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2011 Comprehensive Scientific Report

It gives us great pleasure to present the Ludwig Institute for Cancer Research *2011 Comprehensive Scientific Report*. This report serves as an account of the progress we have made during the past year, an exciting time marked by growth and development as we explore new avenues in cancer research. Although the Ludwig laboratories focus on different aspects of cancer, our researchers share a commitment to improve the quality of life of those affected by cancer.

The comprehensive report details research activities by location and provides an in-depth overview of the work being undertaken at each laboratory within the Institute. Reflected in these reports is the unwavering dedication to our mission of pursuing the scientific efforts required to advance the understanding and control of cancer.

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Director's message

Based on the past experience of the Ludwig Brussels Branch in therapeutic vaccination of cancer patients, we have concluded that the major limiting factor to the efficacy of cancer immunotherapy is the immunosuppressive nature of the tumor microenvironment. Hence our current efforts aim at understanding those suppressive mechanisms and devising strategies to overcome them. We use an integrated approach involving mouse models and human tumor-infiltrating lymphocytes (TILs) to decipher the suppressive mechanisms. We then test therapeutic strategies first in preclinical studies and then in clinical trials combining peptide vaccines with the new treatment. As detailed below, several approaches have now reached the clinical trial stage, namely a galectin-3 inhibitor and local injection of cytokines and Toll-like receptor (TLR) ligands. The two patients already involved in the latter trial showed regression of all their cutaneous metastases. Other programs are currently at the stage of drug discovery, such as the search for inhibitors of indoleamine 2,3-dioxygenase (IDO) or tryptophan-dioxygenase (TDO), a new target recently identified by Ludwig scientists in Brussels. A Ludwig spin-off company named iTeos Therapeutics is being launched to further develop such inhibitors.

A potentially useful strategy for blocking tumor-induced immunosuppression is to inactivate suppressive cytokines *in vivo*. Innovative approaches of "autovaccination" were developed to inactivate endogenous cytokines. Such approaches have also generated unique monoclonal antibodies (mAbs). Some of them have proved very useful in studying the roles of cytokines interleukin-9 (IL-9) and IL-22, which were discovered by Ludwig Brussels researchers, in various models of immunity and inflammation.

We also pursued work on the processing of tumor antigens, the splicing of peptides by the proteasome, the role of intermediate proteasomes and the effect of thymic expression of cancer-germline genes on the immunogenicity of the encoded antigens.

The study of oncogenic mutations in cytokine signaling cascades is another active research area at Ludwig Brussels, with a focus on the Janus kinase 2 (JAK2) V617F and TpoR W515L mutations in myeloproliferative neoplasms (MPNs). New JAK1 mutations were also recently discovered following our long-standing studies of the biology and signaling of IL-9. Some of these JAK1 mutations are also found in human leukemias and represent potential targets for specific inhibitors. A search for small-molecule inhibitors of these mutant signaling molecules has been launched.

Benoît Van den Eynde

Tumor immunology and antigen processing

Benoît Van den Eynde, M.D., Ph.D.

The first research line of the group focuses on the processing of tumor antigens. We study the role of the proteasome and other proteases in production of tumor antigenic peptides that are relevant for cancer immunotherapy. Such antigens are usually presented by major histocompatibility complex class I molecules and are derived from intracellular tumor proteins that are degraded by the proteasome. We described several post-translational modifications that occur during antigen processing and result in peptides whose sequence differs significantly from the sequence of their parental protein. These include the splicing of peptide fragments, which occurs in the proteasome catalytic chamber through a reaction of transpeptidation involving an acyl-enzyme intermediate. We have now described four spliced peptides, two of which are spliced in reverse order. One of these peptides also contains two additional post-translational modifications, resulting in the conversion of asparagines to aspartic acids, through a process called *N*-glycosylation/deglycosylation.

In studying a MAGE-A3-derived peptide whose processing does not depend on the proteasome, we uncovered the function of a cytosolic metallopeptidase, insulin-degrading enzyme, in antigen processing. Insulin-degrading enzyme is involved in degradation of the parental MAGE-A3 protein and is responsible for production of antigenic peptide EVDPIGHLY.

We have also described two new proteasome subtypes that are intermediate between the standard proteasome and the immunoproteasome. They are abundant in normal tissues and produce a unique set of antigenic peptides. We are currently evaluating other functional aspects of these intermediate proteasomes.

Cancer-germline genes such as *MAGE* and *NY-ESO1* encode the most relevant antigens for cancer immunotherapy. Some of these genes are expressed at low levels in the thymus, raising the possibility of central immune tolerance that might reduce the immunogenicity of such antigens. We created a mouse line with knockout of cancer-germline gene *P1A*, and tested whether P1AKO mice develop stronger immune responses to P1A antigens as compared with wild-type mice. We found only slightly stronger P1A-specific immune responses in P1AKO mice, even though these responses were sufficient to induce tumor rejection in defined experimental settings. These results indicate only minimal immune tolerance to antigens encoded by cancer-germline genes and fully confirm their immunogenicity.

We made transgenic mice that developed melanomas with a 70%–80% incidence after tamoxifen injection. These tumors expressed the tumor antigen encoded by cancer-germline gene *P1A*. They were initially highly pigmented and indolent, and later dedifferentiated into unpigmented, highly aggressive tumors. Mice bearing aggressive tumors showed exacerbated systemic inflammation associated with disruption of secondary lymphoid organs, accumulation of immature myeloid cells and immunosuppression. We are currently characterizing this immunosuppression and devising effective therapeutic approaches.

We previously discovered that tumors often resist immune rejection by expressing IDO, a tryptophan-degrading enzyme that is profoundly immunosuppressive. We showed that immune rejection was restored by administration of a pharmacological inhibitor of IDO. In collaboration with medicinal chemists in Namur, Belgium, and Lausanne, Switzerland, we identified several families of new IDO inhibitors that will be further optimized to develop drug candidates. In parallel, we uncovered the role of TDO in tumoral immune resistance. TDO is an unrelated tryptophan-degrading enzyme that is highly expressed in the liver to regulate systemic tryptophan levels. We found that TDO was expressed in a high proportion of human tumors. We showed that TDO-expressing mouse tumors are no longer rejected by immunized mice. Moreover, we developed a TDO inhibitor that, upon systemic treatment, restored the ability of mice to reject tumors. These results describe a mechanism of tumoral immune resistance based on TDO expression and establish proof of concept for the use of TDO inhibitors in cancer therapy. We are preparing the launch of a Ludwig spin-off company, iTeos Therapeutics, which will develop inhibitors of IDO and TDO.

Regulation of T lymphocyte function in tumors

Pierre van der Bruggen, Ph.D.

The group studies the dysfunction of TILs resulting from exposure to galectin-3 produced by tumors, and how this anergy can be reversed by galectin competitor ligands. We also started to examine whether the spontaneous antitumor T cell response of nonmetastatic breast carcinoma patients can be used as a clinical prognostic factor.

