuously grows under the skin with limited interaction with dermal DCs.

Using murine model systems, our laboratory is also interested in investigating immunodominance, especially the observed immunization route-dependent immunodominance hierarchy following intranasally or intraperitoneally IAV administration. Preliminary evidence indicates potential tissue-specific DC subsets and their role mediating differential antigen presentation. The acquired knowledge should be beneficial to future vaccine design.



During the year we published the results of our investigations demonstrating that Influenza A infection enhances cross-priming of CD8+ T cells to cell-associated antigens in a TLR7- and Type I IFN-dependent fashion. We also showed that $CD8^+$ T cells specific to tumor antigens expressed by the directly infected tumor cells and those to the antigens expressed by the uninfected, bystander tumor cells was equally enhanced if they are coinjected. These observations suggest that IAV-infection of tumor cells may be used as a clinical vaccine approach.

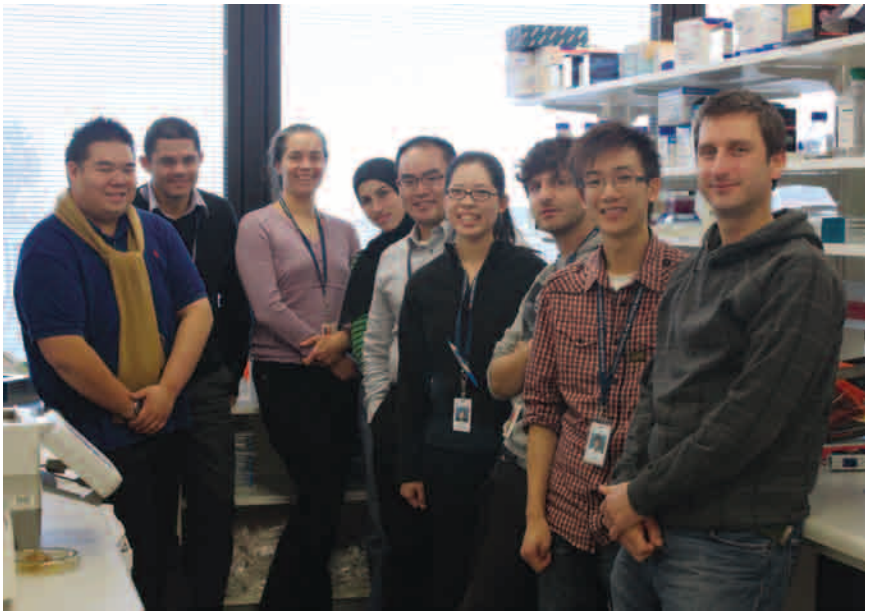
Our investigations determined that the immunoproteasome also plays a 'house-keeping' role. Mice with deficient immunoproteasomes had impaired B cell differentiation, reduced B cell number and defective B cell function, e.g., diminished antibody response to IAV and antibody class switching. An impairment of T cell function was recently observed in these mice. Although similar numbers of T cell bone marrow precursors were observed; their thymic development is significantly impaired. In the immunoproteasome deficient mice, the double-negative (DN) thymocytes appear to have difficulty switching on the nuclear factor kappa B (NFκB) pathway, which is required by the fast developing thymocytes to survive, leading to inefficient transition from DN3 to DN4 stage accompanied with higher apoptosis rate. We have also crossed TCR transgenic mice onto the immunoproteasome deficient background. Our preliminary data also indicate a T cell intrinsic difference for the mature peripheral T lymphocytes. Work continues to elucidate the potential underlying mechanisms.

ONCOGENIC TRANSCRIPTION

John Mariadason, Ph.D.

The Oncogenic Transcription Laboratory investigates the transcriptional changes that drive colon cancer progression. In particular we focus on gene expression changes driven by the histone deacetylase (HDAC) family of transcriptional co-repressors. The laboratory also actively investigates the mechanism by which HDAC-inhibitors 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, which induces a closed chromatin conformation. This decreases accessibility of the basal transcriptional regulatory machinery to DNA, subsequently resulting 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 observations that a number of class I HDACs (HDAC1, HDAC2 and HDAC3) are upregulated in colon cancers and that these HDACs, as well as the class II HDAC, HDAC4, play a pro-proliferative, pro-survival function in colon cancer cells. 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.



Recently, we also gained new insight into the molecular mechanism by which commercially-developed HDAC inhibitors, such as vorinostat induce apoptosis in colon cancer cells. Vorinostat is approved for the treatment of cutaneous T-cell lymphoma and is presently undergoing clinical trial for treatment for a variety of hematological malignancies and solid tumors, including colon cancer. Through screening a large panel of colon cancer cell lines, we 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. 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 discovery of biomarkers predictive of response of colon cancer cells to chemotherapy and targeted therapies. To achieve this, we have assembled and screened a panel of 40 colon cancer cell lines for response to a number of chemotherapeutic and biological agents used in the treatment of colon cancer. With support provided by the Institute and in collaboration with Anamaria Camargo and Sandro de Souza at the São Paulo Branch and Oliver Sieber at the Melbourne-Parkville Branch, we are currently performing whole exome

sequencing of a subset of these cell lines to search for mutation profiles that predict response to each of these treatments. Finally, we are investigating the mechanistic basis for acquired drug resistance in colon cancer and are presently characterizing a series of colon cancer cell lines generated in our laboratory with acquired resistance to chemotherapy and targeted agents.

URO-ONCOLOGY

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

The Uro-Oncology Laboratory studies cancers related to the urinary system: kidney, prostate and bladder cancers. Key aspects of the underlying biology and mechanisms of *de novo* or acquired resistance for these cancers are not well understood. Further advances in the treatment of these malignancies require better understanding of these issues; careful study of clinical tissue and blood samples in the context of annotated clinical outcomes; development of relevant *in vitro* and animal models; and integration of various streams of basic and clinical research in a broad multidisciplinary translational program.

Research in the group during 2010 built upon previous research and included the broad themes of epithelial/stromal interactions (prostate cancer); *in situ* immunology (prostate cancer); mechanisms of resistance to anti-angiogenic and other targeted therapies (renal cell carcinoma); and epigenetic regulation of cancer biology (bladder cancer). Key to these research projects is our ability to work with fresh tissue from patients in various clinical settings.

During 2010 we commenced exploring the mechanisms of escape from abiraterone therapy. This work requires larger amounts of tissue than can be obtained from biopsies or pathological specimens. A heterotopic xenograft method has been established allowing reliable culture of primary samples and expansion to amounts suitable for detailed analysis of genomic and proteomic profiles and sex steroidogenesis.

Other projects within the group include: studies of epigenetic regulation of bladder cancer cell biology where we have shown that cancer-testis antigen (CTAg) expression and various biological functions in transitional cell bladder cancer are regulated by epigenetic mechanisms that are dependent upon both the dose and schedule of demethylating agent treatment; *in vitro* and *in vivo* studies of mechanisms of resistance in renal cell carcinoma which has demonstrated the lack of reliability of volumetric assessments in comparison to molecular markers of target inhibition and functional studies including positron emission tomography (PET).

Clinical programs related to the laboratory involve 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. Two clinical trials for men with prostate cancer that is believed to be confined to the prostate are close to completion and have shown the value of CHOL PET in determining the dominant intraprostatic lesion. This work has been extended in a parallel study of radiotherapy "dose-painting" by DMedSc student Dr. Joe Chang and has been the subject of several awards at international conferences. Further studies of imaging technology in prostate cancer are in planning.

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DIRECTOR'S MESSAGE

2010 saw the departure of two of the Branch's very successful groups. The Angiogenesis Laboratory, led by A/Profs Marc Achen and Steven Stacker, moved to the Peter MacCallum Cancer Centre to establish their Tumour Angiogenesis Program. A/Prof Margaret Hibbs's Signal Transduction Laboratory transferred to Monash University's Alfred Hospital precinct where she formed the Leukocyte Signalling Laboratory. With both of these groups remaining part of Melbourne's vibrant medical research scene, continued collaboration and support of students between these new host institutions and the Branch has been possible.

As a consequence of these changes, the traditional focus of the Branch has now been further consolidated to intestinal cancer with the largest effort directed to malignancies of the colon. Collectively, the remaining four laboratories, which consist of the Epithelial Biology Laboratory (Tony Burgess), the Colon Molecular and Cell Biology Laboratory (Joan Heath and Matthias Ernst), the Ludwig Colon Cancer Initiative Laboratory (Oliver Sieber and Peter Gibbs) and the Joint Proteomics Research Laboratory (Richard Simpson), afford Australia's leading group of basic research scientists working on a single solid malignancy. Together with their colleagues from the Melbourne - Austin Branch, the Peter MacCallum Cancer Centre, Monash and Melbourne Universities, they form the core of a $20m Colon Cancer Program grant from the Australian National Health and Medical Research Council (NHMRC) and play a leading role in the Victorian CCC's colon cancer tumor stream.

The Branch's research laboratories have continued to be successful in attracting collaborative research projects and have included various arrangements with local and global leaders in the pharmaceutical and biotechnological sector. The Branch's involvement in a collaboration with a pharmaceutical company for a project looking at detecting a common B-Raf mutation

associated with cancers paved the way for Melbourne being one of the sites of the world's first clinical trial for a B-Raf inhibitor in colorectal cancer and was among the Branch's translational highlights for the year.

2010 proved to be a productive year for scientists at the Branch who continued to make discoveries of importance, extend and strengthen their networks with national and international collaborators, and to successfully leverage the Institute's generous financial support with external grants at a ratio close to three-in-one. In particular, a success rate of 42% with NHMRC, the primary competitive funding body for medical research in Australia, amounted to twice the national average and was complemented by a string of other grants and fellowships awarded by the Victorian Cancer Agency, the Cancer Council of Victoria, the Cure Cancer Australia Foundation and the Pfizer Medical & Academic Partnership.

The Branch's contribution to basic and translational research continues to be recognized at the highest international levels with publications in *Nature*, *Developmental Cell, PLoS Genetics* and others, alongside numerous presentations at meetings where several junior Institute scientists have received prestigious national and international awards for the quality and innovation of their research. Among the senior leaders at the Branch, Tony Burgess was honored with the *President's Award* by the Victorian Cancer Council for his outstanding contributions to cancer control in Victoria, and Joan Heath was promoted to full Member of the Institute for her internationally recognized scientific achievements.

~ Matthias Ernst

ANGIOGENESIS

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

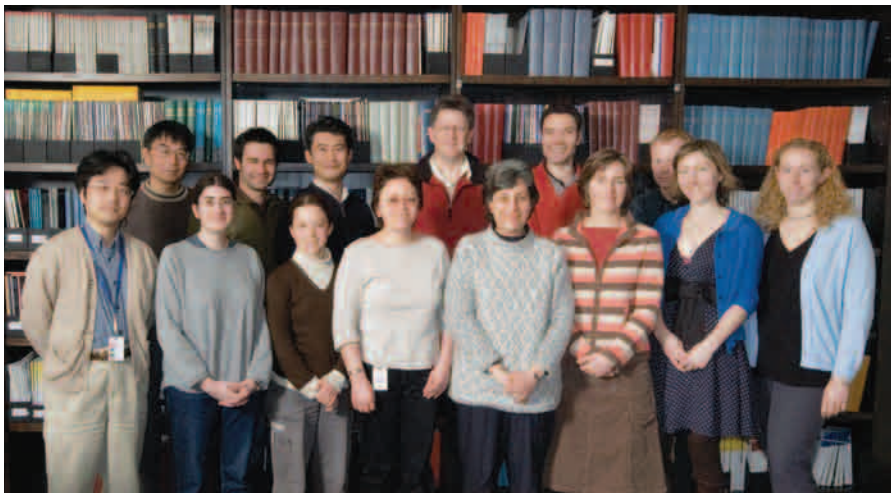
The Angiogenesis Laboratory, which was founded in 1996 and arose from the former Growth Regulation Laboratory headed by Dr. Andrew Wilks, was closed in June 2010 as the Branch changed strategic focus to the translational aspects of research into colorectal cancer. Over its 15 years of operation, the laboratory was involved in the discovery and characterization of growth factors and receptors which play a major role in vascular and lymphatic biology, particularly in relation to cancer.

The laboratory identified new vascular endothelial growth factors and their receptors using PCR-based and bioinformatics approaches. By developing novel bioassays and establishing cell biological techniques in the emerging areas of angiogenesis and lymphangiogenesis, the laboratory was able to define novel mechanisms controlling blood and lymphatic vessel formation, as well as growth factors and receptors with important functions in embryonic development and cancer.

The laboratory revealed that protein growth factors such as VEGF-D are expressed in tumors and, in part, control the capacity of tumor cells to metastasize from a primary tumor, via the lymphatic network, to regional lymph nodes. These findings were important for identifying the role of the lymphatics in tumor metastasis and introduced the concept of inhibitory antibodies that target metastatic spread.

In addition to discovery research, the laboratory was also involved in the development of numerous reagents and assays which have been licensed by the commercial sector and are now being developed as potential anti-cancer therapeutics or diagnostic assays. The laboratory was closely involved with the transfer of much of the Institute's intellectual property relating to angiogenesis and lymphangiogenesis to the Melbourne-based company Vegenics Limited.

In closing the laboratory we are grateful for the years of support from the Institute and interaction with many talented people in the global Institute community. In particular we are indebted to Professor Tony Burgess for his visionary leadership of the Branch and patient mentoring of its staff. In moving to the Peter MacCallum Cancer Centre we will continue our studies in the area of lymphatic biology and cancer metastasis.



COLON CANCER INITIATIVE

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

Our laboratory investigates the genetic, epigenetic and transcriptomic changes that drive colorectal cancer development and progression, with a particular focus on the identification of biomarkers for patient cancer risk and prognosis. Ongoing molecular studies on over 1,000 patients, for whom detailed clinical treatment and follow-up data are available, comprise genome-wide microarray and targeted cancer gene sequencing studies. Molecular data are integrated with epidemiologic data to develop predictors of disease outcome and therapy response. The laboratory also utilizes colon cancer cell lines and animal models to characterize phenotypes associated with molecular signatures and to advance our knowledge of colorectal cancer biology.

In 2010, the laboratory continued extensive molecular profiling studies on patient tumor and matched normal samples including genome-wide assessment of inherited common variants using single nucleotide polymorphism (SNP) microarrays, targeted sequencing of known functional variants in 5FU and oxaliplatin therapy resistance genes

(*TYMS*, *GST*, *XRCC1*, *ERCC1*, *ERCC2*), mutation screening of key colon cancer genes (*APC*, *CTNNB1*, *KRAS*, *BRAF*, *PI3KCA*, *PTEN*, *SMAD2*, *SMAD3*, *SMAD4*, *TGFBR2*, *TP53* and *FBXW7*), genome-wide assessment of tumor-acquired DNA copy number changes and allelic loss, microsatellite instability status (MSI) analysis and array-based gene expression profiling. Bioinformatics pipelines for mutation detection and pathogenicity prediction have been expanded from Sanger to next-generation DNA sequencing data and a novel analysis package for SNP array data (OncoSNP) has been developed in collaboration with the Department of Statistics, University of Oxford. The latter is designed for accurate DNA copy-number identification for complex cancer genomes and is currently being applied to characterize the extent and subtypes of genomic instability in colorectal cancer.

Following our initial work describing the clinical associations and prognostic significance of *BRAF* mutations, further studies have demonstrated the impact of both *BRAF* and *KRAS* mutations on sites of metastatic disease. The presence of *KRAS* mutation, for example, was shown to modify the risk of lung but not liver relapse. These data have direct clinical implications, highlighting the potential of somatic mutations for informing surveillance strategies. A prognostic gene expression signature previously identified for stage B and C colorectal cancer has been taken forward for diagnostic development by an industry partner. The laboratory is also an active collaborator in the international COGENT consortium, and a number of novel colorectal cancer risk loci have recently been identified through this collaboration.

The laboratory has identified a novel marker of the putative epithelial stem cells of the human intestinal tract, PHLDA1. In the human intestine, PHLDA1 protein was shown to be expressed in crypt base cells corresponding to murine Lgr5 expressing epithelial stem cells. Small adenomas showed cytoplasmic staining restricted to undifferentiated neoplastic cells, suggesting that a basic hierarchy of differentiation is retained in early tumorigenesis. Expression was widespread in large adenomas, carcinomas and metastases, with pronounced staining at the invasive margin. Suppression of PHLDA1 in colon cancer cells inhibited cell migration and anchorage-independent growth *in vitro* and tumor growth *in vivo* suggesting its importance in colorectal cancer development.

Our clinicians have continued to collect and expand prospective, comprehensive data information on patients treated for colorectal cancer, which is occurring in parallel with banking of tumor tissue and blood samples from these patients. Industry supported collaborative projects have been initiated, including exploring potential biomarkers in our large cohort of colorectal cancer patients.

A new area of research is on blood biomarkers, with several new projects being initiated in 2010. The potential role of circulating tumor DNA (ctDNA) as a marker of residual or recurrent is being explored in collaboration with the Vogelstein group from the Sidney Kimmel Comprehensive Cancer Center. In a separate project with the Commonwealth Science and Industrial Research Organisation (CSIRO), a panel of blood protein biomarkers has been identified with the potential for use as a screening test for detection of both early and late-stage colorectal cancer. To validate the potential of this panel, CSIRO, the Institute and collaborators at Cabrini, the Western and Royal Melbourne Hospitals are collecting blood samples

and data from a large cohort of patients undergoing colonoscopy, with the final test series to include patients with a normal colonoscopy, with adenomas, with advanced cancer and/or other bowel disease (including diverticular disease, inflammatory bowel disease). Combined with the routine collection of fresh tissue and blood this study continues to build a resource for translational research.

COLON MOLECULAR AND CELL BIOLOGY

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

The Colon Molecular and Cell Biology Laboratory, jointly headed by Matthias Ernst and Joan Heath, aims to understand the genetic networks underpinning gastrointestinal cancer using complementary approaches in mice and zebrafish.

Much effort in the Ernst laboratory is directed towards identifying the molecular mechanisms that link inflammation to tumor promotion. The rationale for this stems from the observation that tumor initiation, triggered by mutations in proto-oncogenes and/or tumor suppressor genes, is generally insufficient for the development of cancers, and that once initiated, cells usually depend on interactions with the tumor microenvironment to progress into a premalignant tumor mass. Accordingly, tumor promotion associated with chronic inflammation of the intestine, stomach and other organs is an enduring process that, up to a point, can be reversed. Of the inflammatory mediators that occur in abundance within the tumor microenvironment and promote the expansion of neoplastic cells, members of the interleukin (IL-)6 family of cytokines are prominent players. Their activities converge on tumor cells through activation of the latent transcription factor Stat3 thereby enhancing survival and promoting proliferation, invasion and induction of an angiogenic switch.

IL-11 is a member of the IL-6 cytokine family and is readily detected in various inflammation-associated pathologies, including gastrointestinal cancers. Similar to IL-6, IL-11 mediates its pleiotropic activities through a receptor complex comprising the ligand binding IL-11Rα and the ubiquitously expressed signal-transducing gp130 receptor β-subunits. Using various combinations of loss- and gain-of-function alleles in mouse models of inflammation-associated gastric cancer (GC) and colitis-associated colon cancer (CAC), we established a novel link between IL-11, the gp130/Stat3 signaling axis and tumor progression. Similarly, growth and maintenance of gastric tumors in *gp130*^{Y757F} mice is also susceptible to systemic administration of IL-11 antagonists or other therapeutic means of systemically attenuating the Stat3 signaling output. Surprisingly, we also found that Stat3-driven gastric tumors in *gp130*^{Y757F} mice develop a high dependence on excessive mTORC signaling that can be exploited therapeutically and which mimics the correlation between excessive STAT3 and mTOR activation observed in human inflammation-associated GC. Thus, the close physical proximity between IL-11 producing cells in the tumor microenvironment and the IL-11 responsive hyper-proliferative neoplastic epithelium provides a strong rationale that inhibition of the IL-11 signaling pathways may represent a novel therapeutic opportunity for the treatment of inflammation-associated (gastrointestinal) cancers.

Given that aberrant activation of Wnt/β-catenin signaling is a common unifying feature of sporadic colorectal cancer in humans, the Ernst laboratory is also interested in understanding the cross talk

between the Stat3 and Wnt pathways and to genetically ascribe signaling thresholds to the various pathologies elicited by these pathways. Specifically, contributions of null and hypomorphic alleles of the *Apc* tumor suppressor allele, the central negative regulator of Wnt/β-catenin signaling that is most frequently inactivated in human colorectal cancer, produce both developmental and patho-physiological phenotypes in mice. To unambiguously define the resulting genotype-to-phenotype relationship, we genetically challenged various mutant *Apc* alleles by restricting intracellular β-catenin expression in the corresponding compound mutant mice. Subsequent quantitation of the resulting Tcf4-reporter activity in mouse embryo fibroblasts enabled us to relate the extent of Wnt/β-catenin signaling to an allelic series of mouse mutants. We found that different permissive Wnt signaling thresholds were required for the embryonic development of head structures, adult intestinal polyposis, hepatocellular carcinomas, liver zonation and the development of natural killer cells. Furthermore, we identified a homozygous *Apc* allele combination with a Wnt/β-catenin signaling capacity similar to that observed in Apc^{min} mutant mice, a widely used model with a germline mutation where somatic Apc loss-of-heterozygosity triggers intestinal polyposis. This enabled us to assess which co-morbidities in Apc^{min} mice arose independently of intestinal tumorigenesis and which as a consequence of it. Collectively, these genotype–phenotype analyses suggest different tissue-specific response levels for the Wnt/β-catenin pathway that regulate both physiological and patho-physiological conditions. Studies in the Heath laboratory are based on the premise that the process of zebrafish intestinal organogenesis is an excellent model for colon tumor biology. We argue that genes that are indispensable during the phase of development (2-3 days post fertilization) when the zebrafish intestinal epithelium is the most rapidly proliferating organ in the embryo may be of particular interest in the context of colon cancer. To test this hypothesis, we identified a dozen zebrafish mutants with striking abnormalities in the size and shape of the developing intestine and, in recent years, we have positionally cloned seven of the underlying mutant genes. We have found that all the cloned genes play roles in essential cellular processes, including nuclear pore formation, transcription, pre-mRNA splicing and ribosomal biogenesis.

One of the mutants that has captured our attention is *caliban (cal)*. This mutant exhibits a growth-arrested intestinal phenotype as a result of a nucleotide variation in a gene that encodes an essential component of the minor class spliceosome. The minor class spliceosome is a complex of small nuclear ribonucleoproteins that is indispensable for the splicing of pre-mRNA molecules containing one or more minor class introns. Minor class introns comprise less than 1% of all introns and are characterized by distinctive consensus splice sites. Whole transcriptome analysis of *cal* and wildtype embryos at 4.5 days post fertilization (dpf) by microarray and RNAseq analysis demonstrates that the major consequences of impaired minor class splicing in *cal* is the global retention of minor class introns and the differential expression of 400 genes, including a sub-set of up-regulated genes that inhibit the cell cycle.

Interestingly, the distribution of minor class introns is conserved throughout evolution and encompasses genes relevant to colon cancer such as the proto-oncogene *BRAF* and the tumor suppressor genes *PTEN* and *LKB1*. Germline mutations in *LKB1* are responsible for Peutz-Jeghers syndrome, an autosomal dominant inherited disorder characterized by intestinal polyposis and a 15-fold increased risk of developing intestinal cancer. To test the hypothesis that aberrant splicing of mRNA molecules containing minor class introns may contribute to tumourigenesis, we analyzed the expression of the human orthologue of our minor class splicing gene in 30 paired samples of human colorectal cancer and adjacent normal tissue. In samples exhibiting a marked down-regulation of the gene, we observed a 2-fold reduction in the production of correctly spliced *LKB1* mRNA. The defective splicing was specific to the removal of the minor class intron; the removal of an adjacent major class intron was unaffected. This prompted our current working hypothesis that impaired minor class splicing efficiency plays a hitherto under-appreciated role in the development of cancer by reducing the activity of tumor suppressor genes.