We first observed that recently activated cytotoxic T lymphocyte (CTL) clones had lost their capacity to bind HLA-peptide tetramers and, upon antigenic stimulation, secreted low levels of cytokines, for example interferon-γ (IFN-γ). These losses of tetramer labeling and function were not due to reduced surface expression of either T cell receptor (TCR) or CD8, which are both essential for tetramer labeling and T cell activation. However, the surface distribution of TCR and CD8 molecules was abnormal when studied by confocal microscopy and fluorescence resonance energy transfer. Whereas TCR and CD8 were colocalized on resting CTLs, they segregated from each other at the surface of recently activated CTLs. Our working hypothesis is that extracellular galectins bind to *N*-glycosylated surface glycoproteins, clustering them into glycoprotein-galectin lattices. Such lattices decrease the lateral mobility of glycoproteins and thereby reduce T cell function. In agreement with this hypothesis, we observed that surface glycoproteins on recently activated CTLs harbored more galectin ligands, and that treating these CTLs with millimolar concentrations of galectin ligand *N*-acetyllactosamine increased TCR-CD8 colocalization and boosted their ability to secrete IFN-γ upon antigenic stimulation.

Galectin-3 and other galectins can be secreted by tumor cells and macrophages, and can be found in high amounts in the tumor microenvironment. Thus we wished to test whether the dysfunctional T lymphocytes that can be found in human tumors can recover their functions after incubation with galectin ligands. Human TILs show impaired IFN-γ secretion and cytotoxicity in contrast to blood CD8 T cells. Interestingly, this dysfunction is correlated with the segregation of surface TCR and CD8 molecules. Treating TILs with galectin ligands or with an anti-galectin-3 antibody restored IFN-γ secretion, cytotoxicity and TCR-CD8 colocalization. This holds true for 80% of the TIL samples tested so far. We are currently trying to understand the very early activation events that are defective in TILs.

Looking forward to clinical applications, we identified two plant-derived polysaccharides that bind to galectins and are in clinical development.

Both boost TIL cytotoxicity and secretion of IFN-γ. A clinical trial should be launched in 2012 in metastatic melanoma patients using a MAGE peptide vaccine and one of these galectin ligands.

Several retrospective studies suggest a correlation between survival of cancer patients with ovarian or colorectal carcinoma and infiltration of their tumors by immune cells. Danièle Godelaine set out to evaluate the frequencies of antitumor CD8 T lymphocytes in the blood of nonmetastatic breast cancer patients who were prospectively recruited in several clinical centers. Blood samples were collected before and after surgery. Frequencies were evaluated by mixed lymphocyte-peptide cultures, carried out with HLA-A2- and HLA-A3-restricted HER2/neu and hTERT peptides, followed by detection of specific cells with HLA-peptide tetramers. Tumors removed at surgery were analyzed by immunohistochemistry for infiltration by immune cells, and fragments were frozen for further genetic analysis of the TCR repertoire. The prospective follow-up of 172 patients will extend over a five-year period. So far, 69 patients have been included and 33 have been screened for frequencies of specific CD8 T lymphocytes. Of the screened patients, 30% have a frequency against the targeted antigens in the range of 3×10^{-6} among blood CD8 T cells, whereas the mean value in healthy donors is 3×10^{-7}. We hope to identify patients with a better prognosis to offer them adapted care, avoiding unnecessary heavy 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 TLR ligands, as well as antibodies directed against inhibitory cytokines such as TGF-β.

Female CBA mice do not reject male skin grafts even though they can mount a specific anti-H-Y cytolytic T cell response. Christophe Bourdeaux and Catherine Uyttenhove tested local approaches to break this tolerance. Repeated local injections of a low dose of IL-12, combined with IFN-α, caused graft rejection in all mice. Like IFN-α, IL-1α, IL-18 and IL-2 could not induce rejection on their own, but synergized effectively with IL-12. A finding of importance for clinical application of this procedure is that several weekly cycles of cytokine treatments are necessary for complete rejection of the grafts.

We tested combinations of agents that are approved for clinical use. Isabelle Jacquemart and Christophe Lurquin observed that repeated local injections of a combination of low doses of IL-2, GM-CSF and IFN-α with TLR7 ligands gardiquimod or imiquimod caused 100% rejection. The crucial components appear to be IL-2 and gardiquimod. Low doses of IL-2, GM-CSF and IFN-α (300 ng IL-2 and GM-CSF, 10^5 U IFN-α and 20 μg gardiquimod per mouse) were also effective when combined with either anti-TGF-β and anti-IL-10 antibody or anti-CTLA-4 antibody.

With Nicolas van Baren of Ludwig Brussels and Jean-François Baurain of the Centre du Cancer, Cliniques Universitaires Saint-Luc, we have launched a clinical trial in which a few patients with superficial lesions of metastatic melanoma receive vaccinations with tumor antigens combined with a local treatment composed of IL-2, GM-CSF, IFN-α and Aldara, a cream containing imiquimod. Two patients have completed the treatment. Both patients showed regression of all their cutaneous metastases. No serious side effects were observed except that repeated injections around the same tumor site were very painful. We are pursuing the trial, but patient recruitment is very slow.

In collaboration with the group of Jean-Christophe Renauld at Ludwig Brussels, we have made significant progress in our efforts to develop autovaccines against cytokines as a tool for studying their functions *in vivo* and to develop a panel of mAbs of mouse origin against mouse and human cytokines with therapeutic perspectives. Self cytokines linked chemically to

a nonself protein or genetically associated with 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 major histocompatibility complex class II membrane proteins and thus attract help from T cells reactive with the nonself structure.

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 and TGF-β1, and the matrix metalloproteinase-9 MMP-9/gelatinase B. We obtained mAbs from these mice, including a mAb against TGF-β1 that inhibits its bioactivity; mAb against IL-17F that abrogates its neutrophil chemotactic activity; a mAb against GCP-2 that we used to demonstrate its essential role in rapid neutrophil mobilization after *Leishmania major* infection; and a mAb against mouse IL-27 that 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–mediated tumor rejection. These projects are developed in collaboration with Jean-François Baurain of the Centre du Cancer, Cliniques Universitaires Saint-Luc, and Pierre Coulie of the de Duve Institute. Previous studies have investigated various therapeutic vaccines containing tumor-specific antigens expressed by 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 only a marginal number of patients. We are following two different approaches to try to improve these results: finding more immunogenic vaccines and combining vaccines with treatments that modify the tumor environment in favor of effective tumor rejection. There is increasing evidence that this environment plays a key role in the inhibition of antitumoral T cell activity.

In an ongoing phase 1 trial, we are testing the safety, immunogenicity and antitumoral effect of a new, promising vaccine called Theravac. Theravac is a recombinant chimeric protein vaccine aimed at targeting dendritic cells (DCs) *in vivo* and forcing them to express the Tyr.A2 melanoma antigen. Theravac is derived from a bacterial toxin that, upon binding to CD11b, is internalized and neutralizes its target DC. The toxin has been inactivated by insertional mutagenesis and coupled to the Tyr.A2 peptide. Preclinical experiments have shown that Theravac can potently activate Tyr.A2-specific cytolytic T lymphocyte. In our trial, patients with tyrosinase-expressing metastatic melanoma are immunized with repeated injections of Theravac, at increasing doses. This project is developed in collaboration with Claude Leclerc at the Pasteur Institute, Paris.