Another intestinal mutant with a markedly hypoplastic intestinal epithelium is *titania*. We identified two independent alleles of *titania* in the gene encoding Periodic tryptophan protein 2 homolog (Pwp2h). As in yeast, zebrafish Pwp2h is essential for the proper endonucleolytic cleavage of the 35S ribosomal RNA precursor, a transcript that contains the sequences of the 28S, 18S and 5.8 S rRNAs embedded in non-coding regions. In *titania* mutants, the levels of

the 35S rRNA precursor are elevated and there is a reduction in the level of mature 18S mRNA. Polysome profile analysis on sucrose density gradients demonstrates a reduced number of correctly assembled ribosomal subunits in *titania*, defects that are likely to impair protein synthesis. Using transmission electron microscopy, we found that the intestinal epithelial cells in *titania* at 4-5 dpf accumulate abundant autophagosomes and autophagolysosomes compared to wildtype cells. Moreover, *titania* embryos display increased conversion of LC3I to LC3II, indicating incorporation of LC3 into autophagosomal membranes and increased autophagic activity. This suggests that impaired ribosome biogenesis in *titania* induces autophagy in order to generate nutrients for the stressed cells. We are currently evaluating whether this has a positive or negative impact on cell survival in the intestinal epithelium. This is of interest because studies in cancer cells suggest that the induction of autophagy may result in both beneficial and deleterious outcomes depending on context. In a positive scenario the up-regulation of autophagy in cancer cells results in the induction of senescence and/or autophagic cell death but in a negative scenario, it may promote their survival.

EPITHELIAL

Tony Burgess, Ph.D.

Improving the understanding of colorectal cancer (CRC) biology and using this knowledge to improve treatments for patients is the aim of the Epithelial Laboratory. To this end, our research involves several major themes important in the development and progression of this disease.

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 CRC; however, the lack of specific reagents has hampered research into their role in the physiology and pathology of the mammalian gut. We have developed apc, axin and specific phospho-β-catenin monoclonal antibodies, reagents and target cell lines to determine wnt ligand affinity and specificity for their cognate receptors and can now measure wnt sensitivity of colon cancer cells with apc, β-catenin, P13K and BRaf mutations.

CRC arises from the stem cell compartment through a series of mutations which allow normal physiological controls to escape. CRC stem cells share many characteristics with normal intestinal stem cells, such as self-replication and differentiation. Accordingly, many of the 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 normal and cancer stem cells; however, little is known about the function of these proteins in the maintenance of the cancer stem cell phenotype. Using well-defined human CRC cell lines, we have modulated the expression of these stem-cell markers by knockdown or inducible overexpression. These experiments allowed us to expand our understanding of 'stemness' associated with several CRC cell lines and the factors that control the balance between self-renewal and differentiation.

APC is a large protein, whose modular structure contains a series of predicted protein-protein interaction domains. In more than 80% of CRC, *APC* mutations

result in the expression of a severely truncated APC protein. Even though APC is clearly an important molecule in CRC, little is known about its structure due to its size and lack of homology with other proteins. We have used classical structure/function analysis to characterize the shape and intramolecular interactions of the full-length and truncated APC protein. The current paradigm for how truncation results in initiation of tumorigenesis is through failure to regulate the 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 its phosphorylation of serine and threonine residues in the N-terminus. Dysregulation of phosphorylation is proposed to result in its accumulation and subsequent activation of Wnt/β-catenin-target genes, which are important in gut development and driving colorectal tumorigenesis. We have 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. We detect populations of N-terminally phosphorylated β-catenin that are not part of the destruction complex, but appear to be involved in cell adhesion or migration.

Phosphoinositol polyphosphates (PIPs) are important components of the plasma membrane and are associated with several signal transduction systems. We have used synthetic PIP analogues to identify PIP binding proteins in CRC cell lines that have different combinations of tumor suppressor and oncogenic mutations, often leading to significant perturbations in the levels and distribution of PIPs. Using affinity capture and proteomics, we identified proteins that bind to different PIPs as a result of oncogenic or growth factor stimulation. We are investigating the role of specific PIP binding proteins in maintaining the transformed state in CRC.

The location, kinetics and differentiation of intestinal cells are regulated by a network which includes wnt, notch, tyrosine kinase and TGF-β signaling. We have initiated a research program to quantitate notch signaling in normal and transformed colonic cells. We are studying the interaction between notch ligands and notch, the kinetics of NICD production and action in both cell lines and colonic crypt cells. We aim to understand the effects of combinations of notch and wnt signaling on the location, self-renewal and/or differentiation of colonic stem cells and transitional cells.

JOINT PROTEIN RESEARCH

Richard Simpson, Ph.D.

The laboratory focuses on cutting-edge analytical protein chemistry and is involved in the development of new systems for proteomic analysis, as well as instrumentation and software for mass spectrometry. The aim of these studies is to increase the flexibility, speed, and sensitivity of protein identification and analysis and to apply these techniques in investigating early oncogenic changes in colorectal cancer.

Epithelial-mesenchymal transition (EMT) describes a process whereby immotile epithelial cells transform into migratory and invasive mesenchymal cells. Implicated as a mechanism used by cancer cells to facilitate metastasis, several EMT molecular events have been identified including the down-regulation of E-cadherin and increased expression of vimentin. However, our understanding of the extracellular microenvironment during EMT progression remains limited. Previously, proteomic interrogation of the secretome from MDCK cells undergoing oncogenic Ras-induced EMT revealed extensive extracellular remodeling, including diminished expression of basement membrane constituents and elevated expression of proteases, protease inhibitors and extracellular matrix proteins associated with cell migration. Molecular alterations occurring at the plasma membrane during EMT were detected using cationic colloidal silica isolation and GeLC-MS/MS. Label-free quantification revealed that MDCK cells undergoing EMT switch from cadherin-mediated (E-cadherin, claudin 4, desmoplakin, desmoglein-2 and junctional adhesion molecule A) to integrin-mediated (α6β1, α3β1, α2β1, α5β1, αVβ1 and αVβ3) adhesion. In addition, the integrin receptor extracellular ligands (collagens I, and V, and fibronectin) were also up-regulated. Conspicuously, Wnt-5a expression was elevated in cells undergoing EMT and transient Wnt-5a siRNA silencing attenuated both cell migration and invasion in these cells. Furthermore, Wnt-5a expression suppressed canonical Wnt signaling induced by Wnt-3a. Wnt-5a may act through the planar cell polarity pathway of the non-canonical

Wnt signaling pathway, as several of the components and modulators (Wnt-5a, -5b, frizzled 6, collagen triple helix repeat containing protein 1, tyrosine-protein kinase 7, RhoA, Rac, and JNK) were found to be up-regulated during EMT. Preliminary examination of extracellular membranous vesicles (exosomes and shed microvesicles) suggests their involvement during EMT progression.

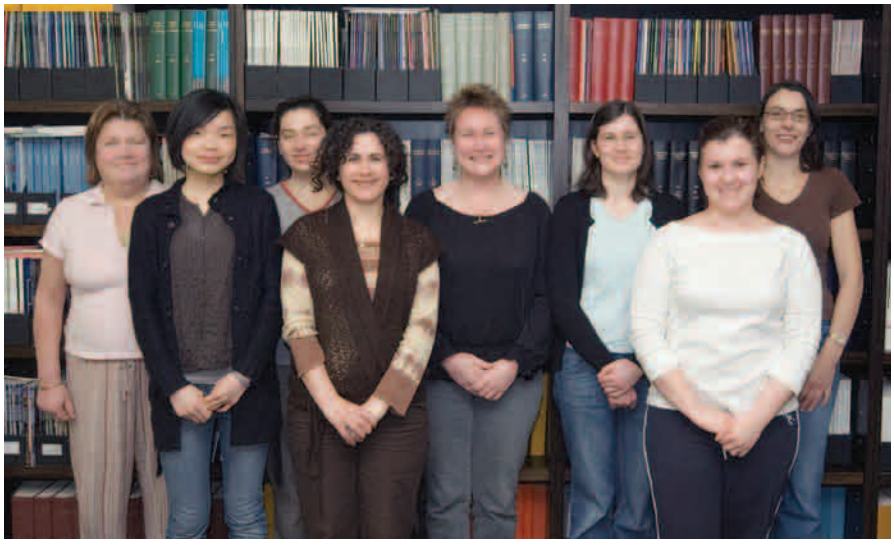
Exosomes are extracellular vesicles 40-100 nm in size that are released from a multitude of cell types and perform diverse cellular functions including intercellular communication, antigen presentation and mediate the transfer of mRNA and miRNA. Utilization of exosomes as cancer vaccines and as cell-free systems to deliver biological molecules including therapeutic drugs and gene therapy agents to tumor cells has attracted considerable interest. Moreover, given that exosomes carry cancer related molecules, they may be an important source of biomarkers for diagnosis and/or therapeutic monitoring. Detailed biochemical and functional analyses of exosomes have been confounded by technical difficulties isolating sufficient quantities of highly purified material for rigorous characterization. We have focused on developing methodologies towards isolating and purifying exosomes from a panel of 20 colorectal carcinoma (CRC) cell lines. CRC cell lines were grown in serum-free conditions and exosomes isolated from concentrated cell culture medium (CCM) using three strategies. Firstly, exosomes were prepared using a series of differential centrifugation steps not exceeding 100,000 x *g*. Secondly, exosomes were isolated by rate zonal centrifugation through a discontinuous gradient. Lastly, exosomes were captured using immuno-affinity with antibody-coated magnetic beads (e.g., anti-A33 mAb). This approach exploited two individual antigens, both of which are potential targets for colon cancer immunotherapy. Although protein profiling, using GeLC-MS/MS, revealed that all four exosome preparations contained the characteristic 'exosome signature' proteins such as Alix, TSG101, HSP70 and the tetraspanins CD63, CD81 and CD9, it is apparent that CRC cells contain two distinct exosome-like populations – one heavily enriched in ESCRT proteins, whilst the other population uniquely contain Golgi proteins. Our data suggest exosomes may be derived by at least two alternate exocytic trafficking pathways - the canonical pathway involving multivesicular body biogenesis and a second, hitherto unidentified, secretory mechanism.

SIGNAL TRANSDUCTION

Margaret Hibbs, Ph.D.

The Signal Transduction Laboratory is focused on the study of signaling pathways in hematopoietic cells to further our understanding of antibody-mediated autoimmune disease and to define pathogenic mechanisms in lung inflammation.

Autoimmune diseases such as systemic lupus erythematosus (SLE) are still managed with general immuno-suppression rather than a specific therapy targeting a causative agent or cell type, making the 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, also providing an opportunity for trialing potential therapeutic agents. We have 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 the generation of pathogenic autoantibodies. Lyn-deficient dendritic cells (DC) 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. We have deleted IL-6 on a Lyn-null background and have found that 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 shows that although Lyn-deficient B cells may be autoreactive, it is their inflammatory environment that dictates their disease-causing potential. Our 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 these two diseases share common susceptibility determinants and it is known that macrophages play an important role in the pathogenesis of both conditions. Macrophages, however, 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 would allow us to determine their signature and identify possible therapeutic targets. However, lung macrophages have been hard to study due to their uniquely high autofluorescence. We have now developed a novel flow cytometry approach which allows the identification and characterization of lung macrophage subpopulations. We are using this method to define subpopulations of macrophages that are recruited to the lungs in response to various pathological stimuli, and in mice harboring inflammatory lung disease. Our 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. The New York Branch is pursuing three targeted approaches to cancer - therapeutic antibodies, antigen-specific vaccines, and enzyme depletion therapy – which 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.

Our 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. Thirteen of our monoclonal antibodies have been licensed to commercial partners for further clinical development. The current focus is on developing antibodies against targets on cancer stem cells and on antibodies that target and interfere with molecules involved in immunosuppression in tumors.

The CT antigen NY-ESO-1, discovered by the New York Branch, is one of the most immunogenic human tumor antigens. Because of its 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 antigen for vaccine development in the Cancer Vaccine Collaborative (CVC), a coordinated network of clinical and laboratory investigators. 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.

The 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. Certain cancers lack the enzyme argininosuccinate synthetase (ASS) necessary to convert citrulline to arginine. We recently found that small cell lung cancer (SCLC) frequently lacks this enzyme and is highly sensitive to arginine deprivation therapy with the enzyme arginine deiminase (ADI) *in vitro* and in mouse models. A Phase II clinical trial of ADI in patients with SCLC has now been initiated.

~ Lloyd J. Old and Gerd Ritter

CANCER CELL SURFACE ANTIGENS

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

A major goal of the New York Branch 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; and 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). Our 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. Thirteen of our monoclonal antibodies have been licensed to commercial partners for further clinical development and more than 10 of these antibodies have entered into clinical trials at Memorial Sloan-Kettering Cancer Center (MSKCC). The therapeutic effects of monoclonal antibodies targeting cancer cell surface antigens are mediated by various distinct functions including antibody dependent cellular cytotoxicity (ADCC), complement-mediated cytotoxicity (CMC), signal agonism or antagonism, and delivery of a "payload" such as toxic small molecules, siRNA, nanotubes and particles with cytotoxic abilities and immunomodulatory factors.

We have now focused on a major challenge in cancer research namely to find and identify markers to define cancer stem cells (CSC), the cells that are considered to initiate cancer and provide a continued source of self renewing cancer cells. The present failure of most current therapies to cure cancers is thought to be 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 represents a major barrier to develop effective CSC targeted cancer therapeutics. We hypothesize 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. A recent advance that has provided a new opportunity to define cell surface targets on CSC has come from the work of Dr. Malcolm Moore at MSKCC. He and his colleagues have developed a novel method to expand CSC in tissue culture, making it possible to obtain for the first time a continued source of CSC from ovarian cancer. This major finding enabled us to obtain sufficient quantities of CSC for immunization and screening for the generation and development of therapeutic antibodies against ovarian cancer stem cells. In collaboration with Dr. Moore's team we have now embarked to generate and characterize novel monoclonal antibodies against ovarian CSC utilizing this novel cell type for immunization of mice and screening of hybridomas. Screening assays, which include mixed hemadsorption assays, FACS, cytotoxicity assays and immunohistochemistry had been adapted for use with these cells and a series of antibodies has been selected for detailed characterization. mAbs are characterized for binding to human normal and cancer tissues, functional features including CMC, ADCC, effects on CSC vitality and growth *in vitro* and *in vivo*, interference with the resistance of ovarian CSC to current chemotherapeutic drugs and for internalization characteristics. In addition, the antigens recognized by these antibodies will be biochemically identified. Those antibodies that selectively recognize CSC will be developed into therapeutic antibodies against cancer. They may be particularly valuable as targeted therapeutic reagents to control the spread of cancer in patients because they recognize antigens on highly clonogenic and tumorigenic cancer stem cells rather than on cancer cells with lower tumorigenic or metastatic potential. In addition we plan to develop these antibodies and the target antigen they recognize into diagnostic tools for early detection of ovarian cancer and for monitoring the success or failure of current therapeutic approaches to control the growth of ovarian cancer. In close collaboration with Sandro de Souza and his colleagues at the LICR São Paulo Branch we also utilize these cells to define the human cell surfaceome of ovarian CSC and to identify additional cell surface targets for development of immunotherapeutics.

SEROMICS AND GRAND SEROLOGY

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

We pioneered high-throughput serological monitoring using a semi-automated ELISA platform for serological responses against a series of tumor antigens as well as using protein microarrays for presence of antibody responses to close to 9000 antigens simultaneously. We also developed analytic tools to interpret and validate these antibody responses and introduced new standards for the field of "seromics", i.e., the scope of antigens defined by serological profiling of protein microarrays. Such analyses should help 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; define biomarkers of cancer for diagnostic or prognostic purposes, but also help predict antibody responses associated with favorable outcome to immunotherapies. We chose to focus our approach by measuring defined antigen-specific responses, rather than random screenings of tumor extracts.

We first applied these tools to the analysis of the ovarian and pancreatic cancer serome and found a major difference among the two groups in the repertoire of antigens recognized. More than 200 proteins were preferentially immunogenic in patients with ovarian cancer compared to 29 in pancreatic cancer. We are currently extending these analyses to other cancer types, notably prostate cancer. We analyzed >180 sera from prostate cancer patients with different stages of disease and looked for immune response to cancer-testis antigens as well as human endogenous retroviral antigen HERV-K. A subset of 42 sera from these prostate cancer patients and from 14 age- and sex-matched healthy donors was analyzed in seromics for association of seroreactivity to retroviral antigens HERV with other antigens. Another major application of seromics and grand serology is to measure changes in antibody profiles following immunotherapy. We have initiated multiple projects in this regard, starting with the immunological effects of CTLA-4 blockade (ipilimumab). We looked first for antibody and T cell responses to NY-ESO-1 and found evidence that NY-ESO-1 immunity is seen in patients with clinical benefit. In order to correlate immune response to NY-ESO-1 with clinical benefit to ipilimumab, we analyzed 144 patients with advanced metastatic melanoma treated at MSKCC and Yale (in collaboration with Jedd Wolchok, Jianda Yuan, Jim Allison, Ludwig Center at MSKCC, Mario Sznol, Yale University), and looked for correlations of NY-ESO-1 immunity with clinical benefit and survival. Our data suggest that integrated NY-ESO-1 immune responses (antibodies, CD8 T cells and CD4 T cells) have predictive value for ipilimumab treatment and argue for prospective studies in patients with established NY-ESO-1 immunity. The findings provide a strong rationale for the clinical use of modulators of immune suppression with concurrent approaches to favor tumor antigen-specific immune responses, such as vaccines or adoptive transfer in cancer patients. We have expanded our seromic profiling to search for additional immunogenic antigens potentially associated with clinical benefit to treatment with anti-CTLA4 and anti-PD1 in patients with melanoma, prostate cancer and colon cancer, to patients with breast cancer who received neo-adjuvant radio- and chemotherapy (before surgery) to study the abscopal effects of radiotherapy on immunogenicity (with Silvia Formenti and Sylvia Adams at New York University). Seromics was utilized to explore sera from different clinical vaccine trials with NY-ESO-1 or MAGE-A4 protein conducted by the Cancer Vaccine Collaborative network in Japan (collaborations with Drs. Nishikawa, Shiku, Wada, Tanemura and Nakayama).

CANCER-TESTIS ANTIGENS

Otavia Caballero, M.D., Ph.D., Achim Jungbluth, M.D., Yao Chen, M.D., Ph.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 about 130 CT gene families, many of them promising vaccine candidates. We have recently created a comprehensive CT database (CTdatabase, http://www.cta.lncc.br/) that integrates heterogeneous

CT antigen related data including basic gene, protein and expression information in normal and tumor tissues as well as immunogenicity in cancer patients. We have deposited our data on the RT-PCR expression analysis of a large series of new CTs in normal and cancer tissues and cell lines. Among our genomic efforts, we sequenced the genomes of a lymphoblastoid (HCC1954BL) and a breast tumor (HCC1954) cell line derived from the same patient and compared the somatic alterations present in both. The lymphoblastoid genome presents a comparable number and similar spectrum of nucleotide substitutions to that found in the tumor genome. KEGG analysis showed that in the tumor genome most mutated genes were organized into signaling pathways related to tumorigenesis. No such organization or synergy was observed in the lymphoblastoid genome. Our results indicate that endogenous mutagens and replication errors can generate the overall number of mutations required to drive tumorigenesis and that it is the combination rather than the frequency of mutations that is crucial to complete tumorigenic transformation. We have also used the genome-wide data from exome and transcriptome sequencing to identify potential tumor suppressor genes, by combining searches for genes with loss of heterozygosity and allele-specific expression. By comparing exome sequences from the two cell lines, we identified loss of heterozygosity events at 403 genes in HCC1954 and at one gene in HCC1954BL. The combination of exome and transcriptome sequence data also revealed 86 and 50 genes with allele specific expression events in HCC1954 and HCC1954BL, which comprise 5.4% and 2.6% of genes surveyed, respectively. Many of these genes identified by loss of heterozygosity and allele-specific expression are known or putative tumor suppressor genes, such as BRCA1, MSH3 and SETX, which participate in DNA repair pathways. Although patterns of somatic alterations have been reported for tumor genomes, little is known on how they compare with alterations present in non-tumor genomes. A comparison of the two would be crucial to better characterize the genetic alterations driving tumorigenesis.

Cancer/testis (CT) genes are most frequently located on the X-chromosome (the CT-X genes). Amongst the best studied CT-X genes are those encoding several MAGE protein families. The function of MAGE proteins is not well understood, but several have been shown to potentially influence the tumorigenic phenotype. In collaboration with a team of investigators from various branches of the Ludwig Institute, we undertook a mutational analysis of coding regions of four CT-X MAGE genes, MAGEA1, MAGEA4, MAGEC1, MAGEC2 and the ubiquitously expressed MAGEE1 in human melanoma samples. We first examined cell lines established from tumors and matching blood samples from 27 melanoma patients. We found that melanoma cell lines from 37% of patients contained at least one mutated MAGE gene. The frequency of mutations in the coding regions of individual MAGE genes varied from 3.7% for MAGEA1 and MAGEA4 to 14.8% for MAGEC2. We also examined 111 fresh melanoma samples collected from 86 patients. In this case, samples from 32% of the patients 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. These results demonstrate for the first time that the MAGE gene family is frequently mutated in melanoma.

Several CT antigens have already proved to be useful biomarkers and are promising targets for therapeutic cancer vaccines. To further identify tumor types or tumor subtypes that may be potentially suitable for immunotherapy with CT cancer vaccines, we have analyzed CT antigen expression in a series of tumor types and when data were available and correlated CT antigen expression with tumor stage and disease prognosis. In collaboration with Munro Neville (London) and A. Goldhirsch and colleagues (Milan) we expanded on our initial study in breast cancer and analyzed by immunhistochemistry 100 selected invasive breast cancers, including 50 estrogen receptor (ER) positive/ HER2 negative and 50 triple negative (TN). We found a significantly higher expression of MAGE-A and NY-ESO-1 in TN breast cancers compared with ER-positive tumors ($P = 0.04$). MAGE-A expression was detected in 13 (26%) TN cancers compared with 5 (10%) ER-positive tumors ($P = 0.07$). NY-ESO-1 expression was confirmed in nine (18%) TN tumor samples compared with two (4%) ER-positive tumors. In another study we performed a comprehensive immunohistochemical study to investigate the protein expression of eight CT genes in 454 invasive ductal carcinomas, including 225 ER/PR/HER2-negative (triple-negative) carcinomas. We found

significantly more frequent expression of all eight CT antigens in ER-negative cancers, and five of them - MAGEA, CT7, NY-ESO-1, CT10 and CT45, were expressed in 12-24% of ER-negative cancers, versus 2-6% of ER-positive cancers ($p<0.001$ to 0.003). In comparison, GAGE, SAGE1 and NXF2 were only expressed in 3-5% of ER-negative and 0-2% of ER-positive cancers. More frequent CT expression was also found in tumors with higher nuclear grade ($p<0.001$ to $p=0.01$) and larger in size (>2 cm). CT antigens are preferentially expressed in hormone receptor-negative and high-grade breast cancer. Considering the limited treatment options for ER/PR/HER2 triple-negative breast cancer, the potential of CT-based immunotherapy should be explored.

We have shown previously that the cancer/testis antigen CT45 is expressed in various epithelial cancers at a frequency of <5% to ~35%. In an analysis of non-Hodgkin B-cell lymphomas and classical Hodgkin lymphoma we found that classical Hodgkin lymphoma has the highest frequency (42/72, 58%) of CT45 expression among all malignancies tested to date, the frequency of CT45 expression in DLBCL is similar to that seen in epithelial cancers, and low-grade non-Hodgkin B-cell lymphomas, including chronic lymphocytic leukemia/small lymphocytic lymphoma, follicular lymphoma, and mantle cell lymphoma, do not express CT45.

CANCER VACCINES AND IMMUNOTHERAPIES

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

We have been conducting a clinical trial of ovarian cancer patients vaccinated with NY-ESO-1 overlapping long peptides (OLP) with or without montanide and poly-ICLC at MSKCC (PI: Paul Sabbatini). This trial is testing for the first time the use of OLP for NY-ESO-1 vaccination, and also addresses the importance of adding toll-like receptor agonists such as poly ICLC to vaccine preparations. We found that OLPs alone were poorly immunogenic compared to OLPs with montanide. This was confirmed on both the T cell and antibody levels, with a majority of patients developing evidence of both CD8 and CD4 T cell responses against NY-ESO-1 during vaccination with OLP+montanide, compared to none in the group without montanide (except for one patient with preexisting immunity to NY-ESO-1). Antibody responses generally appeared later than T cell responses, around week 13 on average after vaccination for antibodies vs. as early as week 4 for CD8 and/or CD4 T cells. Compared to other NY-ESO-1 protein or short vaccines, the OLPs in combination with Montanide appear more consistently immunogenic for generating integrated immune responses (CD4, CD8, Ab), and T cell immunity develops faster. Notably, when adding Poly-ICLC to the vaccine, patients developed far greater levels of immune responses, both humoral and cellular. Occurrence of antibodies could be detected in nearly all patients as early as week 7, and T cell responses to both HLA-class I and class II restricted epitopes were much broader in frequency and variety, and occurred earlier with more consistency. We analyzed the fine specificity of responses, the Th1/Th2 profiles induced, the sensitivity to regulatory T cells, and the avidity of effector cells, and it appears that the combination of OLPs with Montanide and Poly-ICLC is superior in immunogenicity to most other vaccination methods attempted for NY-ESO-1 to date using either peptides or protein. A manuscript is in preparation to describe these findings.

Targeting of protein antigens to cell surface receptors on dendritic cells such as DEC-205 or mannose receptor (MR) is considered to enhance the induction of antigen specific immune responses. In collaboration with Celldex, we have investigated whether this concept is applicable to NY-ESO-1, a cancer-testis antigen widely used in clinical cancer vaccine trials. Two novel targeting proteins consisting of the full-length NY-ESO-1 fused to the C terminus of two human mAbs against the human MR and DEC-205 were constructed and analyzed *in vitro*. We found that antibody-targeted NY-ESO-1 to MR or DEC-205 *in vitro* elicits dual human CD8+ and CD4+ T cell responses with broad antigen specificity from PBLs of cancer patients. Receptor-specific delivery of NY-ESO-1 to APC appears to be a promising vaccination strategy to efficiently generate integrated and broad Ag-specific immune responses against NY-ESO-1 in

cancer patients. The DEC205-NY-ESO-1 construct is now being explored in a phase I clinical trial.

CTLA-4, a molecule expressed by T lymphocytes, when stimulated 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 developed by Dr. Jim Allison (Ludwig Center for Cancer Immunotherapy at MSKCC) and others target CTLA-4 and block this natural breaking process: as a result, immune responses are allowed to continue acting when they should be otherwise 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 patients treated with CTLA-4 blockade immunotherapy. Dr. Jedd Wolchok (Ludwig Center and LICR NYB at MSKCC) has been a clinical lead in the clinical development of CTLA-4 blockade immunotherapy. He and his colleagues recently reported the results from double-blind phase II trial that anti-CTLA4 antibody "ipilimumab" elicited a dose-dependent effect on efficacy and safety measures in pre-treated patients with advanced melanoma and results from a phase III study that ipililumap improved overall survival in patients with previously pretreated metastatic melanoma. These results led to FDA approval of the drug in March 2011.

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.

We are utilizing the SEROMICS approach and ELISA to identify serum autoantibody responses during the course of treatment with immunotherapy in the hope of defining predictive markers of clinical response. In addition we are developing and utilizing a series of strategies and methodologies to assay cellular immune responses in cancer patients. The LICR New York Branch has been a reference and central monitoring laboratory for The LICR-CRI Cancer Vaccine Collaborative and in the past ten years we have been participating or leading the immunological monitoring for more than 65 clinical trials. A major emphasis has been on analyzing sera from melanoma patients treated with CTLA-4 blockade immunotherapy in collaboration with the Ludwig Center for Cancer Immunotherapy at MSKCC, NY (Drs. James Alison and Jianda Yuen). Ipilimumab, a monoclonal antibody against CTLA-4, has been shown to improve survival in advanced metastatic melanoma patients. It also enhances immunity to NY-ESO-1, a cancer/testis antigen expressed in a subset of melanoma patients. As a correlative study, we asked whether immune responses to NY-ESO-1 were associated with clinical benefit to ipilimumab treatment in metastatic melanoma patients (n=144) participating in several clinical trials at MSKCC and Yale University (collaboration with Ruth Halaban and Mario Szol). We first analyzed NY-ESO-1 serum antibody by ELISA in 144 ipilimumab-treated melanoma patients and found 22/140 (16%) seropositive at baseline and 31/144 (22%) seropositive following treatment. These NY-ESO-1-seropositive patients had a greater likelihood of experiencing clinical benefit 24 weeks after ipilimumab treatment than NY-ESO-1-seronegative patients (p=0.02, RR=1.8(1.2-2.8), two-tailed Fisher test). To understand why some patients with NY-ESO-1 antibody failed to experience clinical benefit, we analyzed NY-ESO-1-specific CD4+ and CD8+ T-cell responses by intracellular multicytokine staining in 20 NY-ESO-1-seropositive patients and found a surprising dissociation between NY-ESO-1 antibody and CD8 responses in some patients. NY-ESO-1 seropositive patients with associated CD8+ T-cells experienced more frequent clinical benefit (10/13; 77%) than those with undetectable CD8+ T-cell response (1/7; 14%; (p=0.02, RR=5.4(0.9-33.9), two-tailed Fisher test)), as well as a significant survival advantage (p=0.01, HR=0.2(0.1-0.7), time-dependent Cox model). Our data suggest that integrated NY-ESO-1 immune responses have predictive value for ipilimumab treatment and argue for prospective studies in patients with established NY-ESO-1 immunity. The current findings provide a strong rationale for the clinical use of modulators of immunosuppression with concurrent approaches to favor tumor antigen-specific immune responses, such as vaccines or adoptive transfer in cancer patients. Other experimental evidence for the strong influence of intratumoral immune suppression in cancer comes from our collaborative studies with Dr. Kunle

Odunsi (BRPCI Buffalo). We analyzed the mechanisms of immunosuppression intrinsic to tumor-infiltrating T cells with specificity for NY-ESO-1, and found that LAG-3 and PD-1 were involved in this process. In collaboration with Djordje Atanackovic (Hamburg) we also analyzed T cell responses to the tumor antigen NY-CO-58/KIF2C, originally defined by SEREX, and found that NY-CO-58/KIF2C was able to induce spontaneous CD4+ T cell responses of the Th1-type, which were tightly controlled by peripheral T regulatory cells.

MONOCLONAL ANTIBODY TRIALS

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

Our 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 (mAbs). Thirteen of our mAbs have been licensed to commercial partners for further clinical development and more than 10 of these antibodies have entered into clinical trials at Memorial Sloan-Kettering Cancer Center (MSKCC). Clinical trials 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 include studies with 90-Y-cG250, 111-In-cG250, 177-Lu-cG250 in renal cancer and 124-I-cG250 (phase III for differential diagnosis of renal cancer), and 124-I huA33 in colon cancer for PET imaging. Further, a phase I clinical trial was conducted with Farletuzumab (LK26), a humanized monoclonal antibody against folate receptor alpha, in epithelial ovarian cancer (collaboration with Dr. Paul Sabbatini). Folate receptor α expression is highly restricted in normal adult tissues but upregulated in a wide range of human cancer types, including epithelial ovarian cancer. Farletuzumab, has shown antitumor activity and favorable toxicity in preclinical evaluation. The dose-escalation study included 25 patients and was conducted to determine the safety of weekly i.v. farletuzumab and establish the maximum tolerated dose (MTD). No DLTs or MTDs were encountered. A nuclear imaging sub-study confirmed tumor targeting. There were no objective responses. Stable disease was observed in nine (36%) patients and CA-125 reduction in four. We found that farletuzumab administered as an i.v. infusion at doses of 12.5 to 400 mg/m^2 was generally safe and well tolerated in the management of heavily pretreated patients with epithelial ovarian cancer.

In our pursuit to further improve antibody efficacy in destroying tumor cells and to identify novel mechanism of using antibodies to interfere with tumor cell growth, we have developed antibodies that target cell surface enzymes and inhibit their activity. In collaboration with Christoph Renner, and colleagues (Zurich), Egbert Oosterwijk and colleagues (Nijmegen) and Andrew Scott and colleagues at the Melbourne-Austin Branch, we generated antibodies from a human phage display library that bound to the tumor-associated isoform IX of carbonic anhydrase (G250 antigen) and inhibited the enzymatic activity of CAIX. CAIX is a hypoxia-induced, membrane-tethered enzyme that is highly expressed in many cancers. CAIX has an important role in pH regulation and it may be involved in supporting cancer progression towards more aggressive forms of the disease. The new Fab antibody MSC8 and its corresponding full-length IgG inhibited CAIX activity. Incubation of CAIX-expressing cells with MSC8 IgG produced a lasting inhibitory effect. MSC8 may be used as selective, high-affinity inhibitor of CAIX by targeting a catalytically-active cancer-related protein.

Another strategy that we are pursuing is the use of mAbs 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. We are currently focusing on antibodies against CTLA4 and GITR, two key molecules in immunomodulation (for our anti-CTLA4 related activities see other sections in this report). Activation of GITR by its natural ligand (GITRL) or anti-GITR agonist mAb enhances T-cell responses, inhibits regulatory T-cell (Treg)-mediated suppression and induces tumor immunity in a variety of murine tumor models. As part of the Cancer Vaccine Collaborative clinical network we have initiated a first-in-human dose escalation trial of humanized monoclonal antibody against human GITR (TRX518) in patients with melanoma. The objective is to determine safety and tolerability, pharmacokinetics, and pharmacodynamics, and to explore its immunological activities. As systemic administration of these potent co-stimulatory agents may lead to global T-cell activation and potentially autoimmunity, we are developing more

targeted strategies for these reagents. In collaboration with Drs. Christoph Renner (Zurich), Hiroyoshi Nishikawa (Osaka) and Hiroshi Shiku (Mie), we aim to specifically manipulate the T-cell compartment in the tumor microenvironment by targeting the tumor infiltrating T cells with a bispecific mGITRL-antibody fusion protein construct. mGITRL was linked to a single-chain antibody targeting fibroblast activation protein (FAP) as FAP expression is restricted to cancer-associated fibroblasts (CAFs) found in the stroma of epithelial cancers. The construct bound well to both, target antigen and GITR, was able to co-stimulate CD8+ and CD4+ effector T cells and to overcome Treg-mediated suppression *in vitro*. These studies suggest that targeted tumor therapy with antiFAP-mGITRL fusion protein may induce tumor rejection while minimizing autoimmune side effects.

TARGETED ENZYME AMINO ACID DEPRIVATION THERAPY

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

Besides targeted antibodies and vaccines, 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. Arginine, like asparagine, is considered a non-essential acid, but certain cancer cells, especially melanoma and hepatocellular cancer, lack the enzyme argininosuccinate synthetase (ASS) necessary to convert citrulline to arginine thus making these tumors auxotrophic for arginine. The Mycoplasma-derived enzyme arginine deiminase (ADI) converts arginine to citrulline and tumor cells can be deprived of arginine. Thus, ADI has been developed as targeted biological therapy (ADI-PEG-20) for these types of tumors and has been found to have anti-tumor activity *in vitro* and *in vivo* in mouse models. The New York Branch has played a critical role elucidating the mechanism and in developing ADI for the clinic. 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 better understand 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.

We investigated ASS expression in human small cell lung cancer (SCLC) tumor tissues by immunohistochemistry (IHC) and found that about 45% of SCLC tumors were negative for ASS. We also screened a panel of 10 available human SCLC cell lines for ASS mRNA and protein expression by qRT-PCR and Western immunoblotting analysis, and we detected little or no ASS in 5/10 cell lines. In cell proliferation assays, sensitivity to ADI (ADI-PEG20) was correlated with expression of ASS in SCLC cells. Biochemical analyses of the mechanism of ADI-PEG20 induced cell death in ADI sensitive SCLC cell lines revealed induction of autophagy and cell death through caspase independent mechanisms. Assessment of ADI-PEG20 activity *in vivo* using mice bearing SCLC xenografts demonstrated that ADI-PEG20 treatment of ASS negative SCLC xenografts caused significant and dose dependent inhibition of tumor growth of not only small but also established tumors at doses equivalent to those used in the clinical exploration of ADI-PEG20. Efficacy was significantly greater with SCLC tumors compared to previous studies of melanoma xenografts. These results suggest an important role for ADI-PEG20 in the treatment of SCLC. A clinical trial making use of the ASS deficiency in SCLC has been commenced at MSKCC and Duke University. The eligibility criteria for patient enrollment includes lack or focal expression of ASS in SCLC and all immunohistochemical analyses for patient enrollment are done at the New York Branch. This is a representative example of how the long standing expertise in clinical discovery research in the New York Branch and the coordinated team effort of Branch investigators and external collaborators allowed a rapid follow up on our emerging laboratory discoveries by expediting our studies into investigations in animal models and then directly translating these finding into an early phase clinical trial in patients with small cell lung cancers.

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DIRECTOR'S MESSAGE

It is now more than three years since the Oxford Branch opened in 2007. We have continued to pursue our central goal of identifying key molecular switches that drive cancer progression and cellular heterogeneity, with the aim of benefitting patients. The Branch currently consists of six member track scientists and one clinical associate, all with strong overlapping interests, enabling the Branch to pursue a coherent research strategy while maintaining sufficient diversity to enable cross-pollination of ideas and technologies. In 2010, our research programs were bolstered by the arrival of two new group leaders: Sarah De Val from the University of California San Francisco and Skirmantas Kriaucionis from Rockefeller University.

Transcription factors are molecular switches of cell fate and their target selectivity lies at the heart of cell fate determination. A key research focus is the identification of molecular switches involved in cell growth or death (Professor Xin Lu), stem cells and differentiation (Professor Colin Goding), and tumor vascularization (Dr. Sarah De Val). This is complemented by the research undertaken by other groups at the Branch including Dr. Skirmantas Kriaucionis' work on the epigenetic regulation of gene expression at the genome-wide level.

Protein conformation is a key determinant of the expression and function of all transcription factors, and they are regulated by post-translational modifications. Dr. John Christianson's research is focused on the post-translational regulation of molecular switches. In addition, the work of Dr. Gareth Bond aims to identify molecular genetic signatures of cancer predisposition, progression and response to therapy. The work of Dr. Richard Bryant, a clinical associate, seeks to investigate the molecular events underpinning the progression of prostate cancer and establish new experimental systems with which to study this process.

Our research efforts are now starting to bear fruit with a number of key publications over the past year. We also hosted an LICR-wide genomics workshop in March, and organized and hosted an angiogenesis meeting in April, building upon LICR's strengths in these areas. The Branch's location in the physical center of the University of Oxford's cancer research activities continues to provide new opportunities for collaboration with colleagues working in clinical and basic research.

~ 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. Over the past year, we have continued to investigate how p53 is regulated. By generating and characterizing a Ser312Ala knock-in mouse model, we have demonstrated for the first time that phosphorylation of p53 at Ser312 contributes to its tumor suppressive ability *in vivo*.

Our group's 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. We have previously shown 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 of the assays performed 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.

The main focus of our research has continued to be the elucidation of the biological importance of the ASPP family. In particular, we have focused our attention on the *in vivo* functions of ASPP2 and iASPP using mouse models, and have demonstrated that ASPPs are molecular switches of cell fate (i.e., cell polarization, proliferation/migration and differentiation). The dynamic nature of ASPP localizations, together with their unique structural motifs, make them ideal molecular switches to survey and integrate signals between the cell surface and nucleus, to control transcription and determine cell fate. This is supported by our finding that, in development, ASPP2 binds Par3 to promote epithelial differentiation. Mechanistically, ASPP2 is recruited with Par3 to cell/cell junctions to maintain the integrity of cell-cell junctions and cell polarity, and ASPP2 deficiency results in neuroblastic rosettes that resemble primitive neuroepithelial tumors. We have also shown that ASPP2 suppresses cell proliferation through a novel and p53/p19(Arf)/p21(waf1/cip1)-independent pathway. ASPP2 suppresses the Ras-induced small ubiquitin-like modifier (SUMO)-modified nuclear cyclin D1 and inhibits retinoblastoma (Rb) protein phosphorylation. Nuclear cyclin D1 is also far more potent than the wild type in bypassing Ras-induced senescence. Thus, we have identified SUMO modification to be a positive regulator of nuclear cyclin D1 and revealed a new way by which cell cycle entry and senescence are regulated.

We are engaged in a number of collaborative projects, locally and worldwide. Existing international collaborations continue to be productive. Closer to home, collaborative projects within the Branch have been established with Colin Goding, Sarah De Val, Skirmantas Kriaucionis and John

Christianson, investigating the role of the ASPPs in protein degradation control and blood vessel formation, and the abrogation of the p53 response in melanoma. Collaborations initiated at our host institution with Zoltan Molnar and Yvonne Jones have come to fruition and we look forward to building upon these and other relationships over the coming year.

CELL FATE SWITCHING

Colin Goding, Ph.D.

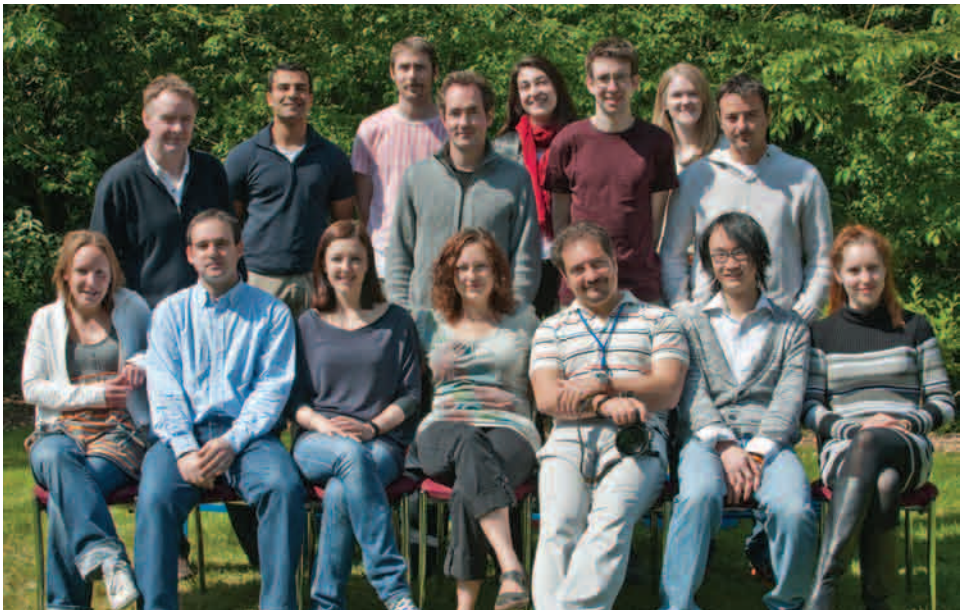
The ability of cells to change or maintain their identity through epigenetic mechanisms underpins development and is misdirected in cancer. Our work is aimed at understanding how this is achieved at the global level via networks of transcription factors and signaling pathways using the melanocyte/melanoma system, and how cells activate genes with kinetics appropriate to a specific signal.

In melanoma, we have identified the microphthalmia-associated transcription factor MITF as a master regulator of melanoma proliferation and invasiveness. This led us to propose a model by which reversible microenvironment variations in MITF expression and activity determine melanoma progression, by a process termed phenotype-switching in which cells can transiently adopt stem cell, proliferative or differentiated cell fates. Although MITF has historically been characterized as a transcription activator, we have identified a key co-repressor complex. We are now examining how the MITF-repressor interaction is regulated since this will have a major impact on melanoma sub-population identity.

A key collaboration with Eric Sahai, using intravital imaging, confirmed that invasive melanoma cells *in vivo* express high levels of Brn-2, a Mitf repressor. However, we have shown that Brn-2 can both activate and repress transcription, and identified key phosphorylation sites that drive the differential subnuclear localization of Brn-2. We also identified a key signal transduction pathway that regulates Brn-2 expression. These data provided key insights into how melanoma progression occurs.

Since MITF levels determine melanoma subpopulation identity, we have initiated a study to determine how the regulation of the epigenetic architecture of the MITF locus relates to its expression. We have also explored the possibility that MITF chromatin loops may be used to detect melanoma subpopulations in patient blood samples, with the aim of developing a more accurate way of detecting metastatic disease and monitoring the effectiveness of novel therapies. Significantly, we have used our knowledge of transcription regulation and normal melanocyte development to identify candidate melanoma stem cells in human tissue and are exploring ways in which these key cells can be isolated. A key aim over the coming years will be the detailed characterization of these cells and the signaling pathways that generate them. Our ultimate goal is to use the knowledge generated to develop therapeutic strategies aimed at the eradication of therapeutically-resistant stem cells from melanomas.

Most current approaches to melanoma therapy are directed at the inactivation of constitutively active oncogenes such as BRAF that drive melanoma proliferation. However, we have discovered that melanomas contain an intact senescence pathway that is suppressed by the activity of the Tbx2 and Tbx3 transcription factors. These proteins also play key roles in determining cell identity in development. We have now identified several cell cycle and stress activated kinases that regulate Tbx2 expression and that regulate Tbx2 expression and have the ability



to interact with co-factors, including Rb1, that enable them to regulate gene expression. The results have enabled us to develop a model to account for both their role in development, and in melanoma invasiveness and senescence bypass. The identification of enzymatic co-factors for the anti-senescence T-box factors, as well as our results have revealed signaling pathways and transcription factors that regulate Tbx2 and Tbx3 expression in melanoma, point towards potential targets for pro-senescence therapy via inhibition of Tbx2/3 expression function.

Finally, our work has led to several close intra-branch collaborations that have been initiated over the past year. In particular, collaborations with Gareth Bond, Xin Lu and Sarah de Val will yield key insights into melanoma predisposition, cell fate determination and vascularization.

REGULATION OF VASCULAR GROWTH

Sarah De Val, Ph.D.

The group was established at the beginning of 2010 and focuses on the transcriptional regulation of vascular establishment and growth, with a particular interest in the transcriptional pathways underpinning the angiogenic switch, and tumor-to-endothelial cell differentiation, in cancer progression. The establishment of an adequate blood supply is a necessary step in the development and spread of solid tumors, whether through rapid proliferation of vessels via sprouting from surrounding regions (the angiogenic switch), differentiation from tumor cells themselves, or a combination of the two. Conversely, the dis-regulated and aberrant structure of the vessels within these tumors make them poor conduits for drug delivery, and result in pockets of cancer cells inaccessible to blood-borne drugs. Consequently, the identification of targets for blocking or modulating vessel growth is an important therapeutic goal for cancer treatment.