In another ongoing clinical trial, melanoma patients are vaccinated with MAGE-3.A1 or NA17.A2 peptide, two tumor antigens.. The patients also receive peritumoral injections with a cocktail of proinflammatory cytokines in one or two superficial metastases. In a mouse model of skin graft rejection, this cocktail can induce effective tissue rejection.

In a recently started clinical trial, melanoma patients receive the same peptide vaccine in association with repeated infusions of an experimental drug called GM-CT-01, a plant-extracted oligosaccharide that binds to and inhibits galectins. Galectin-3 is a protein produced by cancer cells that can

inhibit T cell activation. The Ludwig Brussels group of Pierre van der Bruggen has shown that the anergy that characterizes tumor-associated T cells can be reversed with galectin-3 inhibitors including Davanat.

Our group also 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 tumors, such as T lymphocytes, followed by gene expression analysis. We study inflammatory cells and pathways associated with tumors to understand interactions between tumor and immune cells at the tumor site. We have observed in some tumors the presence of ectopic lymphoid structures, combining high endothelial venules, clusters of T cells and mature DCs, and B cell follicles surrounding follicular DCs. Some follicles contained germinal centers. By molecular characterization of immunoglobulin gene libraries obtained from microdissected follicles, we have confirmed that B cell responses were ongoing in these structures. Our data indicate that adaptive immune responses can occur within the melanoma environment.

Cytokines

Jean-Christophe Renauld, M.D., Ph.D.

The group studies the biology of IL-9 and IL-22, two cytokines discovered by researchers at Ludwig Brussels. We previously showed that IL-9 promotes lymphomagenesis and is used as an autocrine growth factor. However, IL-9-dependent tumor cells often acquire the ability to proliferate autonomously through a series of ill-defined genetic events. We developed an *in vitro* model to characterize the molecular mechanisms underlying this autonomous proliferation. We found that most autonomous tumor cell clones selected in our model acquired activating point mutations in the kinase or pseudokinase domain of JAK1. We identified 25 *de novo* JAK1-activating mutations, including 5 mutations already described in human leukemias. These JAK1 mutations induce constitutive activation of the JAK-STAT pathway, which supports their autonomous proliferation.

Multiple ATP-mimetic inhibitors of JAK are under development. Ruxolitinib, a potent and selective inhibitor of JAK1 and JAK2, has been approved for treatment of intermediate and high-risk myelofibrosis, a form of MPN associated with the JAK2 V617F activating mutation. Laurent Knoops from our group was involved in a phase 3 clinical trial showing that this inhibitor induces a marked reduction in splenomegaly and disease-related symptoms. Treating cancers with ATPcompetitive inhibitors has invariably resulted in the development of inhibitor resistance mutations. Although most JAK1 mutants from our library were sensitive to JAK inhibitors, mutations targeting Phe958 and Pro960 in the hinge region of the kinase domain rendered JAK1 constitutively active but also resistant to JAK inhibitors. Furthermore, mutation of the homologous Tyr931 in the JAK2 V617F mutant found in patients with MPNs also conferred resistance to JAK inhibitors, including Ruxolitinib. Thus, our data provide the first description that some activating JAK mutations not only promote autonomous cell proliferation but also confer resistance to ATP-competitive inhibitors.

IL-22, which was originally identified as a gene induced by IL-9 in T lymphocytes, is produced by TH17 lymphocytes, which are associated with autoimmune and inflammatory processes. In a murine psoriasis model triggered by topical application of the TLR7/8 agonist imiquimod, IL-22-deficient mice or wild-type mice treated with blocking anti-IL-22 antibodies were protected against skin lesion development, showing decreased acanthosis, neutrophil infiltration, pustule development and expression of biomarkers that reflect antimicrobial and hyperproliferative responses of keratinocytes. In contrast with this proinflammatory role in skin inflammation,IL-22 has a protective anti-inflammatory activity in asthma models, as blocking IL-22 *in vivo* increased lung infiltration by eosinophils and bronchial hyperresponsiveness.

The characterization of new innate lymphoid cell populations is currently challenging the dogma that T helper cell subsets represent the main source of cytokines during an immune response. We characterized a new innate lymphoid cell population expressing CD25, CCR6 and IL-7R that represented 1% of spleen cells from Rag2-deficient mice. This population comprises 60–70% $CD4^+$ cells, which produce IL-22, whereas the $CD4^-$ subset coexpresses IL-22 and IL-17. These cells share a transcriptional program with $NKp46^+$ RORγt cells found in intestinal mucosae and play a critical role in antibacterial responses.

In addition to conventional gene-targeting strategies used in our lab to generate mice deficient in IL-9R, IL-22 or IL-22R, we developed novel strategies of anticytokine vaccination leading to production of anticytokine autoantibodies. Neutralizing autoantibodies against IL-9, IL-12 and IL-17 have been induced upon vaccination with the autologous cytokines chemically coupled to immunogenic T helper epitopes. To extend the scope of this strategy, we developed a new vaccination procedure based on DNA vectors encoding the targeted antigenic peptides in fusion with the human CD134L transmembrane protein, allowing for their cell-surface expression. These vaccination methods represent simple and convenient approaches to knock down the activity of soluble regulatory proteins, including cytokines and their receptors, and are currently validated with additional targets in inflammatory models.

Signal transduction and molecule hematology

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

Our major focus is understanding cytokine receptor and JAK signaling in blood formation and the mechanisms by which mutants we identified, such as JAK2 V617F and thrombopoietin receptor (TpoR) W515A, induce MPNs.

Tpo binding to TpoR is thought to impose dimeric receptor conformation(s) leading to hematopoietic stem cell (HSC) renewal, megakaryocyte differentiation and platelet formation. By fusing a dimeric coiled coil in all seven possible orientations to the transmembrane-cytoplasmic domains of the TpoR, we showed that specific biological effects and *in vivo* phenotypes are imparted by distinct dimeric orientations. We identified one orientation that represents the inactive dimeric state and another that is synonymous with a physiologically activated receptor. Several other dimeric orientations were identified that induce proliferation and *in vivo* myeloproliferative and myelodysplastic disorders, indicating that the receptor can signal from several dimeric interfaces. We implicated TpoR in the myelodysplastic syndromes, a group of diseases in which myeloid differentiation is blocked but early progenitors are amplified. One cc-TpoR dimer, denoted cc-TpoR-IV, seems to induce strong cell-to-cell adhesion and effects on HSCs, whereas TpoR induces quiescence. This set of TpoR dimers allows us to investigate the precise signaling mechanisms mediating TpoR effects in HSCs, myeloid progenitors and megakaryocytes.

During the past year we obtained evidence that TpoR is critical for the phenotype induced by JAK2 V617F. We found that weak activation induces essential thrombocythemia, whereas strong activation promotes myelofibrosis. Most patients with MPNs strongly downmodulate TpoR levels in megakaryocyte and platelets. We showed in JAK2 V617F knockin mice and in patients that this effect can be prevented by treatment *in vivo* with JAK2 inhibitors. Signaling experiments showed that at high levels of JAK2 activation, or in the presence of JAK2 V617F, TpoR can exert antiproliferative and senescence-inducing effects that depend on Y626 of the cytosolic domain and on MAP-kinase and STAT3 pathways. TpoR downmodulation occurs as selection against this antiproliferative effect, and this allows continuous megakaryocyte proliferation at stages at which division should stop. Excessive TpoR activation or proliferation at late differentiation stages can induce major megakaryocyte differentiation defects, with pathologic release in the marrow of TGF-β and PDGF, which promote marrow fibrosis.