While numerous studies have examined the signaling molecules involved in vasculogenesis and angiogenesis, the transcriptional mechanisms through which the expression of genes within and downstream of these pathways are regulated remain important questions in vascular biology. To investigate, we are using cellular, molecular, genetic, and transgenic approaches, including the identification and delineation of vascular regulatory elements to identify key *cis*-motifs and *trans*-binding factors, and the analysis of transcription factor expression patterns and function during vessel growth. The questions we are addressing include the manner in which components of vascular signaling pathways are transcriptionally regulated, gene regulation during vascular differentiation, and the transcriptional cascades at work during tumor vasculogenesis and angiogenesis.

To understand how expression of a given gene is regulated, it is crucial to locate and analyze key regulatory elements outside of the proximal promoter region. We have been taking a multi-pronged approach to regulatory element identification, including *in silico* motifs searches, publicly available data detailing histone marks, DNaseI hypersensitivity and ChIP data, and phylogenetic footprinting. For example, the FOX:ETS motif, a novel *cis*-element present in many endothelial gene enhancers, is sufficient to locate vascular enhancers, and is even more effective when used in combination with other information. Confirmation of regulatory regions is primarily through transgenesis. In the last year, we have had considerable success with this program and are identifying an increasing number of enhancers involved in the angiogenic growth associated with tumor vascularization, differentiation of the vasculature, and coronary vessel specification. Important *cis*-motifs

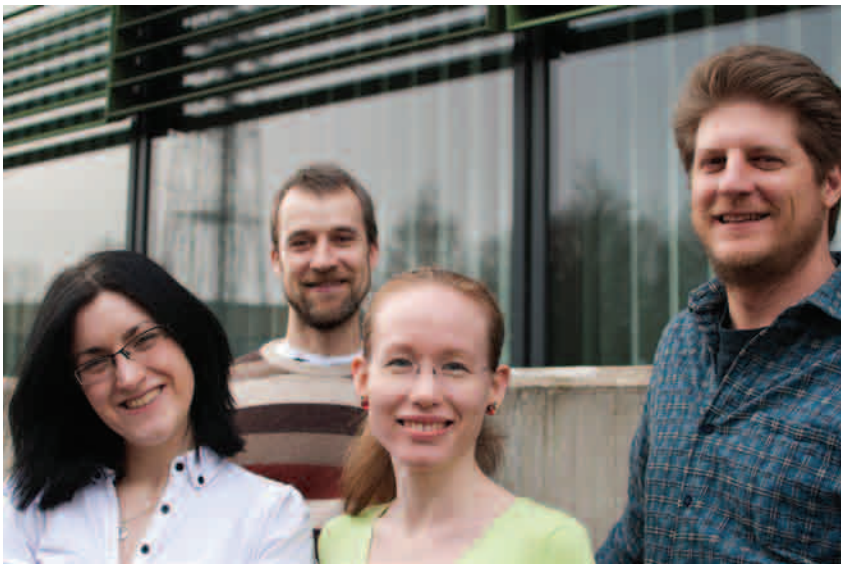
within the identified gene regulatory elements, and potential *trans*-factors, are being located through a combination of phylogenic and DNaseI footprinting, database and literature searches, deletional and mutational analysis *in vitro* and *in vivo*, binding analysis, mass spectrometry and observation of transgene expression in knock-down, knock-out and over-expression models.

The group is also investigating the unsuspected or poorly studied roles of novel transcription factors during vascular differentiation and at the angiogenic front. We are using a combination of gene expression studies and phenotypic analysis of vascular development after gene alteration (including knock-out, knock-down through morpholino or siRNA, and over-expression) in cell and animal models. *In vivo* models include developmental angiogenesis in the mouse and zebrafish embryo, retinal angiogenesis and hanging drop assays to visualize tip- and stalk-cells at the angiogenic front, and tumor vascularization: both histological analysis of spontaneous human and mouse tumors, and implantation of tumors into mouse models with transgenic or altered gene expression.

PROTEIN QUALITY CONTROL

John Christianson, Ph.D.

Approximately one third of the human genome encodes proteins whose biogenesis and maturation occurs through the secretory pathway. Cell surface and secreted proteins/complexes maturing here are important effectors of cellular homeostasis, growth, signaling and motility. Their integrity and abundance are regulated not only transcriptionally, but also post-translationally while in the endoplasmic reticulum (ER). ER-associated degradation (ERAD) is part of a quality control mechanism that ensures fidelity and regulates expression of secreted and integral membrane proteins through selective culling, making it an essential surveillance checkpoint for protein expression in all eukaryotic cells. Our research program aims to understand the role protein quality control in the early secretory pathway plays in the initiation and progression of cancer, with a specific focus on how quality control shapes the cell surface proteome in tumor and metastatic cells. The group employs a systems biology strategy that integrates cell/molecular biological, biochemical and proteomic techniques to characterize the ERAD mechanism, its constituents and its substrates. The long-term goal of this work is to explore quality control mechanisms as a novel point of intervention for cancer therapies.



Appreciation for the complexity and adaptability of the ERAD mechanism is coalescing. More than 40 structurally and functionally distinct proteins have been implicated in ERAD, with many working in synchrony as constituents of transiently forming macromolecular complexes spanning the ER transmembrane axis, in order to degrade the diverse range of proteins with which it is challenged. Our group has been at the forefront of identifying novel metazoan-specific components and mapping the basic functional interactions mediating ERAD. By integrating proteomics and functional genomics, we uncovered more than 70 additional proteins of interest that interact with known components of the ERAD network, a number of which have been functionally validated including UBAC2, TXD16 and FAM8A1. This study represents the most comprehensive mapping of the ERAD network to date and will be an invaluable resource for our group to further elucidate the mechanisms underlying this process.

Insight gained from systematic studies has prompted several questions within the ERAD mechanism. The ER-resident ubiquitin ligase Hrd1 is a core ERAD component but, while not essential for degradation of all substrates, current studies suggest that Hrd1 has a robust, adaptive ability to identify and ubiquitinate substrates with very disparate topologies and features. Other ERAD ubiquitin ligases appear to have a narrower substrate range and we have undertaken the task of determining how substrates are targeted to individual ERAD ubiquitin ligases. Using novel strategies developed in the lab, we isolated and identified novel ERAD substrates and incorporated them to identify cell surface tumor suppressors and oncogenes whose expression is governed by ERAD.

The ER is where the fate of nascent polypeptides is decided, either to fold properly or be degraded. We have previously reported an interaction between the ERAD component and ER lectin OS-9 and the ER-resident, Hsp90 chaperone homologue GRP94. This interaction represents the interface between protein folding and degradation machinery, acting as a platform for the molecular "triage" decisions to be made. The region of OS-9 identified as crucial for GRP94 interaction was also found to harbor several cancer-related mutations, which are currently being investigated with assistance from Benoît van den Eynde's lab in Brussels.

Ubiquitin conjugation is an essential step in targeting ERAD substrates for destruction. Our proteomic survey of essential ERAD components identified AUP1 as a unique interactor of both the Hrd1 ubiquitin ligase and the ubiquitin conjugating enzyme Ube2g2. We have hypothesized that AUP1 acts as a bridge between the two, binding Hrd1 through its transmembrane domain while recruiting Ube2g2 through a highly conserved, C-terminal linear peptide domain. AUP1 also contains a low-affinity ubiquitin-binding CUE domain that appears to be essential for function. AUP1-Ube2g2 is positioned uniquely within ERAD as it scaffolds a secondary ubiquitin-conjugating enzyme for Hrd1 (along with Ube2j1). The potential for two-stroke loading of Hrd1 via Ube2g2 and Ube2j1 offers a mechanism for rapid substrate ubiquitination. Alternatively, AUP1-Hrd1-Ube2g2 may represent a substrate-specific degradation complex that differs from the one formed by Hrd1-Ube2j1. Both of these prospects are being investigated.

EPIGENETIC MECHANISMS

Skirmantas Kriaucionis, Ph.D.

Established in October 2010, the laboratory is interested in understanding the function of DNA modifications in normal and cancer cells. It is well documented that, in addition to multiple genetic abnormalities, cancer cells have a unique epigenetic phenotype. CpG islands of certain tumor suppressor genes get methylated and the rest of the genome becomes hypomethylated. However, the mechanisms resulting in the infidelity of the epigenetic landscape are not currently clear.

It has recently been shown that the presence of a novel DNA modification in differentiated and stem cells, 5-hydroxymethylcytosine (hmC), can be the result of two sequential activities: methylation by DNA methyltransferases followed by hydroxylation by TET family of 2-oxygenases. Recent studies have identified mutations in enzymes from both classes that are often seen in blood cancers. Somatic mutations in DNA methyltransferase DNMT3A were found in 20% of patients with Acute Myelogenous Leukemia (AML). Myelodysplastic syndromes have a frequently mutated (more than 25% cases) 5-methylcytosine oxygenase TET2. In addition, mutations in IDH1 and IDH2 are detected in AML. Normally, IDH1 and IDH2 are involved in making 2-oxoglutarate, which is a cofactor for 2-oxygenases including TET enzymes. The most common cancer related IDH1 and IDH2 mutations cause production of 2-hydroxyglutarate, thought to inhibit the TET family of 2-oxygenases. These

observations indicate the importance of epigenetic mechanisms as suppressors of oncogenesis.

The group is working on elucidating the function of hmC in the genome and the mechanisms responsible for the deposition, recognition and removal of the mark. We are undertaking a number of approaches to quantify and map hmC in various normal and cancer cells together with molecular characterization of TET enzymes. We have established the use of UHPLC to quantify 5-methylcytosine and 5-hydroxymethylcytosine in the genome and are using the technique to characterize various cell types and validate methods that will be used for localizing the modifications in the genome.

The TET family of 2-oxygenases has three members TET1, TET2 and TET3. TET1 (Ten Eleven Translocation protein 1) was initially found to be translocated in AML, creating a fusion with MLL. All three TET enzymes have the conserved 2-oxygenase domain, which can hydroxylate 5-methylcytosine in the genome. We are focusing on the function of TET2 enzyme in myeloid cells by elucidating the pathways that target and modulate its activity, with the goal of revealing the areas of possible intervention.

An on-going collaboration with Nathaniel Heintz's lab at Rockefeller University is focused on describing epigenetic landscapes in terminally differentiated cells using FACS-based separation of nuclei from specific cell types in transgenic mice, which express nucleolus-associated eGFP. The data will provide critical insights into post-mitotic chromatin organization and the function of DNA modifications.

HUMAN CANCER GENETICS

Gareth Bond, Ph.D.

Our 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 better understand the contribution of germline genetics of susceptibility to and progression of cancer, as well as expose potential nodes of intervention that could prove valuable in the prevention and treatment of this disease in humans. 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 among individuals. To overcome this, our approach is to focus on biologically relevant signaling pathways that are likely to be involved in cancer causation and progression. For example, a large body of evidence strongly suggests that the genes that make up the p53 tumor suppressor pathway are central in reducing cancer frequency in vertebrates and mediating the response of commonly used cancer therapies. The p53 pathway is a cellular stress response pathway that is activated upon stresses such as DNA damage and oncogene activation.

Previous work has demonstrated that single nucleotide polymorphisms (SNPs) in the p53 tumor suppressor gene and the MDM2 oncogene can affect p53 signaling, confer cancer risk and survival and suggest that the pathway is under evolutionary selective pressure. This year, in collaboration with Guillermina Lozano's laboratory at the MD Anderson Cancer Center, we provided causal evidence that the G-allele of MDM2 SNP309 (T/G) increases cancer risk in carriers by genetically engineering mouse models carrying either the G- or T-alleles. We showed that homozygous G-allele mice have increased MDM2 levels, decreased p53 levels, and a marked attenuation of the p53 stress response, consistent with the inhibitory role of MDM2 on p53

activity. Importantly, the homozygous G-allele mice have a shorter latency to tumor formation and decreased survival. These data clearly demonstrate that the G-allele of MDM2 SNP309 has a direct impact on the p53 stress response pathway and on cancer risk, demonstrating that inherited genetics of the p53 network could be utilized to further define patients in their abilities to react to stress, suppress tumor formation and respond to therapies. However, very little is known about the germline gene genetic variation of this or other important signaling pathways that are altered in human cancers.

Currently, we are developing multiple approaches to accelerate the identification of functional p53 network SNPs that could further define people with higher cancer risk, faster progression and metastasis, and a poorer outcome. To accomplish this, we are taking advantage of well-characterized traits of known-functional p53 network SNPs, like MDM2 SNP309, to identify other SNPs that affect human cancer. We are mining genomic data sets for SNPs that demonstrate allelic differences in signatures of recent natural selection, cellular chemosensitivities and gender- and age-dependent cancer incidence. For example, the NCI60 cell panel is a panel of sixty cancer cell lines from different tissues of origin. We developed and implemented analytic frameworks to uncover candidate functional SNPs in the p53 network utilizing data derived from the NCI60 human tumor cell line anticancer drug screen. To date, we have utilized the methodology to scan 187 SNPs in 142 p53 stress response genes. Seven SNPs in six genes that exhibit allelic differences in cellular growth-responses to many standard chemotherapeutic agents have been identified. Cells with different genotypes at these loci associate with, on average, a different response for 79 of the 132 agents tested, whereby the homozygotes differ on average 4.9-fold in their respective drug sensitivities. We have observed the greatest differences in SNPs in the YWHAQ and CD44 genes. Importantly, sarcoma patients with the alleles of these SNPs that associate with weaker cellular growth responses to chemotherapeutics, associate with poorer overall survival and an earlier age of diagnosis. Together, these data suggest that allelic differences in cellular chemosensitivities can be used to identify p53 network genes, like YWHAQ and CD44 that harbor SNPs and affect human cancer.

PROSTATE CANCER PROGRESSION

Richard Bryant, Ph.D.

The laboratory's research is focused on identifying key molecular switches involved in the process of epithelial to mesenchymal transition during prostate cancer progression to an invasive and metastatic phenotype. A number of novel potential mediators of epithelial-to-mesenchymal transition have recently been identified including members of the ASPP family of proteins. ASPP proteins were initially identified as regulators of the apoptosis-inducing function of the p53 tumor suppressor protein. Whereas around half of all human cancers contain mutant p53, this abnormality is only found in 10-20% of prostate cancers and tend to be advanced cases of this malignancy. The tumor suppressive function of p53 can, however, be lost through mechanisms other than gene mutation in cancers expressing wild type p53, and aberrant function of members of the ASPP family of proteins may represent such a mechanism. Breast cancer studies suggest roles for ASPP proteins in carcinogenesis, with ASPP1 and ASPP2 being frequently down-regulated in tumors expressing wild type p53, while iASPP is over-expressed in tumors expressing wild type p53 and normal levels of ASPP1 and ASPP2. Moreover, it is becoming increasingly apparent that members of the ASPP family of proteins are involved in a range of epithelial processes including the maintenance of epithelial integrity and, as such, it is possible that aberrant ASPP

protein expression and/or function may promote prostate cancer progression.

p63 is a p53 protein family member expressed by basal cells of normal prostate epithelium, however, this expression is lost during the development of prostate cancer. p63 function is absolutely required for the embryonic development of the prostate gland. We have hypothesized that ASPP proteins regulate p63 function and may be required for normal prostate epithelial development. We have investigated the expression of ASPP proteins in tissue microarrays containing both benign prostate tissue and prostate cancer samples from surgical specimens with associated clinical follow-up data. The nuclear staining intensity of iASPP was typically greater in the prostate cancer samples compared with benign tissue, and the cytoplasmic expression and localization of each of the ASPP proteins differed in the cancer samples compared to benign prostate epithelium. Intriguingly, we observed a significant association between increased diffuse cytoplasmic localization of iASPP and ASPP1 and an adverse clinical outcome in high grade prostate cancer cases. This initial translational data suggest that aberrant ASPP protein expression and/or localization may be mechanistically relevant to prostate cancer progression. In order to test this hypothesis we are currently conducting a number of *in vitro* assays in the laboratory in order to perform a series of experiments with the aim of identifying which aspects of an advanced prostate cancer phenotype are influenced by aberrant ASPP protein function. Initial experiments suggest that inhibition of normal ASPP protein function influences the invasiveness and motility of prostate cancer cells *in vitro,* however, the exact molecular mechanisms underlying these observations remain to be fully elucidated. These experiments are ongoing in the laboratory.

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DIRECTOR'S MESSAGE

The eight research 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 discoveries about the processes that malignant cells use to slacken their genomic integrity; how they hijack signaling pathways to advance their growth and hinder their death; how global gene regulation is orchestrated; and how any of these processes can prompt cancer formation and progression.

Drs. Karen Oegema and Arshad Desai were promoted to Member and earned accelerated promotions to Full Professor in the University. The relationship between the Branch and the University is ever stronger with the continuing efforts of Dr. Don Cleveland as Chair of the Cellular and Molecular Medicine Department of the UCSD Medical School while remaining

a Member of the Institute, and by our joint recruitment of the next UCSD Moores Cancer Center Director. Many postdoctoral fellows and graduate students moved on to independent positions in academia or industry after finishing their training with us.

The Branch is becoming increasingly collaborative with a growing number of publications from more than one of our laboratories. We continue to be very successful at garnering competitive grant support, despite increasingly difficult national circumstances. Our laboratory of Small Molecule Development is assuming a more central role in our experimental design as we become more astute in thinking in this way and several potential inhibitors are well on the way. We are especially fortunate to have recruited Dr. Kevin Corbett from Harvard Medical School who will join us in mid-2011. He is a first class crystallographer who will undoubtedly expand our experimental potential and approaches and we welcome him.

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 in the identification, functional analysis and therapeutic targeting of genetic lesions associated with human cancer.

The *Section of Molecular Cytogenetics* led by Dr. Karen C. Arden, focuses on the PAX3-FOXO1 fusion gene characteristic of the alveolar subtype of the skeletal muscle tumor rhabdomyosarcoma. The expression of the fusion protein alone is not sufficient for tumorigenesis and we have shown that it interferes with myogenic differentiation contributing to the undifferentiated cellular phenotype associated with alveolar rhabdomyosarcoma. The PAX3-FOXO1 fusion protein does this by binding to and destabilizing another transcription factor, EGR1, thereby targeting both proteins for proteasomal degradation. EGR1 directly activates expression of p57KIP2, a member of the cyclin-dependent kinase inhibitor family of proteins known to block G_1/S and is associated with myogenic differentiation. In the absence of EGR1, p57KIP2 is not expressed and the undifferentiated primitive myoblast phenotype is maintained.

In collaboration with Bing Ren, we are actively pursuing the identification of FOXO DNA binding sites and gene targets. While ChIP-Seq methods require exquisitely sensitive antibodies, we have also used an alternate approach by epitope tagging endogenous FOXO1 and FOXO3 genes through homologous recombination. Studies are also underway to understand the role of small noncoding microRNAs in rhabdomyosarcoma. We have created the necessary reagents to perform a large-scale screen to identify miRNAs that are differentially regulated in rhabdomyosarcoma anticipating that this could lead to improved prognostic indicators and provide additional therapeutic targets.

The Section of Human Carcinogenesis led by Dr. Frank Furnari is focused on genetic alterations that drive gliomagenesis. Progress has been made in understanding how cell-extrinsic factors produced from mutant EGFR (ΔEGFR) -expressing cells potentiates heterogeneous growth; the role of this mutant receptor in maintaining tumor growth; and how site-specific tyrosine phosphorylation of the PTEN tumor suppressor mimics gene inactivation and conveys therapeutic resistance to EGFR-directed tyrosine kinase inhibitors as well as predicts poor clinical outcome.

While much is known about interactions between tumor cells and surrounding normal cells, much less is known about those between and among heterogeneous tumor cells within a neoplasm. Strikingly, despite its greater biological activity than wild type EGFR (wtEGFR), only a minority of GBM cells usually expresses the hallmark ΔEGFR lesion, while the remainder expresses wtEGFR. Using mixtures of glioma cells or immortalized murine astrocytes, we demonstrated a paracrine IL6 and/or LIF-mediated mechanism driven by ΔEGFR that can recruit wtEGFR-expressing cells into accelerated proliferation *in vivo*. Such interactions between genetically dissimilar cancer cells could provide novel points of therapeutic intervention.

Although ΔEGFR appears to be an attractive target for anti-glioma therapies, recent clinical experiences with EGFR kinase inhibitors have been disappointing. Since it is not clear whether ΔEGFR is required for maintenance of glioma growth *in vivo*, we developed a tetracycline-regulated ΔEGFR approach, to show that suppression of this receptor attenuates glioma growth. Similar to the clinical experience, tumors in our model system eventually regained aggressive growth after a period of stasis. Mechanistically, we found that a novel gene, kelch domain protein KLHDC8A,

is highly expressed in the tumors that had escaped dependence on ΔEGFR, was expressed in human gliomas and its expression was required for in ΔEGFR-independent tumor growth.

While PTEN mutation can also lead to EGFR-directed therapy resistance, some gliomas retaining wild type PTEN also demonstrate resistance. Investigating modulators of the PTEN/PI3K signaling axis, we have identified PTEN posttranslational tyrosine phosphorylation mediated by src family kinases and fibroblast growth factor receptors (FGFRs) as a means of resistance in gliomas. The clinical significance of this was shown in collaboration with Suely Marie in São Paulo and Paul Mischel at UCLA where a tight association with prognosis of GBM patients and both upfront or acquired resistances to EGFR inhibitors was uncovered. 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 laboratory, that also includes the Section of Structure and Computational Genetics headed by Dr. Christopher D. Putnam, is focused on two research projects using the yeast *Saccharomyces cerevisiae* and other organisms. The first is to elucidate the 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 prevent the accumulation of genome rearrangements like translocations seen 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 and whether the aberrant mechanisms that underlie this can be exploited for therapeutic means.

Work on MMR has primarily focused on the biochemistry and cell biology of MMR. Using limited proteolysis and mass spectrometry, we identified regions of the mispair-binding Msh2-Msh6 complex that undergo nucleotide-mediated conformational changes. We have targeted these regions with engineered cysteines that can lock these regions with a disulfide bond under oxidizing conditions. Biochemical and genetic analysis has demonstrated that locking these regions causes MMR defects *in vivo*, blocks conformational changes, and disrupts the ability of Msh2-Msh6 to convert to a sliding clamp, but does not alter recognition of mispaired DNA or binding of Mlh1-Pms1. In collaborative work with Dr. Arshad Desai, we visualized MMR proteins in living *S. cerevisiae* cells by generating functional MMR proteins tagged with fluorescent markers expressed from their native chromosomal loci. Tagging of Msh2 or Msh6 demonstrated that Msh2-Msh6 forms replication fork-associated foci independent of the level of mispaired bases but dependent upon an Msh6-PCNA interaction. Surprisingly, Mlh1-Pms1, which is the key downstream MMR factor that is recruited to mispair-containing DNA by Msh2-Msh6, formed foci that did not colocalize with Msh2-Msh6 foci. Despite this, the Mlh1-Pms1 foci formation was dependent upon the presence of Msh2-Msh6, and Mlh1-Pms1 foci were present at levels dependent upon the number of mispairs and upon the

proper functioning of downstream MMR processes. These results indicate that Mlh1-Pms1 foci are a MMR intermediate and that Msh2-Msh6 primarily recognizes mispairs as a constitutive component of replication factories and catalyzes Mlh1-Pms1 loading. This view overturns the long-held assumption that the Msh2-Msh6-Mlh1-Pms1 ternary complex is the upstream MMR signal, and suggests that Mlh1-Pms1 or an interacting partner other than Msh2-Msh6 distinguishes the newly synthesized and template strands in replicated DNA. This work has not only resulted in novel assays to probe the temporal dynamics of MMR and MMR defects, but it also is reframing questions and generating new testable hypotheses about eukaryotic MMR for future biochemical and genetic experimentation.