A hallmark of myeloid cancers is represented by constitutive STAT5

activation. We showed that this regulates genes other than the classical STAT5 targets activated by cytokines. We expanded on the identification of miR-28 and its host gene, LIM domain lipoma preferred partner, as novel targets of constitutive STAT5 activation. We also performed ChIP-on-chip analysis of all promoters targeted by STAT5 in a JAK2-dependent manner, and identified several new markers of essential thrombocythemia and myelofibrosis.

Inhibitors of JAK2 are becoming standard therapy in advanced (myelofibrosis) forms of MPNs, but because such molecules do not discriminate between wild-type and mutated JAK2, side effects such as anemia and thrombocytopenia are common. We alanine scanned the helices C of the kinase and pseudokinase domains of JAK2 and identified key residues required for JAK2 V617F activity, but not for cytokine-induced activation of wild-type JAK2. We explored novel mutations in JAK2 pseudokinase and kinase domain and studied the effect of ATP-competitive inhibitors in combination with serine-threonine kinase inhibitors in reducing proliferation of MPN cell lines or primary patients cells. This study has been performed in collaboration with the Experimental Therapeutics Center, A*STAR Singapore, where screening for inhibitors of TpoR W515L and JAK2 V617F are being planned with the support of the Ludwig San Diego Small Molecule Discovery Laboratory.

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Lausanne

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Developmental immunology

H. Robson MacDonald, Ph.D.

Our research in 2011 continued in two main areas. First, we have extended our analysis of the role of inhibitory and activating Ly-49 receptors in T cell development. Using a transgenic model in which prototypic activating (Ly-49D) and/or inhibitory (Ly-49A) receptors are expressed in the T cell lineage together with their common ligand H-2D^d, we have found that ligation of H-2D^d by Ly-49D blocks T cell development at a very early stage by allowing immature thymocytes to bypass the obligatory pre-T cell receptor (pre-TCR) checkpoint in the absence of expression of a TCR-β chain. Subsequent maturation of Ly-49D-induced thymocytes is impaired because they cannot express TCR-$\alpha\beta$, which is essential for positive selection and progression to the mature CD4+ or CD8+ stage. Moreover impaired thymocyte development in Ly-49D transgenic mice can be completely rescued by coexpression of a Ly-49A transgene, demonstrating that an appropriate balance between activating and inhibitory Ly-49 signaling is required for correct T cell development.

Our second major research theme concerns the role of Notch signaling in T cell development. Previous work from our group (in collaboration with Freddy Radtke of École Polytechnique Fédérale de Lausanne) demonstrated that T cell development in the adult thymus is initiated by an obligatory interaction between Notch1 on bone marrow–derived T cell progenitors and the Notch ligand delta-like4 (DL4) on thymic epithelial cells. We have now extended this analysis to the fetal thymus using a mouse model that allows for deletion of DL4 specifically in fetal thymic epithelial cells. We find that fetal thymic development of both $\alpha\beta$ T cells and $\gamma\delta$ T cells strictly depends on DL4-mediated Notch signaling. Thus Notch signaling is essential for T cell development in both the embryo and the adult.

Lymphocyte function

Werner Held, Ph.D.

We are focusing on the identification of factors that impact the elimination of diseased (stressed, infected or transformed) host cells by effector lymphocytes such as natural killer (NK) cells and T cells. NK cell function is profoundly influenced by the hosts' major histocompatibility complex (MHC) class I molecules. A functional adaptation process to MHC class I, which is known as NK cell education, allows efficient NK cell reactions to host cells that lose MHC class I molecules. We have found that education allows NK cells to control primary chronic myeloid leukemia in a murine stem cell transplantation model. NK cell education is mediated by inhibitory receptors specific for MHC class I molecules. In collaboration with G. Leclercq (University of Ghent), we found that inhibitory receptors educate only NK cells and not T cells, suggesting that education is a NK cell–specific process. These analyses have provided further insights into NK cell education and its role in the elimination of leukemic cells following stem cell transplantation.

Molecular immunology

Immanuel Luescher, Ph.D.

In 2011, the following projects were carried out. MHC-peptide multimers containing biotinylated MHC-peptide complexes bound to phycoerythrin streptavidin are widely used for analyzing and sorting of antigen-specific T cells. We developed alternative T cell–staining reagents that are superior to conventional reagents. They are built on reversible chelate complexes of Ni^{2+} nitrilotriacetic acid (NTA) with oligohistidines.

HLA-A2-2xHis6-peptide multimers containing either Ni^{2+} NTA_4-biotin and phycoerythrin streptavidin or phycoerythrin-NTA_4 stained influenza and melan-A-specific CD8+ T cells more efficiently than conventional multimers. Although these complexes were highly stable, they very rapidly dissociated in the presence of imidazole. This allowed sorting of bona fide antigen-specific CD8+ T cells without inducing T cell death and assessment of HLA-A2-peptide monomer dissociation kinetics on CD8+ T cells.

We examined CD8+ T cell responses upon acute lymphocytic choriomeningits virus (LCMV) infection in wild-type (WT) and CD8-β knockout (KO) mice expressing P14 TCR-β chain (P14-β). The primary LCMV-specific CD8+ T cells from both mice were predominantly GP33 specific, and those from P14-β CD8-β KO but not from P14-β WT mice were CD8 independent in terms of cytokine production, cytotoxicity and Db/GP33 tetramer binding. The TCR-α chain repertoire of CD8+ T cells from P14-β CD8-β KO mice was narrower compared with cells from P14-β wild-type mice and remarkably different. Our results argue that CD8-independent CD8+ T cells are preferentially selected and expanded upon LCMV infection in CD8-β KO mice, exhibiting a unique TCR repertoire that translates into noncanonical docking of TCR to MHC-peptide complexes and induction of different TCR signalling.

CD8+ cytotoxic T lymphocytes from CD8-β KO mice exhibit greatly increased Fas/FasL-mediated cytotoxicity that effectively compensates for their blunted granzyme/perforin-mediated killing. We introduced CD8-β in CD8+ T cells from CD8-β KO mice and after activation examined their properties and capacity to eradicate established B16 melanoma tumors. CD8+ T cells from P14-β CD8-β KO (CD45.2+) mice were reconstituted with CD8-β, transferred into C57BL/6 (B6) (CD45.1+) mice and differentiated/activated by LCMV infection. Analysis of Db/GP33 tetramer+, CD45.2+ cells 8 d later revealed that CD8-β reconstituted cells exhibited high perforin/granzyme-dependent cytotoxicity and strongly increased Fas/FasL killing in addition to augmented interferon-γ (IFN-γ) responses. When these cells were transferred into GP33+ B16 tumor–bearing hosts, they

effectively eradicated established tumors; this was not observed in mice receiving cells from P14-β WT and CD8-β KO mice. We conclude that the tumoricidal activity of tumor-specific CD8+ T cells is greatly enhanced by upregulation of their Fas/FasL-mediated cytotoxicity and IFN-γ responses, and that their combined attacks prevent tumor escape.

Translational tumor immunology

Pedro Romero, M.D.

This year, we continued to focus on different aspects of T lymphocyte responses to tumor antigens in the settings of tumor progression and therapeutic vaccination in both cancer patients and preclinical mouse models.

In one study, we have been assessing the role of the microRNA miR-155 in modulating CD8 T cell responses to tumor progression or viral infection. We found that miR-155 expression is intrinsically required for optimal effector CD8 T cell responses. We also showed that miR-155-deficient CD8 T cells cannot efficiently control experimental tumor growth. Conversely, enforced overexpression of miR-155 in tumor antigen–specific CD8 T cells greatly enhanced their ability to control tumor growth. We currently aim to identify possible relevant targets of miR-155 and its exact role in the memory phase of the CD8 T cell response, particularly in the context of therapeutic vaccination against large tumors.

In another study, we want to understand the value of the ratio of effector T (T_{eff}) cells to regulatory T (T_{reg}) cells as a biomarker of vaccination. We use the TCR transgenics OT1 and OT2 as mouse models. Using adoptive transfer of OT-1 and OT-2 x eGFP-Foxp3 TCR transgenic T cells, we are testing the effect of different vaccine formulations on the ratio of T_{eff} and T_{reg} antigen-specific CD8 and CD4 T cells, respectively, in the elicited response. Our results show that the use of peptide alone favors a low ratio because of selective T_{reg} expansion, whereas the addition of Toll-like receptor (TLR) agonists, notably TLR9 agonists, promotes high ratios because of strong T_{eff} expansion. A saponin preparation seems to favor a low ratio. Preliminary results show that high T_{eff}:T_{reg} ratios upon vaccination are associated with better tumor growth control in the therapeutic setting.

Our studies on TLR3 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 1980s using poly(A:U), a double-stranded RNA analog that specifically engages TLR3, strongly suggests the value of TLR3 expression in resected primary tumors as a biomarker of the clinical efficacy of poly(A:U). These results encourage considering the use of TLR3 agonists as antitumor agents in melanoma and breast cancer expressing TLR3.

Finally, this year we concluded a detailed analysis of the melan-A peptide

antigen-specific CD4 T cell response in two small cohorts of metastatic melanoma patients vaccinated with a long synthetic peptide encompassing both class I and class II restricted melan-A T cell–defined antigens. Notably, the vehicle used in these experimental vaccination studies comprised viral-like particles in which the peptide was covalently linked to the repeating monomer making up the particles and also included a type A CpG-ODN, a potent TLR9 agonist. Our results showed that relatively strong CD4 T cell responses were induced in the majority of patients and responding cells showed a Th1-like functional profile.

Clinical-translational oncology

Daniel Speiser, M.D.

We isolated fresh tumor-specific CD8 T cells from metastases of melanoma patients. T cell effector functions were analyzed directly *ex vivo*. Interestingly, although T cells from tumor tissue were functionally deficient, T cells of similar specificity isolated from the blood readily produced IFN-γ and were highly cytotoxic when analyzed directly *ex vivo*. These data demonstrate that functional competent and dysfunctional T cells of identical specificity can coexist in the same host.

Thanks to the availability of many tumor antigen–specific CD8 T cells, we performed gene expression profiling directly *ex vivo*. T cells were prepared from patients after vaccination with melan-A peptide, CpG 7909 and IFA. In parallel, we analyzed *EBV*- and *CMV*-specific CD8 T cells, as reference populations of protective T cells. In peripheral blood, melan-A-specific T cells expressed multiple genes characteristic for T_{eff} cells, with only small differences compared to *EBV*- and *CMV*-specific T cells, indicating that the three CD8 T cell populations are functional effector cells. In contrast, the gene expression profiles of melan-A specific T cells isolated from metastases were very different and showed extended molecular alterations. This expression profile was correlated with the one observed in so-called exhausted T cells from mice chronically infected with LCMV clone 13. In contrast, the gene profile of the T cells from melanoma metastases was largely different from murine “anergic” T cells. These data suggest that mechanisms of exhaustion are responsible for the functional deficiencies of human T cells in melanoma metastases. However, further investigations are needed to validate and refine these findings. Further studies particularly need to elucidate mechanisms underlying the fact that T cell dysfunction in metastases is reversible, and that effector cells in circulation and exhausted cells in the tumor environment coexist in the same host.

Gene expression profiles indicate that functional T cell deficiency is due to alterations in multiple molecular pathways. These pathways may represent potential targets for cancer therapy. Recently we studied the newly discovered inhibitory receptor BTLA (B and T lymphocyte attenuator, CD272). We demonstrated that this receptor significantly inhibited human CD8 T cell responses. BTLA-mediated inhibition was particularly marked in tumor-specific T cells. This occurred when BTLA interacted with its ligand herpesvirus entry mediator, which is expressed by tumor cells *in situ*. We found that melanoma cells often expressed herpesvirus entry mediator *in vivo*, even more frequently than ligands of other inhibitory pathways. Interestingly, immunotherapy with CpG-based vaccines but not other vaccines induced BTLA downregulation and thus made T cells

partially resistant to this inhibition. Therefore, BTLA-mediated inhibition can be partially reversed by vaccination with peptides plus CpG 7909. More recently, we characterized the expression of further inhibitory receptors, including CTLA-4, PD-1, KLRG-1, 2B4, CD160, LAG-3 and TIM-3. Our data demonstrate that T cells in melanoma metastases coexpress many of these receptors, and that all of their ligands are present *in situ*. These data provide a rationale for improving immunotherapy of cancer by combining powerful T cell vaccines with treatments blocking T cell suppression in metastases.

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Melbourne-Austin

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Director's message

The scientific laboratory programs at the Melbourne-Austin Branch comprise themes in signaling pathways, transcriptional regulation of cancer, cancer immunology and tumor biology. Our papers in major basic and clinical research journals over the past year reflect these themes. Important discoveries made during the year include the role of phosphatase and tensin homolog (PTEN) in induction of intestinal adenoma and carcinomas; the phenotypic plasticity of melanoma, which contributes to resistance to therapy; and the structural interplay between growth factor receptors and intracellular signaling pathways. These discoveries are relevant to cancer biology and have potential clinical applications.

Our translational research into antibody and signaling therapeutics, cancer biomarkers and cancer vaccines, as well as clinical trials for proof-in-concept and phase 2 trials, plays a major part in Ludwig activities. Notably, a phase 1 bioimaging study with ABT-806, a Ludwig monoclonal antibody (mAb) against the epidermal growth factor receptor (EGFR) and licensed to Abbott Laboratories, began in 2011 with Melbourne-Austin as the leading global clinical site.

We continue to be very successful in our applications for competitive grant support. During 2011 a particular highlight was an AUD $2 million grant from the Australian Cancer Research Foundation to purchase state-of-the-art molecular imaging equipment for our animal models.