Identification of genes and pathways that cells use to prevent genome instability 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. We have developed assays that probe the role of long repetitive elements in chromosomal rearrangements as well as ligation-dependent PCR assays for the yeast genome that allow probing of copy number changes more rapidly and cost-effectively than microarray-based analyses. We have additionally begun to probe why specific pathways play larger roles in suppressing repeat sequence-mediated rearrangements than single copy sequence-mediated rearrangements. By combining genome-wide data developed for *S. cerevisiae*, we have used systems biology-inspired bioinformatics approaches to identify genes and pathways enriched for roles in the maintenance of genome stability. We have been leveraging this analysis in two different ways. First, we have developed the genetic and robotic infrastructure and are beginning to identify genetic interactions in *S. cerevisiae* that lead to increased genomic instability, as our analysis of current genome-wide data indicates that genetic interactions that cause growth defects are poor surrogates for understanding this problem. Second, we are collaborating with Dr. Sandro José de Souza in the laboratory of computational biology, São Paulo Branch to determine if the human homologs of these genes are mutated or show aberrant expression in cancer cell lines and cancers being analyzed by cancer genomics initiatives. Our goal is to understand potential mechanisms for cancer development and progression and identify novel targets for cancer therapy.

CELL BIOLOGY

Don W. Cleveland, Ph.D.

The laboratory 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.

Chromosome missegregation has long been associated with cancer. Key kinases essential for chromosome segregation and mitosis are the Aurora kinases. In the last year, the laboratory identified an Aurora kinase/protein phosphatase 1 phosphorylation switch that mediates chromosome congression and spindle attachment. Aurora kinases A and B were

shown to phosphorylate a single residue on the kinetochore motor CENP-E. PP1 binds CENP-E via a motif overlapping this phosphorylation site and binding is disrupted by Aurora phosphorylation. Phosphorylation of CENP-E by the Auroras at spindle poles disrupts binding of PP1, but activates CENP-E for towing of initially polar chromosomes toward the cell center. Kinetochores on such chromosomes make subsequent stable attachment to spindle microtubules only after dephosphorylation of the major microtubule capture complexes by PP1 that rebinds to, as is delivered by, CENP-E. Thus, an Aurora/PP1 phosphorylation switch modulates CENP-E motor activity as an essential feature of chromosome congression from poles and localized PP1 delivery by CENP-E to the outer kinetochore is necessary for stable microtubule capture by those chromosomes.

Like DNA, centrosomes are duplicated only once each cell cycle. The duplication process is governed by polo-like kinase 4 (Plk4). Recognizing that abnormalities in number of microtubule organizing centers (centrosomes) can promote errors in spindle formation that lead to subsequent chromosome missegregation and extra centrosomes are associated with many cancers, in the past year the laboratory identified how centrosome over-replication is blocked: kinase-mediated, autoregulated instability of Plk4 acts to self-limit Plk4 activity so as to prevent centrosome amplification.

Regarding the mechanisms underlying the adult motor neuron disease ALS, a proportion of disease is caused by mutations in superoxide dismutase (SOD1). With conformation specific antibodies, the laboratory demonstrated that misfolded mutant SOD1 binds directly to the voltage-dependent anion channel (VDAC1), an integral membrane protein imbedded in the outer mitochondrial membrane. Direct binding of mutant SOD1 to VDAC1 was shown to inhibit conductance of individual channels when reconstituted in a lipid bilayer, thereby establishing a direct link between misfolded mutant SOD1 and mitochondrial dysfunction in inherited ALS.

Mutation in two functionally related DNA/RNA-binding proteins, TDP-43 and FUS/TLS, were identified in the past two years as causes of ALS. With isogenic cell lines expressing wild type or ALS-linked TDP-43 mutants and fibroblasts from a human patient, the laboratory used pulse chase radiolabeling of newly synthesized proteins to determine that ALS-linked TDP-43 mutant polypeptides are more stable than wild type TDP-43. Affinity purification and quantitative mass spectrometry, in collaboration with the Zhou laboratory, were used to determine that TDP-43 complexes not only with hnRNP family proteins, but also components of Drosha microprocessor complexes, thereby uncovering a previously unknown role for TDP-43 in microRNA biogenesis. Moreover, a fraction of TDP-43 was found complexed with FUS/TLS, an interaction substantially enhanced by TDP-43 mutants. Taken together, abnormal stability of mutant TDP-43 and its enhanced binding to normal FUS/TLS imply a convergence of pathogenic pathways from mutant TDP-43 and FUS/TLS in ALS.

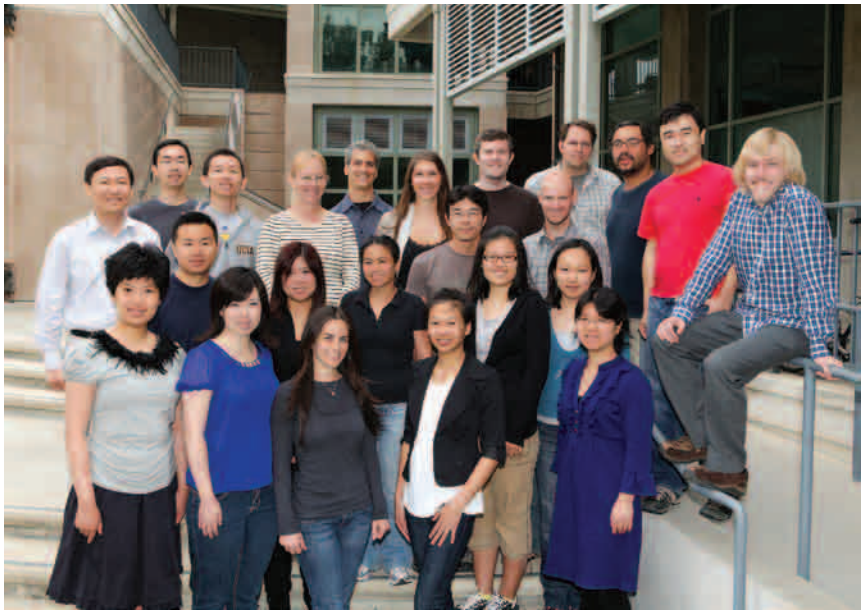
CHROMOSOME BIOLOGY

Arshad Desai, Ph.D.

The laboratory is focused on understanding the mechanisms that distribute the genome during cell division. Specifically, we are investigating the epigenetics of centromere identity and the coupling between mechanics and checkpoint signaling at the kinetochore-microtubule interface during chromosome segregation. Accurate segregation of chromosomes

is essential to prevent aneuploidy. The interface between chromosomes and microtubules is also the target of anti-mitotic chemotherapeutic drugs, such as taxol. A major project area is focused on the conserved KNL-1/Mis12 complex/Ndc80 complex (KMN) protein network, comprised of 9 interacting proteins, that we showed in earlier work provides the core microtubule-binding activity of the kinetochore and acts as a scaffold for spindle checkpoint signaling. In the past year, we extended analysis of the KMN network to meiosis, where homologous chromosomes, rather than sister chromatids, are segregated. Our efforts revealed a new kinetochore-independent mechanism operating during anaphase of oocyte meiosis to separate chromosomes . We also discovered the key step in silencing the spindle checkpoint in human cells, based on analysis of a conserved protein family that targets the dynein motor complex to kinetochores. This work sheds light on the function of the second conserved microtubule-binding activity of kinetochores, resident in the dynein-dynactin complex, in chromosome segregation.

In parallel to an investigation of mechanics and checkpoint signaling, we are studying the mechanisms that specify kinetochore formation at a localized site on chromosomes. The specification event involves formation of specialized chromatin containing a histone H3 variant called CENP-A. The specialized chromatin domain containing CENP-A is proposed to be propagated by DNA replication and replenished during early G1 to epigenetically maintain centromere identity. We have shown that deposition of CENP-A occurs *de novo* following fertilization in *C. elegans*. The distribution of the centromeric histone variant genome-wide in *C. elegans* was analyzed which revealed a relationship to germline gene expression. This effort is leading to a transgenerational epigenetic inheritance model for centromere identity. We are also pursuing mechanistic studies of CENP-A-specific loading machinery. Finally, we contributed to collaborative efforts to annotate the *C. elegans* genome as part of the modENCODE project.



GENE REGULATION

Bing Ren, Ph.D.

The laboratory's research has been focused on understanding the nuclear processes that determine cell fate and differentiation in mammalian cells. To achieve this goal, we have been investigating two related problems:

Identifying the transcriptional regulatory sequences in the genome

As part of the NIH mouse ENCODE project, we have been working to generate maps of *cis*-regulatory elements in the mouse genome. The approach involves the identification of transcription factor binding sites and chromatin modification status genome-wide using ChIP-seq. Applying this method to ten adult and embryonic mouse tissues has led to the identification of over 300,000 *cis*-regulatory elements including a large number of novel promoters, enhancers and insulator elements. Analysis of these sequences confirm, in a genome-wide scale, that the activities of enhancers are largely restricted to specific cell types or tissues, while the majority of promoters or insulator binding sites are broadly utilized. We also find a significant number of alternative promoters utilized by genes in different tissue types. By correlating the chromatin state at enhancers with promoter usage, we are able to assign target genes for a significant fraction of enhancers. Bioinformatic analysis further identifies potential transcription factors that control tissue specific gene expression.

The lab also participates the modENCODE consortium, and has generated chromatin modification maps and RNA polymerase

II binding sites throughout the Drosophila genome in ten development stages. The resource has led to the annotation of promoters, enhancers and insulator elements in the fly genome, significantly expanding our understanding of the regulatory logic in this model organism.

In collaboration with Len Pennacchio's lab, we have also identified tissue specific enhancers involved in regulating gene expression in embryonic heart tissues in the mouse. In contrast to enhancers that direct forebrain or midbrain specific expression, the heart enhancers identified are generally less conserved in sequence, suggesting rapid evolutionary divergence of regulatory programs controlling heart specific gene expression in the mammals.

Epigenetic mechanisms regulating pluripotency, lineage commitment and tumorigenesis

As part of the NIH Roadmap Epigenome project, we have generated comprehensive epigenome maps for the human embryonic stem cells (ESC) and fibroblasts. Analysis of these epigenomic profiles revealed dramatic differences of chromatin landscapes between the pluripotent and lineage-committed cell types. For example, there is significant expansion of H3K27me3 and H3K9me3 chromatin domains in the differentiated cells, which tend to affect genes coding for developmental regulators and lineage-specific functions. It suggests that formation of such large, special chromatin domains is a critical step in cellular differentiation.

Working together with Drs. Andy Simpson, Bob Strausberg, Otavia Caballero, Anamaria Aranha Camargo and other Ludwig investigators, the lab has begun to characterize the genomic and epigenomic changes in breast cancer cells, to determine whether genetic and epigenetic factors play a role in tumorigenesis. The first phase of this Institute-wide collaboration has led to the identification of loss of heterozygosity (LOH) in many potential tumor suppressor genes in breast cancer cells. More recently, we have been able to obtain base resolution DNA methylomes, transcriptomes, as well as several chromatin modifications for a breast cancer cell line (HCC1954) and the primary human mammary epithelial cells. Preliminary analysis has revealed large scale, widespread DNA hypomethylation in the cancer cell genome that is closely associated with transcriptional silencing, in particular many known tumor suppressor genes. This result is surprising since DNA methylation has largely been considered a mechanism to repress gene expression. We demonstrate here that loss of DNA methylation was tightly coupled to gain of repressive chromatin marks, such as H3K27me3 or H3K9me3.

In summary, we have made significant progress towards understanding the *cis*-regulatory elements in the mammalian genome and the Drosophila genome. The information produced has provided novel insights into processes regulating tissue-specific gene expression. Additionally, we have gained considerable knowledge about the epigenomic landscapes in pluripotent and lineage-committed cell types, finding evidence for the involvement of several key epigenetic processes during normal development and tumorigenesis.

MITOTIC MECHANISMS

Karen Oegema, Ph.D.

The laboratory uses the nematode *C. elegans* as a model system to study cell division. Research in the lab is currently focused in three major areas: (1) the molecular mechanics of cytokinesis; (2) centriole duplication and function; and (3) the functional genomics of cell division.

Highlights in two of these areas are summarized below.

The role of the centrosomal asters during cytokinesis

Cytokinesis, which partitions the mother cell into the two daughter cells, is accomplished by constriction of a contractile ring that forms around the cell middle. To ensure that each daughter cell obtains a single genomic copy, cytokinesis is tightly coupled to chromosome segregation. After segregating the chromosomes, the anaphase spindle signals to the cortex to position the cleavage furrow so that it bisects the spindle midzone, a set of microtubule bundles that forms between the separating chromosomes. Furrow formation is coordinately directed by the centrosomal asters and spindle midzone. In work published in 2010, we explored the contribution of the asters by analyzing the consequences of altering interaster distance during the first cytokinesis of the *C. elegans* embryo. Delaying aster separation led to a corresponding delay in furrow formation, but yielded a single furrow that ingressed at a normal rate. By contrast, disrupting midzone-based signaling results in a furrow that formed with normal timing, but which ingressed at a reduced rate. Simultaneously delaying aster separation and disrupting midzone-based signaling led to failure of furrow formation. We conclude that signaling by the separated asters: 1) couples furrow formation to anaphase onset by concentrating contractile ring proteins on the equatorial cortex in a midzone-independent manner; and 2) refines spindle midzone-based signaling to restrict furrowing to a single site.

Generating a functional gene network for the C. elegans essential gene set

A current challenge is to translate the parts lists generated by genome sequencing into functional maps of the pathways that execute cellular processes. Our goal has been to do this for the *C. elegans* essential gene set. *C. elegans* has 20,000 genes of which 900 are essential for embryo production or events during the first two embryonic cell divisions. These genes represent the set of genes essential for basic cellular processes common to all cells. Five hundred of these genes were previously profiled in a high-content screen that used timelapse Differential Interference Contrast (DIC) microscopy to film the first two divisions of embryos depleted of individual gene products. A major complication of this prior screen was the onset of sterility following RNAi, which resulted in an inability to assay function for a large number of genes. To fill the gap caused by the sterile phenotype, we profiled the 554 sterile genes using a high-content assay based on high-resolution imaging of the syncytial *C. elegans* gonad— a complex tissue whose architecture depends on a broad spectrum of interacting cellular processes. Phenotypes were parameterized by scoring for 94 possible defects. To generate a computational network, we collaborated with Kris Gunsalus and Fabio Piano at New York University. A centerpiece of this work was the development of a quantitative measure, which we call the Connection Specificity Index that compares phenotypic profiles and considers network context to assign a value between 0 and 1 to each connection. Values close to 1 indicate that the A and B phenotypes are similar and shared by only a small number of genes. Values close to 0 indicate that the phenotypes are dissimilar or are shared by many genes. The framework we developed allowed us to combine our analysis of the sterile collection with the data from the prior timelapse embryo-filming screen to generate an integrated network of 819 essential *C. elegans* genes that can be viewed at multiple levels of functional resolution. The analysis demonstrates that multi-parametric phenotypic profiling in a complex tissue in a multi-cellular organism can yield functional maps with a resolution equivalent to genetic interaction-based profiling in simple model eukaryotes. The manuscript describing these findings, which will be accompanied by access to a comprehensive phenotypic database and an online interactive version of the gene network, is currently in press at *Cell*.

PROTEOMIC BIOLOGY

Huilin Zhou, Ph.D.

The laboratory is focused on two areas of investigation: 1) the regulation of DNA damage checkpoint activation; and 2) the identification and characterization of pathways controlled by the DNA damage checkpoint to suppress genome instabilities.

Mutations to many DNA damage checkpoint genes cause elevated genome instabilities frequently observed in cancer cells. In particular, genetic studies in yeast have demonstrated that Mec1 and Tel1, the orthologs of mammalian ATR and ATM, respectively, have a key role in suppressing genome instabilities. The

question is how the Mec1/Tel1 family of protein kinase suppresses genome instabilities. To address this question, most Mec1 and Tel1 substrates in yeast have been identified. These substrates function in several nuclear processes, including DNA replication and repair, telomere maintenance, chromosome segregation, chromatin remodeling, RNA transcription and processing, and the DNA damage checkpoint itself. Given the complexity of the processes that Mec1 and Tel1 substrates are involved in, we aim to develop a mechanistic understanding of how Mec1 and Tel1 regulate genome maintenance by pursuing the following investigations.

Mechanisms of DNA damage checkpoint regulation.

A major function of Mec1 and Tel1 is to activate a downstream effector kinase Rad53 for cell cycle arrest in response to DNA damage. Understanding how Rad53 is activated by Mec1 and Tel1 should provide insights into how Mec1 and Tel1 function. To study the mechanism of Rad53 activation, a biochemical system to measure the activation of Rad53 was developed. Rad9 and Mrc1 are two key adaptor proteins that are required and sufficient for Rad53 activation *in vivo*. We previously reconstituted Mrc1 dependent Rad53 activation. Recently, we reconstituted Rad9 dependent Rad53 activation by Mec1 and Tel1 and found that Rad9 promotes Rad53 via an oligomerization-based mechanism, which is distinct from Mrc1. In parallel, we used an *in vivo* approach to study how Rad9 controls Rad53 activation during the cell cycle and found that multiple CDK phosphorylation of Rad9 acts redundantly and is required for Rad53 activation. Moreover, the BRCT domain of Rad9, a phosphoprotein-binding domain found in many DNA repair proteins including BRCA1, has an essential role in Rad53 activation. These findings have provided insights into the role of the BRCT domain in the DNA damage checkpoint activation and further in mediating the functions of Mec1 and Tel1.

The roles of Mec1 and Tel1 in telomere maintenance.

We are interested in understanding how Mec1 and Tel1 suppress genome instabilities. Of particular interest is their regulation of telomeres for the following reasons. First, genetic studies have shown that mutation to *TEL1* causes shorter but stable telomeres, while mutation to *MEC1* has little effect on telomere length. Remarkably, mutation to both kinases causes severe telomere erosions. Because telomere dysfunction can cause chromosomal fusion, breakage and rearrangements, the regulation of telomeres is a major function of Mec1 and Tel1 in genome maintenance. Second, a number of Mec1 and Tel1 substrates implicated in telomere maintenance have been identified from our proteomic screens. We have characterized their genetic interactions with either *MEC1* or *TEL1*. Third, we have determined the substrate specificity of Mec1 and Tel1 and found they redundantly phosphorylate most of their substrates.

To understand how Mec1 and Tel1 regulate telomeres, we are pursuing several directions. First, using mass spectrometry the phosphorylation of each Mec1/Tel1 substrate implicated in DNA double stranded break repair and/or telomere maintenance has been characterized. Second, we generated phosphorylation-defective mutations to each of the genes encoding these substrates and are examining their phenotypes in telomere length control either by itself or in combination. Third, we are using quantitative mass spectrometry to identify the associated proteins of the BRCT domains in the yeast proteome in an effort to understand how signals from Mec1 and Tel1 are used to control their substrates. Together, these studies should provide important insights into how Mec1 and Tel1 regulate telomeres and ultimately genome stability.

SMALL MOLECULE DISCOVERY

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

The drug discovery industry is presently facing extraordinary challenges, including the inherent difficulty and mounting expense of the research and development process, increasingly stringent FDA regulation, as well as the recent economic downturn. To help the Institute address the progressively greater need for innovative new medicines, the laboratory was formed to develop and execute an Institute-wide, systematic, 'virtual' small molecule drug discovery process. By complementing the efforts of Institute basic and clinical scientists with outsourced research capabilities, the laboratory seeks to efficiently identify small molecules that target proteins found by Institute investigators to be important in cancer. This past year, the group has built an automated screening laboratory and developed the internal infrastructure/external relationships required to perform multiple aspects of small molecule drug discovery, including high-throughput biochemical and cell-based screening, structure-based drug design, medicinal chemistry, and *in vitro*/*in vivo* pharmacokinetics. These resources, which are available to all Institute investigators and collaborators, have enabled the laboratory to make substantial progress towards the generation of potent and selective inhibitors of several different kinases, including polo-like kinase 4 (PLK4) and maternal embryonic leucine zipper kinase (MELK).

Polo-like kinase 4

Aneuploidy or an aberrant chromosome number is a common feature of cells from human cancers. Although some cancer cells may be stably aneuploid, many exhibit some degree of chromosomal instability (CIN), an accelerated rate of chromosomal gain/loss during cell division. Recent studies have demonstrated that the supernumerary centrosomes frequently observed in cancer cells can trigger CIN. Experiments in worms, flies and mammals have indicated that polo-like kinase 4 (PLK4), the most divergent member of the polo family of serine/threonine kinases, is a conserved, essential driver of the biogenesis of centrioles (which recruit pericentriolar material to form mature centrosomes). Using high-throughput biochemical and cellular assays and molecular modeling, the laboratory identified compounds with nanomolar affinity for PLK4 and >100-fold selectivity relative to several structurally-related kinases in biochemical assays. These inhibitors block centriole duplication in cells and significantly compromise the growth of multiple tumor cell lines *in vitro*. The team will be testing these small molecules, which exhibit good *in vitro* pharmacokinetic properties, in xenograft proof-of-concept studies in the near future.

Maternal Embryonic Leucine Zipper Kinase

Under normal circumstances, the expression of maternal embryonic leucine zipper kinase (MELK), a member of the AMP-activated kinase (AMPK) subfamily of serine/threonine protein kinases, is largely restricted to proliferating progenitor cells. Intriguingly, MELK is also expressed at high levels in aggressive brain cancers (such as glioblastoma multiforme) as well as colon, breast, ovary and lung tumors. Suppression of MELK expression inhibits the proliferation of human glioblastoma and medulloblastoma cells, in part due to enhanced apoptosis. Hence, inhibition of MELK may be a novel approach for the treatment of brain and other cancers, potentially through the modulation of tumor progenitor cells. In the past year, the laboratory has screened multiple small molecules against MELK and has identified two distinct scaffolds, both of which significantly inhibit MELK activity in biochemical assays. The group has also discovered a rat neural progenitor cell line in which MELK expression is regulated by differentiation. These tools will used to perform chemical biology experiments to dissect MELK function.

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DIRECTOR'S MESSAGE

The Branch is now in its fifth year at the Hospital Alemão Oswaldo Cruz and we are continuing to make important discoveries in HPV infections, neurobiology and cancer genomics. Branch members played important roles in scientific societies, journal editorials and scientific committees and were very successful in obtaining external grant support. Collaborative interactions among our research groups, Ludwig Centers, other Ludwig Branches and international investigators have increased. Many investigators and postdoctoral fellows trained at the Institute have moved on to independent positions in academia.

In 2016 the Ludwig royalty stream, which largely funds the research of the Branch, will end and the Branch will cease to function as an independent research unit. The process of transitioning to this new operating model is already underway and in the near future all research groups will relocate to different host institutions. The Molecular and Cellular Biology Group headed by Dr. Vilma Martins was the first to relocate to the International

Research and Teaching Center at the Hospital A.C. Camargo in November. The research groups will continue to receive Institute support during this transition but will eventually seek local funding to underwrite their research activities. We are optimistic about the future and grateful to the Institute for its many years of support.

~ Anamaria Camargo

VIROLOGY

Luisa L. Villa, Ph.D.

The Virology Group focuses on several areas:

Natural History of HPV Infection in Men: The HIM Study

We are conducting a large international (US, Mexico, Brazil) prospective study of men, the HPV Infection in Men Study (HIM), following approximately 4,000 men aged 18 – 44 years and 450 men aged 45 – 70 years every six months for four years. By collecting samples from the genitals, anal canal, oral and skin samples, we are contributing novel data about HPV infections in these different anatomical sites. Recruitment of eligible men was concluded in September 2009, reaching a total of 4292 men (1443 – Brazil; 1426 – United States; 1423 – Mexico). Retention rates for the past two years are above 85%. HPV prevalence in different anatomical sites and risk factors for infections have been published. Recently, incidence and clearance of genital HPV in this cohort were published in the *Lancet*. Analyses of relevant information on the natural history of HPV infections and risk of oral, genital, anal disease in males are underway.

HPV and tumor microenvironment

The behavior of the tumor is dictated by the interplay between the different cell types that compose the tumor mass and their interactions with tumor stroma. Tumors are infiltrated by cells from the immune system that can exhibit both anti- and pro-tumoral activities depending on the cell types involved (CTL vs. Tregs and/or M2 macrophages) and the pattern of cytokines produced (IFNg vs. IL-10 and TGFβ). Understanding the molecular events underlying the recruitment of the different immune cells could be important to the development of anti-tumor therapies. We are currently investigating the phenotype of myeloid recruited to the tumor area and the migration of myeloid cells from tumor to peripheral lymphoid organs in the HPV-TC1 tumor model. These are also ongoing studies, where we intend to reveal how E6/E7 expressing cells control gene expression and the phenotype of myeloid cells. Furthermore, we are analyzing the expression of cytokines in the spleen of mice bearing tumors or not in order to identify possible systemic effects of the tumor. We are also studying the effect of HPV16 oncoproteins in the regulation of apoptosis mediated by Tumor Necrosis Factor Related Apoptosis Inducing Ligand (TRAIL). Furthermore, we are analyzing the effect of these proteins in keratinocytes expression of different Toll-like receptors (TLRs) and their activation by different ligands. Another recently published 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 we observed that 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.

Molecular markers of HPV-related disease

We have previously shown that 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. We observed a significant trend between hK7 positivity

and severity of cervical disease suggesting that hK7 might be a useful biomarker for high-grade cervical dysplasia and cancer. Similar analyses have been performed using other biomarker candidates, with promising data for superoxide dismutase-2 (SOD-2) expression in invasive cervical disease. These observations have recently been extended to a large series of penile carcinoma with preliminary indications of the association of higher expression of SOD-2 and lymph node metastasis. Further analyses shall indicate the value of this marker in the prognosis of cervical and penile neoplasias.

MOLECULAR AND CELLULAR BIOLOGY

Vilma Martins, Ph.D.

The laboratory is focused on deciphering the physiological functions of the cellular prion protein (PrP^C) and in particular its association with the extracellular matrix proteins vitronectin (VN) and laminin and the co-chaperone stress inducible protein 1 (STI1). The group previously demonstrated that PrP^C modulates neuronal survival and plasticity as well as memory formation. In addition, recent data suggest the participation of PrP^C in tumoral processes. It is our main objective to understand how the interaction between these proteins can be explored as therapeutic targets in neurodegenerative diseases such as prion diseases and Alzheimer´s Disease as well as in tumors.

PrP^C is a glycosylphosphatidylinositol (GPI)-anchored protein, which suggests that the transduction of PrP^C-mediated extracellular signals requires interaction with integral transmembrane proteins. Indeed, the identification of PrP^C interacting proteins is important for mapping PrP^C-signaling components. Experiments were conducted to address the PrP^C-LN interaction using a gamma1 chain LN peptide (gamma1 peptide) that mimics the PrP^C binding site on LN. The PrP^C-gamma1 peptide complex activates phospholipase C, protein kinase C, and ERK1/2, and promotes Ca^2+ mobilization from intracellular stores. Phage display, coimmunoprecipitation and colocalization experiments have all shown that the group I metabotropic glutamate receptors, mGluR1 and mGluR5, associate with PrP^C and transduce signaling responsible for neuronal plasticity.

A similar approach was taken to identify the transmembrane receptor that is activated upon formation of the PrP^C-STI1 complex. In hippocampal neurons, PrP^C-STI1 engagement induces Ca^2+ influx and increases the concentration of intracellular Ca^2+. A series of Ca^2+ channel inhibitors were used to identify candidates that are involved in PrP^C-STI1-induced Ca^2+ influx. A specific inhibitor of the alpha7 nicotinic acetylcholine receptor (a7nAChR), alpha-bungarotoxin, blocked PrP^C-STI1-mediated signaling, neuroprotection and neuritogenesis. Importantly, when a7nAChR was transfected into HEK293 cells, it formed a functional complex with PrP^C and restored PrP^C-STI1-mediated signaling. Together, these results led us to suggest that PrP^C may function as a cell surface platform for the assembly of signaling modules promoting the coupling of specific transmembrane receptors for mediating neurotrophic properties. They also identify novel targets for modulating the effects of PrP^C in neurodegenerative diseases.

Neuronal survival, plasticity, and memory formation are closely correlated with control of protein synthesis. The experiments performed showed that STI1 increased PrP^C-dependent neuronal protein synthesis through phosphoinositide-3 kinase, the

mammalian target of rapamycin and the ERK1/2 pathways. This process is essential for the neurotrophic activities of PrP^{C}. The STI1-induced increase in protein synthesis was also observed in synaptosomes, which indicates that STI1 can enhance translation of preformed mRNA, and further suggests that STI1 may affect local protein translation at synapses. Strikingly, the translational stimulation mediated by PrP^{C}-STI1 binding was disrupted in neuronal cell lines that were infected with the converted PrP^{C} infectious isoform (PrP^{sc}). These data indicate that modulation of protein synthesis is critical for PrP^{C}-STI1-mediated neurotrophic functions and suggest that the impairment of this process during PrP^{sc} infection may participate in neurodegeneration.

Our screening and reporting of prion diseases in Brazil has continued over the past year with the support of the Brazilian Ministry of Health, which recognizes our group as a reference center for the genetic analysis of prion diseases. From September 2009 to August 2010, 39 cases of suspected Creutzfeldt-Jakob disease (CJD) were reported, which represents a similar number of cases reported from 2008-2009. The number of reported cases increased every year from 2005 to 2009. However, the cases reported in the last 12 months were still substantially less than the expected 180 cases per year, the expected incidence rate of prion disease (1 in 1,000,000) based on the estimated population of Brazil and data in other parts of the world. Therefore, the group is working with the Ministry of Health to increase the reporting of suspected cases of CJD.

MOLECULAR BIOLOGY AND GENOMICS

Anamaria Camargo, Ph.D.

The Molecular Biology and Genomics Group has two complementary programs: Clinical Genomics and Functional Genomics.

The Clinical Genomics program is focused on colon and breast cancer and benefits from a strong collaboration with a renowned group of surgeons led by Dr. Angelita Gama from the Hospital Alemão Oswaldo Cruz and with researchers from the Brazilian Network for Cancer Research, respectively, as well as from the availability of a next-generation sequencing platform available at the Branch.

In 2010, as part of the Colon Cancer Initiative, we were able to define a gene expression signature to predict response to neoadjuvant chemoradiation in rectal cancer patients and to identify cell surface proteins mutated during colon cancer progression. We are now focusing on the role of neoadjuvant therapy in selecting resistant tumor cell subpopulations and in characterizing genetic alterations related to treatment failure. In 2011, we are planning to sequence the coding exons (exome) of rectal tumor samples before and after neoadjuvant chemoradiation and to look for genetic alterations enriched in post-treatment samples. In a related work, done in collaboration with Dr. John Mariadason from the Melbourne-Austin Branch, we will sequence the exome of a panel of 12 colon cancer cell lines with distinct susceptibility patterns to drugs used for colon cancer treatment in 2011.

As part of the Breast Cancer Initiative, we sequenced the genome of a breast tumor cell line and of a lymphoblastoid cell line derived from the same patient. This project was carried out in collaboration with research groups in Brazil (LICR São Paulo Branch, National Laboratory for Computational Science and University of São Paulo) and the United States (LICR San Diego and

New York Branches and the J. Craig Venter Institute). By comparing the set of mutated genes found in the tumor and lymphoblastoid cell lines, we provided important insights into the normal mutational processes and into the functional implications of the accumulation of somatic mutations in tumor genomes. In 2010, we also started a collaborative project with a clinical group from the Perola Byington Hospital in São Paulo and Washington University to characterize genetic alterations related to treatment failure in patients with advanced breast cancer submitted to neoadjuvant therapy with estrogen blockers.

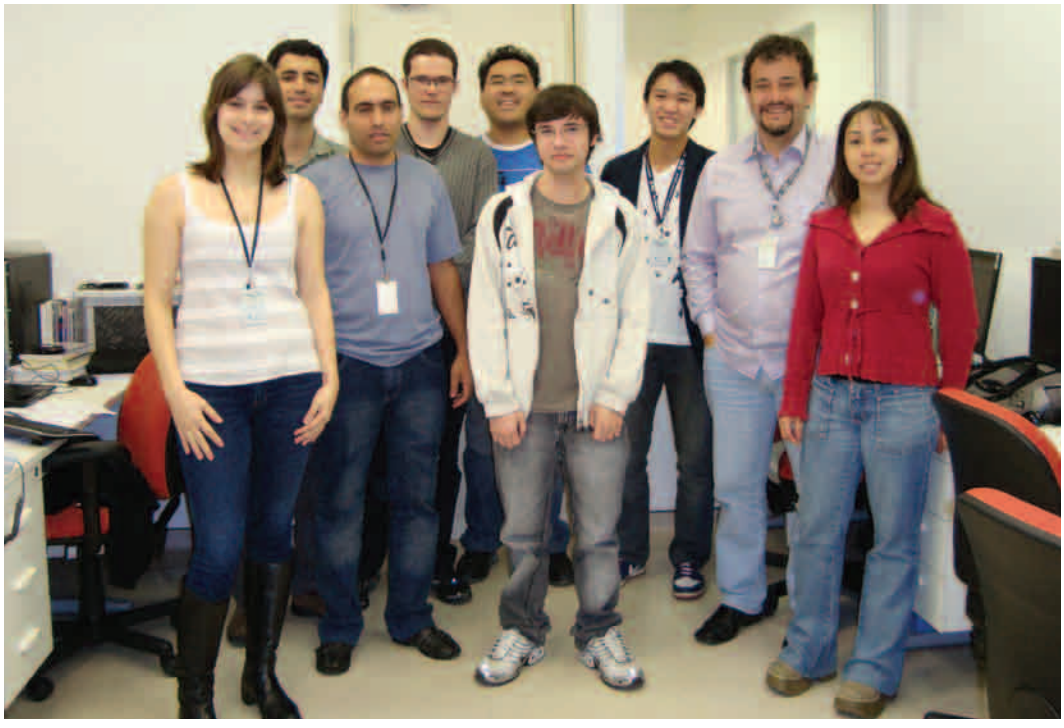
The functional genomics program continues to explore the role of two genes (ADAM23 and SIGIRR) differentially expressed in breast tumors. The ADAM23 gene is epigenetically silenced in breast tumors and promoter hypermethylation has been shown to be strongly associated with poor disease outcome. Ablation of ADAM23 gene using RNAi resulted in enhanced invasion, migration and adhesion modulated by αvβ3-integrin activation. In 2010, we addressed the biological relevance of tumor heterogeneity in relation to ADAM23 gene expression. Using both *in vitro* and *in vivo* assays we were able to demonstrate that ADAM23 positive and negative cells cooperate to enhance metastatic spread and colonization.

SIGIRR was identified as an upregulated gene in c-erbB2 positive tumors. This gene belongs to the IL-1R superfamily and acts as a decoy receptor that negatively modulates IL-1R and TLR4 signaling. We demonstrated that SIGIRR downregulates the secretion of pro-inflammatory cytokines such as IL-8, TNFα and CXCL2 and lymphocyte recruitment indicating an important role in immune suppression and evasion. Interestingly, we found that ablation of SIGIRR using RNAi promotes significant morphological changes in breast tumor cell lines which are accompanied by loss of E-cadherin expression and upregulation of transcription factors involved in EMT. The unexpected role of SIGIRR in EMT will be further explored in 2011.

COMPUTATIONAL BIOLOGY

Sandro José de Souza, Ph.D.

The group continued to use computational resources to explore different aspects of cancer genomics. A key component of these efforts is a network of collaborators within and outside the LICR structure. In 2010 we continued a very fruitful



interaction with Dr. Lloyd Old and his group at the New York Branch and started a new collaboration with Dr. Richard Kolodner at the San Diego Branch.

Cancer Genomics continues to be our major focus. Together with the New York Branch, we are exploring the human cell surfaceome, a collection of genes coding for cell surface proteins. A database containing integrated information about the human surfaceome was developed and used to explore next-generation sequencing data. The group participated in a multi-centric effort to sequence the genome of a cancer cell line (HCC1954) and a normal lymphoblastoid cell line (HCC1954-BL) derived from the same patient. Together with other groups in Brazil and the US, the group was responsible for all major bioinformatics analyses for this project. A clear distinction between the two genomes is the number of chromosomal rearrangements (very high in the tumor genomes and almost absent in the normal genome). Interestingly, the number of somatic point mutations was almost similar in both genomes. Using a systems biology-based approach, we obtained a series of data that allowed us to suggest that somatic mutations in the normal cell are random while somatic mutations in the tumor cell act synergistically to promote tumorigenesis.

Significant efforts were also directed to an ongoing collaboration between the Computational Biology group, the Molecular Biology and Genomics group and the group of Dr. Angelita Gama from our host institution, the Hospital Alemão Oswaldo Cruz. Together with these two other groups, we continue to manage a colorectal tumor bank that today contains thousands of samples and clinical information from more than a thousand patients. Within this initiative, we have deep sequenced and analyzed the transcriptome of dozens of rectal tumor patients searching for an expression signature that would predict response to radio-chemotherapy. Furthermore, genome sequences obtained from primary colon tumors and the corresponding liver metastasis are being compared to better understand tumor evolution in this type of cancer. Finally, the complexity of the human transcriptome continued to attract our attention, especially regarding alternative splicing.

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DIRECTOR'S MESSAGE

The most important change in 2010 was recruitment of new groups to the Branch. Rickard Sandberg joined as an Assistant Member in mid-2010. He had already established a laboratory at the department of Cell and Molecular Biology at the Karolinska Institutet after a very successful postdoctoral period at MIT in Boston. Rickard is combining genome-wide experimental and computational techniques in his research that aims at understanding general principles of mammalian gene expression programs. A particularly important focus is to elucidate gene regulatory principles in early pluripotent embryonic cells. Johan Holmberg was recruited as a project leader. He is employed as a group leader at the Karolinska Institutet and during the year became affiliated with the Branch where his lab is focusing on how pluripotency factors contribute to glioblastomas. His group is also interested in the important question of how mature cells of the brain maintain their differentiated identities.

Together these two additional groups will greatly strengthen our research programs. The new and existing groups have both unique and common interests and I am very excited about the potential synergistic interactions that are already beginning to emerge. I am also pleased with how groups are continuing to attract external funding. Susanne Schlisio received funding from several sources including the Swedish Cancer Fund and the Swedish Children's Cancer Fund. Both Jan Stenman and Johan Holmberg attracted significant grants and Rickard Sandberg received a grant awarded to promising young investigators from the European Research Council (ERC Starting Grant). The number of postdocs and students has increased due to additional external funding and we anticipate this will further strengthen our research programs in the coming year. We are very enthusiastic and excited about our future progress as the relatively new and young groups continue to build and develop their research programs.

~ Thomas Perlmann

GENE EXPRESSION

Thomas Perlmann, Ph.D.

The laboratory focuses on understanding the development of specific types of neurons. Under the cell fate decision process a regulatory network must be sufficiently flexible to allow cell type specification. Later in development such a regulatory network becomes increasingly robust to support the maintenance of the terminally differentiated state, which under physiological conditions is very stable. We aim at understanding 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; and how pathological changes of a network can lead to cellular dysfunction and cancer.

In regenerative medicine a strong focus is directed at the development and understanding of pluripotency of both embryonic stem (ES) cells and iPS cells that can be made from adult somatic cells. There is hope that these pluripotent stem cell lines can be used both in cell therapy and as tools to model human disease in cell culture. However, these advancements will depend on methods that allow therapeutically interesting cell types to be generated from stem cells. We have previously identified a key transcription factor (Lmx1a) that is critical for the generation of dopamine (DA) neurons, cells that degenerate in patients with Parkinson's disease. We also showed that Lmx1a, after expression in ES cell-derived neural progenitors, could lead to a remarkably robust method to generate transplantable DA neurons from stem cells. During 2010 we extended these studies and showed that additional cell types, including serotonergic-, visceral motor- and somatic motor neurons can be generated by a similar strategy. Due to the efficiency of this method, we were able to make highly enriched cultures of different types of neurons amenable to global transcriptome analysis that allowed us to map differential gene expression in a way that has not been possible to achieve by any other method. The experiments showing how transcription factors can induce distinct cell fates also provided general principles for how highly efficient generation of specific neuron types can be generated from stem cells. These studies will be published in 2011.

Recent advances in induced cellular reprogramming emphasize that the terminally differentiated state is not irreversible. Nonetheless, the phenotype of differentiated cells is very stable. Thus, an important but as yet largely unresolved problem is to understand how the differentiated state is stably maintained. The lab is focusing on this problem by studying the role of the transcription factors that continue to be expressed in differentiating and mature DA neurons. We are using both gain- and loss-of-function experiments in neurons cultured *in vitro* and in conditional knockout studies in mice. The results give important clues of how differentiated cells are maintained and we anticipate that these findings will have important implications for disorders such as Parkinson's disease and cancer.

For several years we have studied a group of orphan nuclear receptors (belonging to the NR4A family). One of these receptors (Nurr1) has been studied because of its critical role in developing DA neurons. However, very little is known of how NR4A proteins function in other cell types in which they are expressed. Recently it was shown that NR4A receptors play important roles in myeloid stem cells and loss of NR4A function is linked to the appearance of myeloid cancers. The loss of NR4A proteins is also associated with increased sensitivity to DNA damage. In a series of experiments we have elucidated a new and unexpected role of NR4A receptors in

DNA double strand break repair. We found that NR4A proteins become localized to DNA repair foci and participate directly in the repair process by a mechanism that is dependent on NR4A phosphorylation by DNA-dependent protein kinase but entirely uncoupled to their functions as transcription factors. The results have implications for understanding the basic pathway for DNA double strand break repair and also suggest one possible activity that may influence how NR4A proteins are linked to cancer.

STEM CELL BIOLOGY

Jonas Muhr, Ph.D.

The goal of the laboratory is to unveil general principles that underlie the regulation of stem and progenitor cells in the central nervous system. Critical goals include the characterization of how neural stem cells (NSCs) are maintained in an undifferentiated/proliferative state and how they are committed to differentiation and generate post-mitotic neurons and glial cells. A second goal is to use this knowledge to unveil molecular similarities and differences in the regulation of NSCs and stem cells contributing to the establishment and growth of glioma.

To address these questions, the group concentrated on High-Mobility-Group (HMG) proteins of the Sox transcription factors family. This is a class of architectural non-histone proteins involved in gene regulation, maintenance of chromatin structure and have vital regulatory roles in various stem cell populations. Focusing on NSCs, the group has previously shown that while one group of Sox proteins (including Sox1, Sox2 and Sox3) maintain neural cells in a self-renewing progenitor state, another set of Sox proteins (including Sox5, Sox6 and Sox21) have the opposite function and promote NSCs to differentiate into post-mitotic progeny. A third group of Sox factors (including Sox4, Sox11 and Sox12) are expressed in differentiating neurons and both are required and sufficient for the induction of neuronal protein expression. The diverse activities achieved by these different Sox proteins have raised the question about their target gene selection. Last year the laboratory collaborated with Rickard Sandberg and his co-workers and performed chromatin immune precipitation combined with deep sequencing analysis (ChIP-seq) to identify and compare genome-wide targets of Sox proteins acting at different stages of neural lineage differentiation. These analyses show that Sox proteins, acting at different stages of neurogenesis, bind and activate genes in a stage-specific manner. However, the data reveal that an important function of Sox proteins is also to pre-bind genes that later are induced by sequentially acting Sox factors. Thus, the orderly fashion by which different gene programs are expressed during neural lineage progression is coordinated by sequentially acting Sox transcription factors.

Our finding that Sox5, Sox6 and Sox21 can promote NSCs to exit the cell cycle and promote differentiation may suggest a possible role in preventing tumor formation in the brain. The group set up a model system in mice allowing Sox5, Sox6 and Sox21 to be ablated in stem cells of the adult brain to examine if these genes possess tumor suppressor activities. Gene ablation is combined with lenti virus-mediated expression of H-Ras and AKT oncogenes. Since H-Ras and AKT induce tumors only inefficiently, loss of a tumor suppressor is also required in this mouse glioblastoma model. Preliminary results indicate that the loss of Sox21 in the presence of H-Ras and AKT expression leads to a dramatic phenotype with

glioma formation in the majority of examined mice. This phenotype is even more prevalent under conditions when Sox5, Sox6 and Sox21 are simultaneously removed. The finding that Sox5, Sox6 and Sox21 can prevent tumor formation is notable as it is one of the first examples of how lineage specific transcription factors can prevent NSCs from uncontrolled cell growth associated with cancer.

DEVELOPMENT AND DISEASE

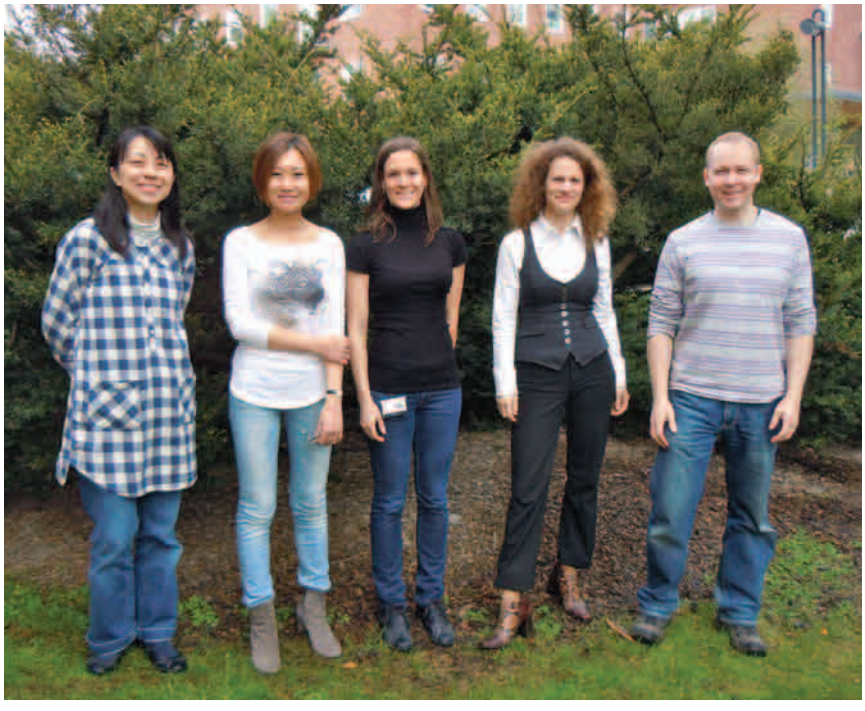
Jan Stenman, Ph.D.