In conjunction with our host institution, Austin Health, we obtained AUD $44.88 million in funding for the completion of the Olivia Newton-John Cancer and Wellness Centre. It will house a full range of oncology clinical (inpatient and outpatient) services, clinical trial and research facilities, and almost 6,000 square meters of laboratories and administration space. Completion of stage 1 of this comprehensive cancer center is scheduled for June 2012, and stage 2 for June 2013. This is an exciting time for the Ludwig Melbourne-Austin, as this new space will enhance our research capacity and enable our discovery and clinical programs to flourish and significantly contribute to the Ludwig mission.

Andrew M. Scott

Tumor targeting

Andrew M. Scott, M.D.

The Tumour Targeting Laboratory focuses on targeting and molecular imaging of tumors, and exploring receptor-based signaling pathways responsible for cancer cell growth. We are pursuing immunological approaches to enhance cell-killing mechanisms of recombinant antibodies, and undertaking clinical trials of novel antibodies, small molecules and imaging ligands.

The laboratory has a major focus on the biology and signaling pathways involved in erbB family members of receptors. Our research into the mechanism of action and cellular function of the mAb 806, which targets a conformationally exposed epitope of erbB1 (EGFR), has continued over the past year. We have also been engaged with Abbott Laboratories for clinical development of mAb806 and ^{111}In-labeled ABT-806 (ABT-806i) for bioimaging. Our research has also extended to identifying similar conformational epitopes in other erbB receptors, and generating and characterizing novel antibodies against these targets.

Our collaborative research into the ephA3 receptor–binding antibody IIIA4 with Martin Lackmann (Monash University) continues with exploration of the biochemical and biologic sequelae of EphA3 function in mouse models, and development of a humaneered form of the antibody, which entered a first-in-human study in Australia in late 2011.

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-Leʏ humanized antibody hu3S193 in phase 1 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 of hu3S193 that are 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 complement-dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity is being correlated with C1q and FcgRIII binding properties, respectively. We have also identified strategies for successful linkage of short interfering RNA to STAT3 to antibody (hu3S193), and achieved knockdown of STAT3 in cell systems. This is currently being extended to *in vivo* models, which we are using to understand the impact of STAT3 knockdown on signaling pathways and tumor growth.

As a partner of the Victorian Cancer Biologics Consortium, the laboratory has

been actively involved in the development of PEG-AVP0458, a PEGylated recombinant diabody targeting the TAG-72 pancarcinoma antigen. It is designed to deliver toxic payloads to targeted tumor tissue. A phase 1 safety and biodistribution study of PEG-AVP0458 radiolabeled with ^{124}I in patients with advanced ovarian and prostate cancer is scheduled to begin in 2012.

During 2011 our clinical trial program completed a protocol with anti-CAIX antibody cG250, combined with sunitinib malate (marketed as Sutent), in patients with advanced or metastatic renal cell carcinoma. This trial explored molecular expression of CAIX and targeting of renal cell carcinoma by cG250 in these patients, and evaluated the efficacy of the combination treatment. In collaboration with Abbott Laboratories, our clinical trial program began a phase 1 imaging trial of ABT-806i in patients with advanced cancer. We also continued our imaging study of anti-DR5 antibody CS-1008 in patients with metastatic colorectal cancer in collaboration with Daiichi Sankyo Pharma Development.

Positron emission tomography

Andrew M. Scott, M.D.

The Centre for Positron Emission Tomography at Austin Hospital has developed an integrated clinical and research positron emission tomography (PET) program and is the largest academic PET center in Australia. Many of the research studies combine quantitative PET measurements of tumor metabolism with detailed protein profiles and gene arrays to compare *in vivo* analyses with proteomic and genetic characteristics of tumors.

During 2011 we began construction of a solid-targetry laboratory to produce long-lived PET isotopes (for example, ^{124}I, ^{64}Cu, ^{89}Zr and ^{86}Y) that will be used for protein-based molecular imaging studies in animal models and human trials. The Ludwig oncology clinical PET program continued with ^{18}F-FDG PET studies for oncology patients and 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. In cancer patients, we have also been quantitatively assessing pharmacokinetics and pharmacodynamics (metabolism, hypoxia and blood flow) of novel mAbs using ^{124}I-antibody, ^{18}F-FDG, ^{18}F-FMISO and $H_2{}^{15}O$ PET.

Molecular imaging of tumor metabolism and evaluation of new therapy pharmacodynamics remain a major focus of our research program. Studies of hypoxia in metastatic colorectal carcinoma and glioma, using ^{18}F-FMISO PET and ^{18}F-FDG PET, have demonstrated patterns of anaerobic and aerobic glycolysis that predict response to standard therapy and provide new insights into cancer metabolism. With Ian Davis and clinical collaborators, we have demonstrated the ability of ^{11}C-choline to stage prostate cancer more accurately than standard imaging tests in newly diagnosed patients, and to assist in radiotherapy treatment planning in patients with large primary prostate cancers. We have developed pioneering treatment strategies for mesothelioma patients using PET-directed radiotherapy with radiation oncologist Malcolm Feigen. These strategies have shown high response rates and improved overall survival.

We have also extended our animal model PET/SPECT/CT/Doppler microscopy platforms to investigate nanoparticle biodistribution, study antibody pharmacokinetics and pharmacodynamics, and develop novel PET hypoxic probes. Integrating multiple molecular imaging technologies, including PET, CT/MRI and optical imaging, remains a focus of our imaging research program.

Cancer immunobiology

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

This program integrates clinical and laboratory teams to better understand immune responses against cancer and to develop new cancer therapies based on this knowledge. In previous years, the Cancer Vaccine Program has focused on the immunotherapy of cancer. This led to detailed studies of antigen expression in melanoma and to investigations of the poorly understood mechanisms of heterogeneity in melanoma. The research focus has broadened to include extensive studies of cellular subpopulations in melanoma. To reflect this, the name of the laboratory was changed in 2011 from the Cancer Vaccine Laboratory to the Cancer Immunobiology Laboratory.

The cancer testis antigen NY-ESO-1 has been a model antigen for our laboratory and clinical research. Analyses from our previous phase 1 and phase 1/2 studies enabled characterization of tumor antigen expression and patient immunological profiles. These studies included evaluation of major histocompatibility complex class I downregulation, quality and specificity of the immune response (including fine specificity of epitopes generated through vaccination compared with those presented by cancer), and the presence of regulatory or immunosuppressive mechanisms in the tumor microenvironment, including antigen-specific regulatory T (T_{reg}) cells.

Although vaccination can generate robust immune responses, clinical impact remains elusive. In addition to potential immune-evasive mechanisms, cellular heterogeneity within the tumor population probably contributes to escape from therapeutic control. In preliminary studies, we identified subpopulations of tumor cells that arise as a result of phenotypic switching from a proliferative to an invasive state. The plasticity that enables transformation between these subpopulations may explain how tumor cells evade therapy. To further characterize these subpopulations, cells have been isolated on the basis of functional assays, such as resistance to chemotherapeutic drugs and retention of a dye that dilutes upon cell division, thereby defining slow cycling. Analysis of gene expression in these cells is enabling identification of potential novel therapeutic targets. The first results of these studies have identified epithelial-to-mesenchymal transition as key process in the heterogeneity, chemoresistance, motility and invasiveness of melanoma cells. We are evaluating the molecules that mediate this process as new targets for the clinic.