The group was established in the last quarter of 2008. The overall goal is to understand the molecular regulation and biological functions of Wnt signaling in development and disease. The Wnt family of proto-oncogenes is secreted glycoproteins essential for normal development of organisms ranging from the fly to humans. For example, it was previously reported in *Science* that Wnt signaling regulates both the formation of blood vessels in the central nervous system (CNS) and the early differentiation of the blood-brain barrier (BBB). In adult animals and humans, alterations of Wnt signaling are critical to many diseases, such as malignances. In an effort to understand how active Wnt proteins are produced and secreted; how Wnt protein and pathway activity is regulated; and the function of Wnt proteins in specific biological contexts, particularly in the CNS vasculature the group is:

- performing large-scale screens to identify novel regulators of Wnt protein and pathway activity using both cell based assays and the Xenopus model system; and
- studying the role(s) of canonical Wnt signal transduction in the central nervous system vasculature during development and disease.

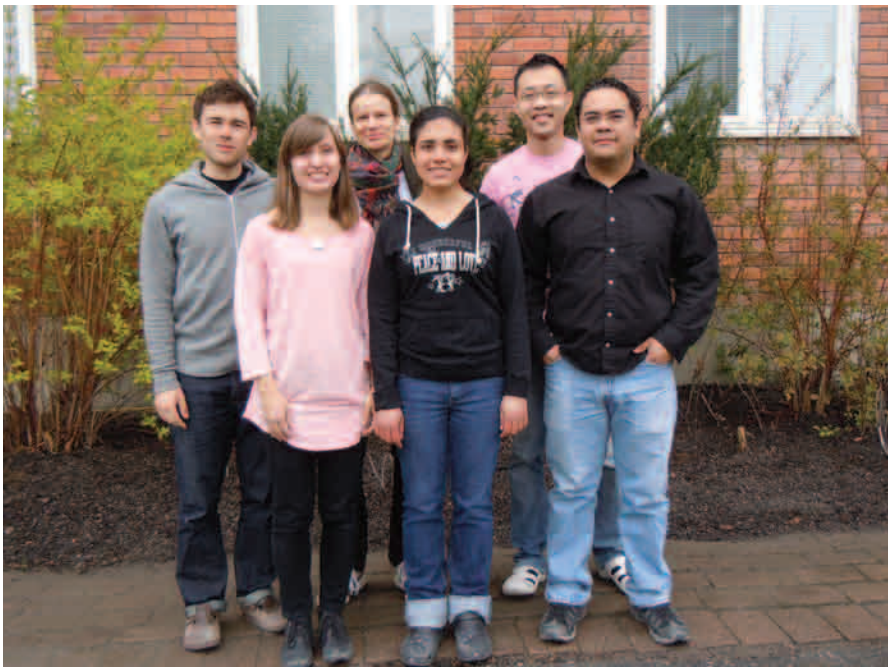
In order to develop specific approaches to prevent and cure diseases caused by aberrant Wnt pathway activity, a more complete understanding of this signaling pathway is necessary. An important step towards realizing this goal is to identify and characterize novel regulators of Wnt protein and pathway activity. During the past year, the group initiated a number of screening projects. Using the powerful Xenopus model system, which during the past decades has been extensively used to unravel general mechanisms of Wnt signaling, the group performed a large-scale screen for regulators of Wnt signaling pathway activity. Furthermore, as Wnt protein maturation and secretion are two processes that are particularly poorly understood, the group developed and validated two screens to preferentially identify regulators of these important processes.

The CNS vasculature is quite distinct from the more porous vasculatures permeating other organs. 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 BBB differentiation *in vivo*. As the cellular and molecular mechanisms by which Wnt signaling regulates these processes remain unclear, the group is attempting to address these questions using several *in vitro* and *in vivo* approaches. For example, the group aims to identify the downstream targets of *Wnt7a/b* and determine the role(s) these play in CNS vascular development. During the past year, the group characterized a new mouse model, and continued the development of new mouse models to allow for more advanced *in vivo* direct Wnt target screens.



In brain tumors, the BBB is disrupted resulting in vasogenic brain oedema, which is a major cause of morbidity. 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 have observed that beta-catenin accumulates in the vasculature associated with certain brain tumors. Coupled with earlier work, this suggests a role for canonical Wnt signaling in neovascularization. The group will explore whether neovascularization or normalization of the dysfunctional tumor vasculature can be prevented by modulating canonical Wnt activity in the tumor endothelium.

During the past year, the group successfully established a mouse glioma tumor model. This model uses transposase-mediated integration of transposon expression constructs into the genome resulting in ectopic expression of oncogenes and allows the group to generate brain tumors on various genetic backgrounds and study the functional consequences of manipulating Wnt signaling in the brain tumor vasculature. The group is working on carefully characterizing the tumor model and is able to verify Wnt activity in brain tumor endothelial cells by generating brain tumors in a Wnt reporter mouse strain.



OXYGEN SENSING AND CANCER

Susanne Schlisio, Ph.D.

The research focus of the laboratory concerns the mechanisms by which disruption of oxygen-sensing pathways can lead to cancer. Oxygen-sensing is mediated partly via prolyl hydroxylases that require molecular oxygen for enzymatic activity. The work specifically focuses on how prolyl hydroxylases execute apoptosis in neural precursors during development and how disruption of this process can lead to certain forms of nervous system tumors. Accordingly, research in the lab is focused in three major areas:

(1) direct impact of cancer metabolism in EgIN hydroxylase functioning;
(2) molecular mechanism of how prolyl hydroxylase EgIN3 executes apoptosis in neural precursors during development; and
(3) how failure of developmental apoptosis mediated by EgIN3 predisposes to certain forms of nervous system tumors.

A long-term goal is to understand how alteration in metabolism can contribute to the pathogenesis of cancer. EgIN hydroxylase activity is dependent upon metabolites (2-oxoglutarate) that take part in the Krebs cycle. Inactivation of the Krebs cycle enzyme such as succinate dehydrogenase, a bona fide tumor suppressor, leads to enzymatic inactivation of the EgIN prolyl hydroxylase through the accumulation of succinate. The group recently identified how inactivation of the tumor suppressor succinate dehydrogenase blunts neuronal apoptosis through inactivation of the proapoptotic prolyl hydroxylase EgIN3. We are now working to link the function of the prolyl hydroxylases with cancer metabolism.

To identify novel oxygen sensing mechanisms, the group is attempting to identify novel EgIN3 hydroxylation

substrates. It was recently demonstrated that EglN3 mediated apoptosis requires hydroxylation activity and is independent of HIFa regulation. Therefore identification of novel substrates will reveal mechanisms of how the prolyl hydroxylase EglN3 executes neuronal apoptosis. In the last two years, the group established a substrate trap that allows stabilization of the transient enzyme substrate interaction. Using this substrate trap, affinity purification coupled with mass spectrometry will help reveal novel EglN3 substrates. A genome-wide loss of function screen was completed to identify EglN3 downstream targets required for apoptosis function. An initial pilot screen proof of concept for their approach was derived and a novel tumor suppressor called KIF1Bb identified. This gene encodes a kinesin motor protein located on chromosome 1p36.2, a region of the genome that is frequently deleted in neural crest derived tumors including neuroblastoma. The current screen includes a new and more complex short hairpin library provided by the Broad Institute. Using this library, additional hits required for EglN3 mediated apoptosis were identified. Regarding the identification of the potential tumor suppressor KIF1Bb, the group identified additional missense mutations in melanomas and continues to investigate how this kinesin induces apoptosis. Interestingly, its kinesin domain is indispensible for apoptosis function. Large-scale affinity purification coupled with mass spectrometry identified KIF1Bb binding partners, specifically interacts with the minimal region that is necessary and sufficient for apoptosis function. This work was performed in collaboration with Ulf Hellman at the Uppsala Branch for mass spectrometry and peptide sequencing. One such binding partner called DHX9 was identified and further evaluated in functional assays. It is important to note that DHX9 acts as a critical mediator of KIF1Bβ-induced apoptosis, assayed by loss and gain of function approaches. Localization studies revealed that cytoplasmic DHX9 locates towards the nucleus upon KIF1Bb induction and that nuclear localization of DHX9 is neccesary for KIF1Bb mediated apoptosis. Subsequently, the group is trying to understand what gene expression signature is mediated by DHX9 in the context of KIF1Bb induction.

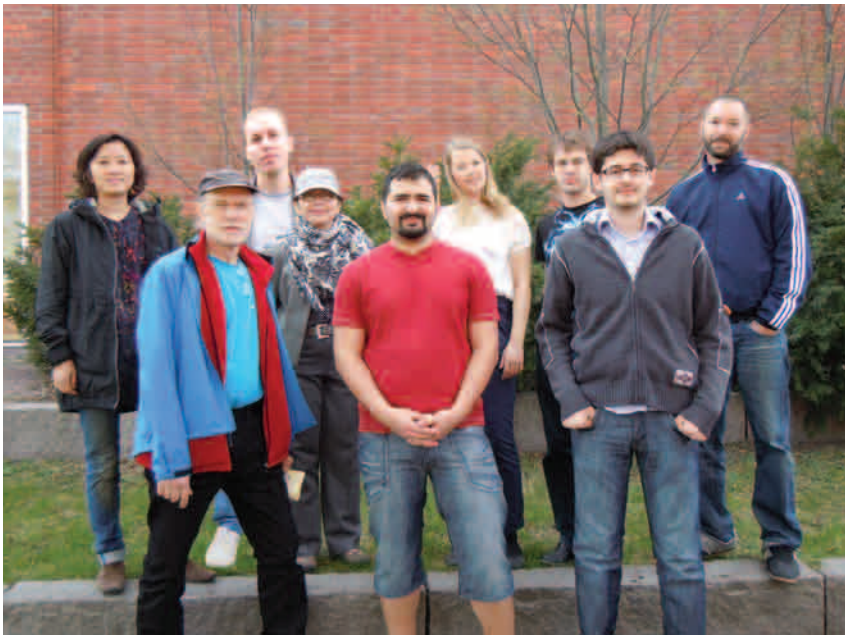
A goal is to inactivate this gene in model organisms to determine if loss of KIF1Bb by itself or in collaboration with other oncogenes promotes neuroblastoma development or other forms of neural crest derived tumors. The group observed that complete loss of KIF1Bb results in early embryonic lethality has begun to conditionally inactivate this gene in neural crest precursors.

COMPUTATIONAL GENOMICS

Rickard Sandberg, Ph.D.

The long-term goal of the group is to reveal general principles of mammalian gene expression programs using a combination of genome-wide experimental and computational techniques.

Currently, the group is studying global gene expression through an *in vivo* differentiation process with single-cell resolution. To this end, a single-cell RNA-Seq protocol for genome-wide transcriptome mapping from individual cells (or ~10 pg of total RNA) has been implemented. This protocol gives better transcript coverage than previously published methods and can be used for transcript quantification and analyses of RNA processing. The group is now applying this method to studies of gene regulation in mouse preimplantation development, an *in vivo* system with



natural single-cell resolution, where the totipotent zygote will cleave, divide and give rise to the three lineages (trophectoderm, epiblast and hypoblast) of the blastocyst. The aim is to address how gene expression patterns are regulated as cells differentiate and in particular how pluripotency is specified and restricted *in vivo* by comparing pluripotent epiblast cells to closely related hypoblast and to trophectoderm cells. To further characterize the transcriptional regulation in preimplantation embryos, the group is mapping enhancers in blastocyst-derived cell lines (embryonic, primitive endoderm and trophoblast stem cells) through ChIP-Seq towards p300 and enhancer-associated histone marks. Combined with the above mentioned single-cell transcriptomes, the lineage-specific enhancers will be associated with transcription and mined for *cis*-regulatory motifs to better define the gene regulatory network that determine preimplantation development.

The group is also interested in post-transcriptional gene regulation, and recent evidence supports a role for chromatin structure and histone modifications in the regulation of pre-mRNA splicing. Together with Shalini Oberdoerffer at the National Cancer Institute (NIH, USA), the group analyzed the role of DNA methylation and CTCF protein binding in pre-mRNA splicing. Using experiments on the *Ptprc* gene and genome-wide data, it was demonstrated that DNA methylation can affect pre-mRNA splicing, through CTCF dependent RNA polymerase pausing.

Another long-term aim of the lab is to develop computational techniques that make better use of the massive amounts of genomic, epigenetic and transcriptome data available in public repositories. The group has set up a computational infrastructure for RNA-Seq, ChIP-Seq and other genome-wide data that is capable of analyzing hundreds of samples in parallel by distributing processor and memory intensive parts using in-house developed programs. Having access to large amounts of transcriptome data, the group is exploring the importance of post-transcriptional regulation with increasing proliferation and in cancer grading. In a collaborative project with Jonas Muhr, the group analyzed genomic patterns in the binding of Sox transcription factors to enhancers in embryonic stem cells, neural progenitors and post-mitotic neurons. Combining these binding patterns with RNA-Seq and microarray data revealed that Sox factors pre-bind enhancers that will be activated at succeeding stages of development.

CNS TUMORS AND DEVELOPMENT

Johan Holmberg, Ph.D.

The core question addressed by the group is how the generation and subsequent maintenance of differentiated neural cells is orchestrated and how errors in this process are involved in the formation of brain tumors. The group is exploring the possibility that expression of transcription factors (TFs), associated with embryonic stem (ES) cell self-renewal and pluripotency, is a key feature of self-renewing brain tumor cells that distinguishes them from neural stem cells and less malignant tumor cells. Together with Jonas Muhr, the group has previously shown that the combined expression of Oct4, Klf4, Sox2 and Nanog is a hallmark of glioblastoma multiforme (GBM), with Oct4 and Klf4 expression exclusively restricted to grade IV GBM tumors. In order to explore whether the

presence of Oct4 and Klf4 is a cause or consequence of increased malignancy, the group is now targeting their expression in patient derived primary tumor cells with lentiviral shRNA vectors. To fully appreciate the possible role for tumor formation, of these TFs *in vivo*, the group is using immunodeficient NOD-SCID mice as a model for intracranial xenograft transplantation. Upon transduction of the tumor cells with shRNA prior to transplantation, they also include a *luciferase* expression vector. This enables continuous monitoring of tumor growth with an IVIS bioluminescence camera. The group has successfully generated intracranial tumors from all the primary tumor cells at their disposal and are currently transplanting cells with shRNA targeting the above mentioned TFs. This loss of function approach will be complemented with a gain of function approach, wherein the above mentioned TFs will be overexpressed in glioma cells of a lower malignancy grade (II and III) and then assayed for acquisition of GBM features.

The process of neurogenesis is characterized by a gradual loss of progenitor properties and acquisition of specific neuronal traits. This is accompanied by a progressive restriction of alternative cell fates. Several studies have focused on how a cell acquires neuronal properties. However, less is known about how progenitor properties are shut down and how gene programs necessary to acquire alternative cell fates are permanently silenced. In collaboration with Dr. Bracken at Trinity College, Dublin, the group is exploring the role of the putative tumor suppressor Chromodomain Helicase DNA Binding protein 5 (Chd5) in promoting and maintaining a neuronal identity, partly through the repression of genes characteristic for progenitors or alternative cell fates. Earlier studies suggest that Chd5 is a key tumor suppressor gene of the chromosomal region *1p36*, a region frequently lost in neuroblastoma. The analysis shows that Chd5 is predominantly expressed in differentiated neurons but also in progenitor cells during terminal differentiation. To address whether Chd5 is necessary for terminal neuronal differentiation *in vivo*, the group is currently targeting Chd5 expression with shRNA in developing mouse telencephalon through *in utero* electroporation. In a complimentary approach, the group is utilizing a mouse ES cell based model for cortical neurogenesis. The preliminary results indicate that Chd5 is essential for proper neurogenesis, both *in vitro* and *in vivo* and that without Chd5 expression neural progenitors fail to differentiate. To better characterize the genetic response to loss of Chd5, the group is performing microarray analysis at different time points during the transition from neural progenitor to differentiated neuron, comparing wild type cells with cells wherein Chd5 is knocked down or overexpressed. To gain a more direct mechanistic understanding of how Chd5 performs its function, the group will perform ChIP-Seq assays at similar time points as the array. As the high levels of Chd5 are maintained in fully differentiated neurons, we are exploring a possible role for Chd5 in maintaining a differentiated neuronal identity with shRNA targeting Chd5 in differentiated cells derived from the ES cells described above. If the cellular response to deletion of Chd5 is loss of mature neuronal identity the genetic events will be analyzed with expression microarrays. Identified factors are candidates for further gain- and loss-of-function experiments. Depending on the results obtained *in vitro*, the group plans to address the *in vivo* role of Chd5 expression in mature terminally differentiated neurons, through the generation of a conditional mouse knockout model.

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DIRECTOR'S MESSAGE

Cancer cells are characterized by perturbations in signaling pathways that regulate cell growth, survival, differentiation and migration. The aim of the work is to elucidate the molecular mechanisms that regulate these events and we expect that this research will make it possible to develop means for better diagnosis, prognosis and treatment of cancer patients.

Researchers at our Branch study two growth regulatory factors, 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 have important roles during the embryonal development in the formation of different kinds of mesenchymal cell types. Overactivity of PDGF is implicated in autocrine as well as paracrine stimulation of tumors and the goal is to elucidate the molecular mechanisms of signal transduction via PDGF receptors and explore the clinical utility of PDGF antagonists.

UPPSALA BRANCH

TGFβ family members act via heteromeric complexes of type I and type II serine/threonine kinase receptors and have important roles during the embryonal development. Most often, the members of this family inhibit cell growth, but they also stimulate matrix production and induce apoptosis. TGFβ is initially a tumor suppressor. In later stages of tumor progression, TGFβ becomes a tumor promoter through its ability to induce epithelial-mesenchymal transition which links to increased migration and metastasis. Additional pro-tumorigenic effects of TGFβ include its suppressive effect on the immune system and its ability to stimulate angiogenesis. Our objectives are to explore the molecular mechanisms whereby TGFβ acts and investigate the possibility that selective TGFβ antagonists can be made to inhibit the protumorigenic effects of TGFβ while leaving its tumor suppressor effects unperturbed. The ultimate goal is to explore the clinical utility of such antagonists.

The role of the microenvironment in tumorigenesis is another line of research undertaken at the Branch. We are exploring the role in tumor progression of the large polysaccharide hyaluronan and its receptor CD44, and their interactions with growth factor receptors. As in prior years, Branch groups are supported by expertise in proteomics and mass spectrometry.

~ C.-H. Heldin

PDGF TRANSLATIONAL RESEARCH

Carina Hellberg, Ph.D.

The research is focused on PDGF receptors as cancer drug targets, and on the mechanisms of modulation of PDGF β-receptor signal transduction.

Tyrosine phosphorylation of proteins is essential in signal transduction pathways that regulate cell growth, adhesion, migration and differentiation, and is controlled by kinases as well as tyrosine phosphatases. Growth factors promote the growth and survival of tumor cells, often by signaling through receptor tyrosine kinases. Tyrosine kinase receptors also induce tumor vascularization, which is necessary for tumor growth and metastasis. These findings have led to the development of anti-tumor drugs that specifically target tyrosine kinases. Further understanding of the molecular mechanisms underlying tumor formation should generate a new wave of target-specific drugs.

We have shown that targeting tumor vasculature by combining inhibition of PDGF and VEGF receptors reduces the growth of B16 melanoma tumors. We now demonstrate that inhibition of c-Kit selectively sensitizes melanoma cells to paclitaxel. However, when grown *in vivo*, B16 tumors do not respond to paclitaxel treatment, not even when combined with treatment by the c-Kit inhibitor imatinib. Instead, a combination of imatinib and vatalanib, a VEGF receptor inhibitor, sensitizes these tumors to low doses of paclitaxel.

After ligand stimulation, PDGF β-receptor signaling is terminated by receptor dephosphorylation in parallel with receptor internalization and degradation. Receptor signaling can therefore be modulated by altering the rate of receptor dephosphorylation or trafficking. We have identified oncogenic H-Ras as a regulator of receptor internalization and signal transduction. Expression of oncogenic H-Ras altered the route of ligand-induced internalization of PDGF receptors and caused an increase in both the amplitude and duration of receptor phosphorylation. The increased PDGF receptor phosphorylation was associated with increased survival signals. Since PDGF-BB also increased the anchorage-independent growth of H-Ras transformed fibroblasts, it is possible that the altered receptor trafficking augments cell transformation.

Screening for protein tyrosine phosphatases (PTPs) that regulate PDGF β-receptor phosphorylation and signal transduction has identified the receptor-like PTP LAR as a positive regulator of PDGF β-receptor phosphorylation. Inhibition of c-Abl kinase in LAR knock-out fibroblasts reverted this phenotype, indicating that LAR promotes PDGF β-receptor activation by inhibiting c-Abl, which can act as a negative regulator for PDGF receptor activation.

To investigate the role of T-cell phosphatase in tumor cell biology, novel substrates were identified. The glycolytic enzyme pyruvate kinase M2 (PKM2) interacted with the T-cell phosphatase. Since PKM2 mediates the shift to aerobic glycolysis in tumor cells, the "Warburg effect", the possibility that T-cell phosphatase regulate tumor cell metabolism was investigated. HeLa cells where the T-cell phosphatase had been knocked down displayed increased PKM2 activity compared to the control cells. The intracellular concentrations of several glycolytic intermediates were also affected, indicating that this phosphatase could participate in the regulation of aerobic glycolysis.

PDGF SIGNAL TRANSDUCTION

Johan Lennartsson, Ph.D.

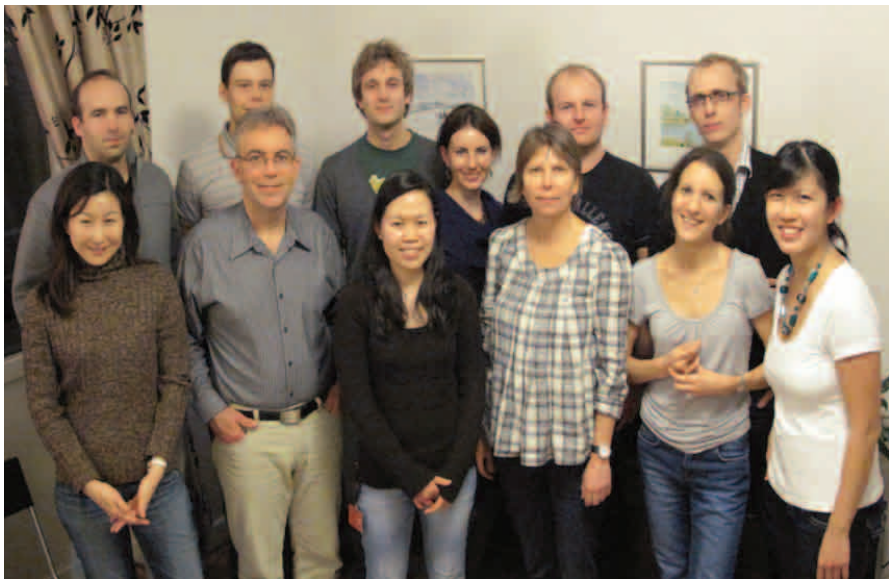
Our aim is to elucidate signaling mechanisms of PDGF receptors. A major goal of our work is to understand the complex network of regulatory events that control PDGF-induced activation of different MAP kinase pathways, in particular the Erk1/2 and Erk5 pathways. The biological consequence of Erk1/2 activation is dependent on the magnitude as well as the temporal pattern of activation and we have investigated if the major signaling pathways activated by the PDGFR can modulate the activation of Erk1/2. We found that Src kinase activity was important for the initial phosphorylation of Erk1/2; inhibiting Src kinases caused a delay in Erk1/2 activation. Similarly, inhibition of phospholipase Cγ (PLCγ) also caused a delayed onset of Erk1/2 phosphorylation, which was caused by lack of protein kinase C activation. However, interfering with phosphatidylinositol (PI) 3-kinase or the GTPase activating protein (GAP) for Ras increased the strength of Erk1/2 activation, suggesting that these pathways normally function to dampen Erk phosphorylation. When we investigated which step in the Erk1/2 MAP kinase cascade the various pathways influenced the activation, we found that in all cases this occurred at the level of Ras activation. The *c-fos* gene is an established downstream target of Erk1/2 and we observed that the effects on Erk1/2 activation by interfering with different signaling pathways correlated well with changed kinetics or amplitude of c-fos gene expression. In conclusion, cross-talk with other PDGFRβ-induced signaling pathways is important for fine-tuning the pattern of Erk1/2 activation.

MKP3 negatively modulates PDGF-induced Akt and Erk5 phosphorylation as well as chemotaxis

MAP kinase phosphatase-3 (MKP3), also known as DUSP6, is a dual specificity phosphatase considered to selectively dephosphorylate Erk1/2. We found that in NIH3T3 fibroblasts, MKP3 levels are regulated in an Erk1/2- and PI3-kinase-dependent manner in response to PDGF treatment, but independently of Erk5 expression. Silencing of MKP3 expression did not affect PDGF-BB-induced Erk1/2 or p38 phosphorylation; however, the basal levels of Erk1/2 and p38 phosphorylation were elevated. Furthermore, we found that the PDGF-BB-mediated activation of Erk5 and Akt were enhanced when the MKP3 expression was reduced. In concurrence, inhibition of Mek1/2 using the inhibitors CI-1040 blocked the PDGF-BB-induced MKP3 expression while it enhanced Akt and Erk5 phosphorylation. Functionally, we found that MKP3 silencing did not affect cell proliferation, but did enhance the chemotactic response towards PDGF-BB. Although both Akt and Erk5 have been linked to increased cell survival, we were unable to detect any change in the ability of PDGF-BB to protect the NIH3T3 cells from starvation-induced apoptosis. However, we observed an increase in apoptosis in untreated cells with reduced MKP3 expression. Our data indicate that there is negative cross-talk between Erk1/2 and Erk5 that involves MKP3, and that PI3-kinase, in addition to promoting Akt phosphorylation, negatively modulate Akt, in a MKP3-dependent manner.

TGFβ SIGNALING

Aristidis Moustakas, Ph.D.



We investigate transforming growth factor β (TGFβ) and bone morphogenetic protein (BMP) signaling pathways and aim at elucidating new mechanisms of their regulation. We also explore the AMP-regulated kinase (AMPK) family, transcription factors and micro-RNAs that are involved in epithelial-mesenchymal transition (EMT), tumor cell invasiveness and cancer stem cell self-renewal.

Regulation of TGFβ/BMP signaling

TGFβ family ligands mediate their biological effects via receptor serine/threonine kinases, Smads, MAP kinases and Rho GTPases, by regulating expression of many genes in a cell type-dependent manner. TGFβ signaling is finely tuned via negative regulation such as receptor and Smad de-phosphorylation and ubiquitination.

We have reported on new mechanisms of Smad regulation that operate at the chromatin level. PARP-1 (poly(ADP-ribose) polymerase is a nuclear enzyme involved in gene transcription. TGFβ signaling leads to the rapid formation of nuclear complexes between PARP-1 and Smads. PARP-1 ADP-ribosylates Smad3 and Smad4, which dissociate from DNA, thus controlling the duration of Smad complex residence on DNA. In addition, Smads can associate with the chromatin insulator protein CTCF in the nucleus. We identified the Smad-CTCF protein complex is the *H19/Igf2* imprinting control region, suggesting that TGFβ may be involved in the epigenetic control of gene expression.

We also focused on cytoplasmic regulatory mechanisms of TGFβ signaling. The tumor suppressor kinase LKB1 forms complexes with Smad4 via the adaptor protein LIP1, phosphorylates Smad4 in its DNA-binding domain and inhibits binding of Smad4 to DNA. LKB1 can negatively regulate both TGFβ and BMP signaling, by acting on the common Smad4 of these pathways. A new line of research in collaboration with Peter ten Dijke's laboratory, explains why TGFβ causes cell death in a cell type-dependent and growth condition-dependent manner. The key regulatory protein is the mitogen and stress-activated kinase (Msk) 1, which is activated by TGFβ and counteracts the apoptotic response.

In collaboration with the EU-funded network of excellence "ENFIN" (Zhike Zi, University of Freiburg, Edda Klipp, Humboldt University Berlin, Xuedong Liu, University of Colorado at Boulder), we performed quantitative analysis of Smad signaling in response to TGFβ and established the importance of threshold ligand levels and the oscillatory behavior of the pathway.

Epithelial-mesenchymal transition and regulation of tumor-initiating cells

TGFβ can positively contribute to cancer progression by promoting EMT, tumor cell invasiveness and metastasis. EMT is also linked to the generation of unique progenitor cells, carrying stem-like capacities within tumors.

We previously established a molecular pathway downstream of TGFβ, involving the nuclear factor HMGA2 and its downstream targets Snail and Twist. Our new work showed that PARP-1 regulates the EMT response to TGFβ, while LKB1 counteracts the mesenchymal transition promoted by TGFβ. In collaboration with Vassilis Gorgoulis at the University of Athens, we analyzed the crosstalk between mechanisms that control EMT and molecules that regulate genomic stability during cancer progression. We also explore the roles of p53 and the AMPK family member salt-inducible kinase 1 (SIK1) in controlling tight junction dissolution during EMT.

We have also progressed into mechanisms by which HMGA2 and Snail regulate the self-renewing and tumor-initiating potential of cancer cells of the breast and brain. We extended previous work on the crosstalk of TGFβ and Notch pathways, by establishing a new role of these pathways during invasiveness of renal carcinomas in

collaboration with Håkan Axelsson at the University of Lund. Finally, we completed two lines of collaboration with Christos Stournaras at the University of Crete, analyzing mechanisms by which TGFβ and BMP regulate actin dynamics during EMT. The above findings have direct relevance to the mechanisms by which TGFβ acts as a pro-metastatic factor and direct our research towards novel therapeutic approaches.

APOPTOTIC SIGNALING

Maréne Landström, M.D., Ph.D.

We explore the molecular mechanisms whereby TGFβ activates non-Smad signaling pathways leading to migration, invasion and apoptosis of tumor cells. Our long-term goal is to develop novel therapeutic strategies and identify potential novel tumor biomarkers.

The type I TGFβ receptor (TβRI) recruits the E3-ligase TRAF6

We have elucidated a molecular mechanism for a non-Smad signaling pathway of TGFβ. Thus, TβRI has a consensus binding site for the ubiquitin ligase TRAF6; the binding of TRAF6 leads to Lys63-dependent ubiquitination and activation of the kinase TAK1, which in turn activates the p38 MAPK pathway. We are investigating the molecular mechanisms whereby TRAF6 determines the specificity of cellular responses induced by TGFβ.

Inflammation in relation to tumor biology

There is a close link between chronic inflammation and tumor progression. We want to understand the underlying molecular mechanisms for how inflammatory cytokines can promote tumor progression. Moreover, we are investigating the detailed molecular mechanisms for activation of the TAK1 – p38 MAP-kinase pathway by inflammatory cytokines, such as TNF-α and IL-1, and have explored the importance of Lys63-linked polyubiquitination of TAK1, for its activation by TNF-α and IL-1β. We have found that Lys34 in TAK1 is a major acceptor for Lys63-linked polyubiquitin chains also in these pathways, leading to activation of NFκB and induction of an inflammatory response which would promote tumor progression *in vivo*.

Development of novel treatment strategies for patients with advanced prostate cancer

There is an urgent need to develop improved therapeutic strategies for patients with advanced and metastatic prostate cancer, as no efficient therapy is currently available. The small gold compound aurothiomalate (ATM) is used for treatment of rheumatoid arthritis and has been found to cause growth inhibition of ovarian and non-small-cell lung cancer. In our search for novel treatments of advanced prostate cancer, we have investigated possible effects of ATM. Interestingly, ATM can specifically induce apoptosis of prostate cancer cells while normal primary prostate epithelial cells are not affected by ATM. We found 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. We anticipate that our studies will add ATM to the future therapeutic options for treatment of patients with advanced prostate cancer. We are also investigating whether inhibition of specific kinases, activated by TGFβ in the non-canonical Smad signaling pathway, could be used to treat prostate cancer.

Does Smad7 act as a bridge between TGFβ and Wnt signaling?

Signaling molecules downstream of TGFβ and Wnt receptors regulate cell fate and proliferation in tumors. We have previously reported that 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). Furthermore, with the use of siRNA and anti-sense techniques, we have shown that Smad7 expression is required for TGFβ-induced stabilization of β-catenin. Interestingly, we have identified Smad7 and p38 as regulators of the activity of glycogen syntetase kinase-3β (GSK-3β), causing stabilization of β-catenin, which is crucial for TGFβ-induced cell migration of prostate cancer cells.

Smad7 target genes

Smad7 is a nuclear protein and TGFβ stimulation of cells leads to an export of Smad7 to the cytoplasm, where it binds to the activated TβRI. We have explored the possibility that Smad7 also has a nuclear function, and have found that Smad7 regulates gene transcription.

MOLECULAR PATHOLOGY

Kohei Miyazono, M.D., D.M.S.

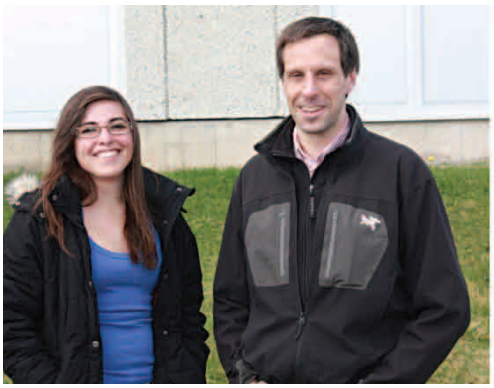
We are investigating the mechanisms of regulation of gene expression by TGFβ family members. TGFβ family cytokines regulate a variety of cellular processes, including differentiation, proliferation, migration and cell death in a cell-type specific and context-dependent manner. Recent technological advances in high-throughput analyses of transcriptional regulation by use of massively parallel sequencing and tiling microarrays enable us to determine regulatory mechanisms of such context-specific transcriptional regulation. We focus on the differences in Smad family binding genomic regions in different cell types, and analysis of angiogenesis-related factor(s) downstream of TGFβ family signaling pathways.

Analysis of Smad family binding regions by chip/ChIP-sequencing

We have identified Smad2/3 binding regions in normal human epidermal keratinocyte cell line HaCaT using promoter tiling array and several co-regulatory factors which bound to Smad2/3 binding genomic regions and affected global TGF-β-induced transcription. We extended the analysis to determine the differences in Smad2/3 binding regions in some other cell lines, and successfully determined Smad2/3 binding regions in HepG2 hepatoblastoma cells. We analyzed the extent of overlap of Smad2/3 binding regions between HepG2 and HaCaT cells, and found a HepG2-specific regulatory factor which cooperated with Smad2/3 by helping their target promoter recognition. Based on these findings, we will continue to reveal the mechanisms of changes in TGFβ responses in the cells in some pathological situations, including cancer.

Regulatory mechanisms of angiogenesis by TGFβ family signaling

Perturbations of TGFβ family signaling pathways have been implicated in diverse developmental changes and some diseases, including cancer, ectopic ossification, and cardiovascular diseases. Mutations in *ENG*, *ACVRL1* (also known as ALK-1) or *SMAD4* have been shown to cause hereditary hemorrhagic telangiectasia (HHT), which is a multisystemic vascular disorder characterized by epistaxis, telangiectasia, and arteriovenous malformation. *ACVRL1* and *ENG* encode endothelial-specific receptors for TGFβ and BMP-9/10 and transduce their signals through BMP-specific receptor-regulated Smads (Smad1/5/8). These facts indicate that BMP signaling in endothelial cells is implicated in pathogenesis of HHT. However, molecular mechanisms of BMP signaling in endothelial cells and its direct target genes have not been characterized. We determined Smad1/5 binding genomic regions in endothelial cells by ChIP-sequencing, and analyzed their characteristics. We identified novel target molecules of Smad1/5, and analyzed their roles in endothelial cells. We are currently collaborating with Dr. Johan Ledin's group at the Evolutionary Biology Centre (EBC) of Uppsala University to reveal the *in vivo* relevance of our findings using the zebrafish angiogenesis model. We also collaborate with the Genome Science Division, Research Center for Advanced Science and Technology (RCAST), University of Tokyo; Department of Molecular Pathology, Graduate School of Medicine, University of Tokyo; and the TGFβ Signaling group, the Protein Structure group and the Cancer Signals group at LICR.



CANCER SIGNALING

Peter ten Dijke, Ph.D.

We are interested in the detection, identification and functional characterization of signaling complexes and intermediates that distinguish cancer cells from normal cells, or that differ during the different stages of tumor progression. These complexes might function as diagnostic and/or prognostic markers and become targets for therapeutic intervention. In our current work, we focus on components of the TGFβ-Smad and MAP-kinase – AP-1 pathways, two pathways that play critical roles in tumor progression and interact on multiple levels.

Induction of breast cancer cell invasion by TGFβ

We have developed a TGFβ-dependent invasion assay system consisting of spheroids of MCF10A1 normal breast epithelial cells (M1) and RAS-transformed (pre-)malignant derivatives (M2 and M4) embedded in collagen gels. Both basal and TGFβ-induced invasion of these cell lines were found to correlate with their tumorigenic potential; M4 showing the most aggressive behavior and M1 showing the least. TGFβ-induced invasion in premalignant M2 and highly malignant M4 cells was inhibited upon specific knockdown of Smad3 or Smad4. Interestingly, matrix metalloproteinase (MMP) inhibitors mitigated TGFβ-induced invasion of M4 cells, while leaving basal invasion intact. In line with this, TGFβ was found to strongly induce MMP2 and MMP9 expression in a Smad-dependent manner. This collagen-embedded spheroid system therefore offers a valuable screening model for TGFβ/Smad- and MMP2- and MMP9-dependent breast cancer invasion.

TGFβ activates mitogen- and stress-activated protein kinase-1 (Msk1) to attenuate cell death

We observed that TGFβ can activate mitogen- and stress-activated kinase 1 (Msk1). Knockdown of GADD45, a Smad4-induced upstream regulator of p38 MAP-kinase prevented TGFβ-induced p38 and Msk1 activity. Msk1 functionally regulated pro-apoptotic BH3-only BCL2 proteins, as Msk1 knockdown reduced Bad phosphorylation and enhanced Noxa and Bim expression, leading to enhanced TGFβ-induced caspase-3 activity and cell death. This finding suggested that Msk1 represents a pro-survival pathway bifurcating downstream of p38 and antagonizes the established pro-apoptotic p38 MAP-kinase function.

In situ proximity ligation detection of c-Jun/AP-1 dimers in breast cancer cells

Genetic and biochemical studies have shown that selective interactions between the Jun, Fos, and activating transcription factor (ATF) components of transcription factor activating protein 1 (AP-1) exhibit specific and critical functions in the regulation of cell proliferation, differentiation, and survival. Until recently, no methods were available to detect endogenous AP-1 complexes in cells and tissues *in situ*. Therefore, we validated the proximity ligation assay (PLA) for its ability to specifically visualize and quantify changes in endogenous c-Jun/c-Fos, c-Jun/ATF2, and c-Jun/Fra1 complexes. We found that aggressive basal-like breast cancer cells can be discriminated from much less invasive luminal-like cells by PLA detection of c-Jun/Fra1 rather than of c-Jun/ATF2 and c-Jun/c-Fos (2; Fig. 3). Also in tumor tissue derived from highly metastatic basal-like MDA-MB231 cells, high levels of c-Jun/Fra1 complexes were detected. Together, these results demonstrate that *in situ* PLA is a powerful diagnostic tool to analyze and quantify the amounts of biologically critical AP-1 dimers in fixed cells and tissue material.

MATRIX BIOLOGY

Paraskevi Heldin, Ph.D.

The aim of our work is to explore the mechanism whereby hyaluronan via its receptor CD44 promotes tumorigenesis, including the regulation of hyaluronan synthases (HASs) by posttranslational modifications, the molecular mechanism of signaling via CD44 and the functional importance of its interactions with growth factor receptors.

Regulation of HAS by posttranslational modifications

The extensive accumulation of hyaluronan

that occurs in various malignant and inflammatory conditions is correlated to the severity of the pathological condition. Recently, we have delineated the downstream signaling pathways through which PDGF-BB stimulates hyaluronan synthesis in human dermal fibroblasts, and now focus on regulation of the activity of HAS proteins.

We have demonstrated that HAS activation and stability is regulated by ubiquitination. By transfection of pairs of differently tagged HAS isoforms in COS-1 and CHO cells, followed by immunoprecipitation and immunoblotting, we showed that HAS2 forms both homodimers and heterodimers with other HAS isoforms, and the dimeric configuration is important for the activity of HAS2. We also demonstrated that HAS2 is mono-ubiquitinated at Lys190 (K190), which is conserved among all HAS isoforms and resides in their glycosyltransferase domain; mutation of K190 inactivates the enzymatic activity of HAS2. These findings demonstrate for the first time that HAS2 activity is regulated through mono-ubiquitination at K190 and oligomerization.

Elucidation of the molecular mechanism of signaling via CD44

CD44s is a transmembrane receptor with FERM-, ankyrin- and PDZ-binding motifs in its intracellular part. CD44 has been proposed as an important marker for breast cancer-initiating cells, and its aberrant expression is associated with persistent inflammation and malignant transformation. We and others have demonstrated that there is a cross-talk between CD44 and growth factor receptors, including the receptors for PDGF-BB, TGFβ, hepatocyte growth factor and epidermal growth factor.

To elucidate the molecular mechanisms underlying its multiple functions, we used a peptide-based pull-down assay to identify proteins that interact with CD44. Several interaction partners were identified, including proteins involved in cytoskeletal reorganization, transcription, endocytosis and intracellular transport. An endogenous complex between CD44 and one of the interacting proteins, the actin binding protein IQGAP1, was demonstrated in several normal and transformed cell types.

Role of hyaluronan-CD44 interactions for breast cancer metastasis

The adhesion of tumor cells to microvascular and lymphatic endothelium is a pre-requisite for their intravasation into vasculature and subsequent extravasation into secondary organs. Bone metastasis is prevalent for advanced breast cancer. A critical step in the metastatic process of breast cancer cells, and any other carcinoma, is remodeling of the basement membrane. Our aim is to explore the role of hyaluronan in the molecular mechanisms that underlie the degradation of basement membrane and translocation of tumor cells.

To study whether endogenous hyaluronan production is involved in the invasion of a clone of the breast cancer cell line MDA-MB-231 that forms metastasis in bone, we used an *in vivo*-like basement membrane model. We found that knockdown of HAS2 completely suppressed the invasive capability of these cells, by the induction of tissue metalloproteinase inhibitor (TIMP)-1 and dephosphorylation of focal adhesion kinase. This study provided new insights into a possible mechanism whereby HAS2 and hyaluronan production can promote breast cancer invasion.

PROTEIN STRUCTURE

Ulf Hellman, Ph.D.

The Protein Structure Group has solid experience in peptide synthesis, general chromatography work and MALDI TOF mass spectrometry.

Peptide synthesis and connected affinity chromatography

The synthetic peptides modified with phosphorylations, acetylations, oxidations or other chemical groups at a given position, have been most useful for groups at the Branch. The peptides produced are used to generate anti-peptide antibodies, as substrates or inhibitors, or as ligands in affinity chromatography experiments. We now also carry out affinity purification of anti-peptide antibodies in collaboration with other groups at the Branch. Another development in our group is that we, in collaboration perform affinity based searches for interacting partners using immobilized ligands (i.e., a modified synthetic peptide carrying a given motif). The combination of the biotin-streptavidin system and magnet beads has proved powerful. Promising results are now being confirmed in *in vivo* cell experiments.

MALDI mass spectrometry

We use 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), installed in March 2007 offers a high sensitivity and accuracy for both MS and especially for MS/MS, user friendliness and peptide sequencing possibilities.

■ Sample preparation for mass spectrometry

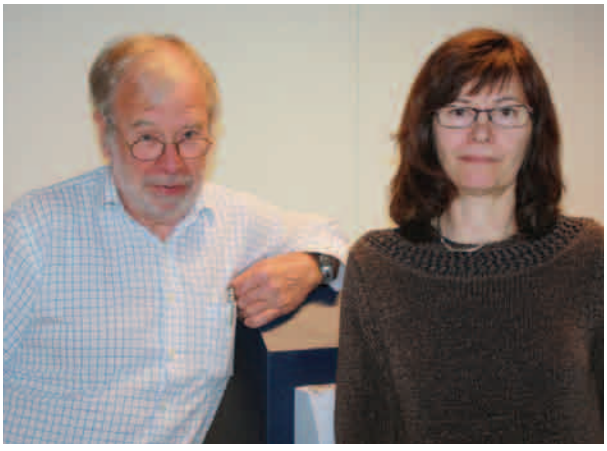
Practically all of the samples for analysis by MALDI-TOF-MS are delivered as bands or spots from one- or two-dimensional SDS-PAGE gels. The proteins in the gel pieces are prepared for MS analysis most often by in-gel tryptic digestion. Coomassie-visible material is analyzed directly after proteolysis; silver-stained material requires a work-up by concentration and desalting on micro columns (i.e., C18 µZipTip). This, combined with other improvements, allows us to get significant identities of minute amounts of sample – typically we identify proteins represented by single weak silver stained spots from one 2D gel.

■ Peptide mass fingerprinting (PMF) and analysis of post translational modification (PTM)

The peptide masses from an in-gel digest are used to scan a sequence database. Even if a significant match is found, we often confirm the identity by subjecting a few peptides to fragment analysis by PDS followed by MS/MS search. Phosphorylated peptides, highly important but notorious for low sensitivity by MALDI TOF MS, are enriched using pipette tips covered with TiO_2/ZrO_2 particles. These have proven to increase the sensitivity significantly. Over the past few years, we have carried out interesting proteomics projects with groups at the Karolinska Hospital looking for proteins that are significantly changed in various tumors. We also spend a fair amount of effort to determine PTMs, of proteins, including methylation, acetylation, ubiquitinylation and phosphorylation.

■ Post source decay (PSD) based peptide sequencing by MALDI-TOF/TOF-MS

Fragment analysis of peptides by MALDI-TOF/TOF-MS using PSD is a straightforward technique. The TOF/TOF technology allows complete PSD spectra to be generated in seconds, so it is possible to quickly analyze several peptides from one digest. Should we fail to identify the target protein (i.e., if it is not represented in any database), the identity may be established by determining the amino acid sequence of a few tryptic peptides and use of BLAST homology searching. This combines extremely well with the sulfonation derivatization protocol, rendering tryptic peptides acidic at the N-terminus, leading to easily interpreted spectra, as they comprise a unique series of y-ions. Hence, an amino acid sequence can unambiguously be determined faster, cheaper and more sensitive than was ever possible using chemical Edman degradation.



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