Our collaborative relationships with pharmaceutical companies, including CSL, Bristol-Myers Squibb, Sirtex Medical, Roche and GlaxoSmithKline, continue to provide basic and translational clinical research opportunities.

We embarked on a collaborative project with CSL to identify novel antibodies to target melanoma cells using phage display technology. In 2011, Ludwig continued its long-standing collaboration with GlaxoSmithKline on studies of BRAF and MEK inhibitors *in vitro* on melanoma cell lines; melanoma gene signatures and the impact of adjuvants on NY-ESO-1 cross-presentation *in vitro.*

Current clinical research projects include playing an international leadership role in a multicenter phase 1 trial of a new vaccine against NY-ESO-1 and a study with a MAGE A3 vaccine in patients with resected hepatocellular carcinoma. This study will start in 2012 and will be performed throughout Asia and Australia by the Asia-Pacific Hepatocellular Carcinoma Trials Group. Two other phase 1 clinical trials were designed and planned in 2011, one evaluating a combination therapy with ipilimumab in stage III/IV melanoma and the other evaluating a combination of intrahepatic embolization and a radiosensitizer in patients with uveal melanoma and liver-only metastases.

Joint Austin Ludwig oncology unit

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

The Joint Austin Medical Oncology Unit was established to enable translational clinical research by Ludwig clinical investigators. Hui Gan and Tom John focus on phase 1 drug development and lung cancer, respectively. Ian Davis and Niall Tebbutt contribute clinical evaluations of new treatments for urological and gastrointestinal cancers, respectively. Immunotherapy of melanoma remains the major focus of the unit led by Jonathan Cebon.

Hui Gan has continued to expand the phase 1 trial portfolio at Austin Health, with an emphasis on novel targeted therapies. We have successfully begun a phase 1 bioimaging study using ABT-806, a novel mAb against EGFR that was developed at the Institute and licensed to Abbott Laboratories. Gan received a prestigious Victorian Cancer Agency grant to foster collaborations between Austin Health and neighboring centers to enhance phase 1 clinical trial activity. Gan contributes to a translational research program in head and neck cancer focused on identifying new therapeutic targets (in collaboration with Ludwig São Paolo) and biomarkers.

Tom John has undertaken a series of projects focusing on melanoma, breast and lung cancer. This includes further investigation of an earlier finding that expression of cancer testis antigen NY-ESO-1 predicts response to chemotherapy in lung cancer. His current research focuses on methylation markers in lung cancer and determining whether these can be induced through demethylating drugs. In collaboration with researchers at St. Vincent's Hospital, John is investigating histological subtypes in lung cancer. He has demonstrated that cancer testis antigens are associated with improved outcome in stage III melanoma, and is currently focusing on the validation of a melanoma gene signature. Both of these projects are being run under the auspices of the Melbourne Melanoma Project.

Ian Davis has continued as Australian lead investigator for several clinical protocols to develop new drug treatments for urological cancers. Among these is a trial for MDV3100, a novel androgen receptor antagonist, in patients with metastatic castration–resistant prostate cancer before chemotherapy. A previous trial of this drug in which the unit participated, involving postchemotherapy patients, has recently been reported and shows a survival advantage for the drug. The broader Austin Uro-oncology Group, led by Davis, continues to perform a wide range of clinical trials across the various types of genitourinary cancer and has a broad spectrum of collaborators on and off campus.

Niall Tebbutt has continued research in gastrointestinal cancers focused on novel biologic therapies and predictive biomarkers. Accrual to the investigator-initiated RADichol study involving everolimus in advanced cholangiocarcinoma was completed, and results are expected next year. Translational work evaluating VEGF-D as a predictive biomarker for bevacizumab in advanced colorectal cancer was presented at the American Society of Clinical Oncology Annual Meeting. Additional work evaluating the predictive and prognostic effects of *k-ras* and *b-raf* mutations in patients with advanced colorectal cancer treated with bevacizumab and the phase 2 DUX study evaluating dual targeting of EGFR in chemotherapy-refractory colorectal cancer were published in the *Journal of Clinical Oncology*.

T cell

Weisan Chen, Ph.D.

Research in the T Cell Laboratory focuses on T cell biology and vaccine development. Laboratory activities are divided between human T cell research and murine models of influenza infection and tumor antigens to elucidate mechanisms associated with immunodominance; interaction between T cells with different antigen specificity; interaction between T cells and antigen-presenting cells; and antitumor immunity in the presence and absence of innate signals. The laboratory also continues to monitor patient T cell responses to cancer vaccines from clinical trials conducted at Ludwig Melbourne-Austin.

In the human system, our laboratory has continued studying T_{reg} cell epitopes specific to antigen NY-ESO-1 using a novel CD3-downregulation method recently developed in our lab. We have shown that many late-stage melanoma patients who received the NY-ESO-1/ISCOMATRIX vaccine had both NY-ESO-1-specific effector T (T_{eff}) cells and T_{reg} cells induced. We also showed that CD4+ T_{eff} and T_{reg} cells can recognize the same T cell epitopes and that a high percentage of antigen-specific T_{reg} cells are detected in tumor sites. Such knowledge is important for improving vaccine designs and T cell monitoring.

Although influenza A virus (IAV) has been studied for many decades, cellular immunity has not been well characterized. We have established a novel systematic approach to identify the most immunodominant CD8+ T cell epitopes from IAV-experienced individuals. Results showed that existing computer prediction algorithms are not accurate, especially when predicting immunodominant T cell epitopes. We have now shown that the most immunodominant CD8+ T cell responses in HLA-A2-negative, healthy individuals also focus on epitopes derived from the IAV nucleoprotein. We also showed that many immunodominant epitopes would not have been predicted by any available program as they either do not contain typical motifs or are too long. Similarly, using a transgenic mouse system that express human HLA-B7 and our systematic approach, we have identified the most immunodominant CD8+ T cell epitopes presented by this HLA molecule. Data indicate that HLA-transgenic mice might be useful in epitope discovery. The knowledge could help in developing a universal T cell–based vaccine to IAV.

We have recently generated an orthotopic murine cutaneous melanoma model. B16 melanoma cells are inoculated in the skin dermal layer, ensuring their interaction with dermal dendritic cells (DCs) and Langerhans cells. Compared with the widely used subcutaneous model, the biggest difference

is that cutaneous melanoma forms early metastasis in draining lymph nodes whereas the subcutaneous tumor continuously grows under the skin with limited interaction with dermal DCs. We have now shown that skin-derived CD103+ DCs play a major role in cross-presenting tumor derived antigens.

Our laboratory also showed that the immunoproteasome plays a "housekeeping" role. Mice with deficient immunoproteasomes show impaired B cell differentiation, reduced B cell number and defective B cell function, such as diminished antibody response to IAV and antibody class switching. We have now made a similar observation for T cells in these mice. Although T cells have similar a number of bone marrow precursors, their thymic development and survival are significantly impaired. More specifically, because of reduced proteasome-dependent degradation of the inhibitor of nuclear factor κB, the nuclear factor κB signaling pathway in DN3 cells is impaired in immunoproteasome-deficient mice, leading to inefficient transition from DN3 to DN4 stage accompanied with higher apoptosis rate.

Oncogenic transcription

John Mariadason, Ph.D.

Colorectal cancer is one of the leading causes of cancer-related death in the Western world. Although treatment of this disease has significantly improved in the past decade, the five-year survival rates for patients with nonresectable metastatic colon cancer remains <15%. Strategies to improve outcomes for patients with this disease are urgently needed. The Ludwig Oncogenic Transcription Laboratory focuses on this overarching goal in two ways: discovering new treatment targets for colon cancer and streamlining the use of existing therapies through the identification of biomarkers that predict drug response. We describe two areas that were actively investigated in 2011 below.

Class I and II histone deacetylases as potential treatment targets for colon cancer

Our laboratory has previously demonstrated that three members of the histone deacetylase (HDAC) family of transcriptional corepressors are upregulated in colon cancer, and we have demonstrated that these enzymes play a proproliferative/prosurvival role in colon cancer cells. These findings suggest that inhibition of HDAC activity may be a potential mechanism of inhibiting the growth and survival of colon cancer cells. Notably, several compounds which inhibit HDACs have been developed, with two agents, vorinostat and romidepsin, which are approved for the treatment of cutaneous T-cell lymphoma. Consistent with the protumorigenic activity of HDACs, we identified multiple colon cancer cell lines which are highly sensitive to HDAC inhibitor–induced apoptosis. We then compared the gene expression changes induced by HDAC inhibitors in colon cancer cell lines sensitive and resistant to these drugs, and identified a set of 48 genes, comprising multiple immediate-early and stress response genes, preferentially induced by these drugs in sensitive colon cancer lines. Our work this year has focused on understanding the mechanisms by which induction of immediate-early genes contributes to apoptosis, and to determining whether HDAC inhibitors also induce this transcriptional response in other tumor types, particularly hematological malignancies such as cutaneous T-cell lymphoma in which HDAC inhibitors are clinically used. We are also investigating whether other agents which can induce similar transcriptional responses can be used to enhance the apoptotic activity of HDAC inhibitors.

Intestinal-specific inactivation of PTEN leads to intestinal tumorigenesis

The PI3K/PTEN signaling pathway is constitutively activated in a high percentage of colon cancers, due to either activating mutations in the

PIK3CA gene or mutational or epigenetic inactivation of the negative regulator of PI3K signaling, PTEN. To directly investigate the importance of this signaling pathway in intestinal tumorigenesis, we specifically hyperactivated PI3K signaling in the mouse intestinal epithelium by targeted deletion of the gene encoding PTEN in this tissue. We observed that while intestinal-specific PTEN deletion did not have a major effect on cell fate determination or proliferation in the small intestine, 19% of animals developed small intestinal adenomas and adenocarcinomas at 12 months of age. These tumors demonstrated strong pAKT and nuclear β-catenin staining, indicating simultaneous activation of the PI3K/AKT and Wnt signaling pathways. The long latency of tumor formation and the presence of nuclear β-catenin in these tumors suggest that PI3K signaling probably serves to facilitate tumor promotion in the intestinal tract, subsequent to spontaneous activation of β-catenin/TCF signaling. These findings further establish PTEN as a bona fide colon cancer tumor suppressor gene, and suggest that inhibition of PI3K signaling may have therapeutic benefit in treatment of colon cancer.

Uro-oncology

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

The Uro-oncology Laboratory investigates prostate cancer (PC). PC is understudied, largely because of the paucity of relevant commercial cell lines and the difficulty of culturing fresh prostate cancer cells *in vitro* or in animal xenografts. Clinical observations highlight key aspects of PC behavior that require better understanding, such as why PC preferentially metastasizes to bone; why some apparently similar PCs behave aggressively whereas others do not; why some advanced PCs remain sensitive to androgen receptor signaling whereas others become independent of it; how the immune system interacts with PC; and what changes in the sex steroid milieu are associated with treatment. Further advances in treatment of PC 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 built on our previous PC clinical, basic and translational research, and included themes of epithelial-stromal interactions, *in situ* immunology, mechanisms of escape from effective androgen receptor blockade, and effects of sex steroids on immune function. Key to these research themes is the ability to work with fresh tissue from PC patients in various clinical settings.

Through exploring prostate-stromal interactions we aim to determine how PC metastasizes, and why bone is the preferred site. Despite access only to tiny clinical tissue samples, we can enrich for PC epithelial cells, obtain autologous matched cancer-associated fibroblasts and infiltrating immune cells, compare these with concurrent blood samples, and expand and maintain cells for sufficient periods to perform key assays. We have uncovered evidence of possible autocrine loops; separated PC cells based on function and subsequent phenotypic analysis and begun to explore functional differences between basal and luminal cells. We are planning relevant *in vivo* experiments to determine the functional implications of these findings. This work could potentially identify new therapeutic targets on cells other than malignant epithelial cells, and allow identification of new predictive and prognostic markers.

Exploring PC immunology *in situ* is a complex project. It involves detailed assessment and comparison of immune cell subsets in terms of phenotype and function in blood and in small amounts of available PC tissues using multicolor flow cytometry. We have determined T_{reg} cell numbers and proportions in benign and malignant prostate tissue and, paradoxically, we

have detected activation of CD8+ T cells within tissues despite the presence of T_{reg} cells. The functional status of these cells will be determined.

Mechanisms of escape from abiraterone therapy are being explored using a heterotopic xenograft method that allows reliable culture of primary PC samples and expansion to amounts suitable for detailed analysis of genomic and proteomic profiles and sex steroidogenesis.

We are also making detailed immune parameter measurements in men undergoing androgen deprivation therapy to study the effects of sex steroids and specific sex steroid receptor antagonists on immune function.

Urological Clinical programs included ^{18}F-FDG and ^{11}C-choline PET, with completion of two clinical trials in PC. This work has been extended in a parallel study of radiotherapy "dose-painting" and has received several awards at international conferences.

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Director's message

During 2011, the Ludwig Parkville Branch has seen the move of the successful Joint Proteomics Research Laboratory, led by Richard Simpson, to La Trobe University, Melbourne, while the Walter and Eliza Hall Institute of Medical Research has agreed to take on sole responsibility for the Joint Proteomics Services Facility. This marks the successful transition of an integral core facility that has been part of Ludwig Melbourne-Parkville since its inception and now continues to provide services to the broader research community in Melbourne and farther afield.

Our research focuses on intestinal cancer, with the largest effort directed to the colon. This work continues to provide novel and exciting insights into the role of genomics, cellular interactions and protein functions in this disease and is complemented by preclinical models and clinical trials. Together with our colleagues at Ludwig Melbourne-Austin, Ludwig researchers in Parkville, including the Epithelial Biology Laboratory (Tony Burgess), the Colon Molecular and Cell Biology Laboratory (Joan Heath and Matthias Ernst) and the Ludwig Colon Cancer Initiative Laboratory (Oliver Sieber and Peter Gibbs), remain Australia's leading group of basic and clinician scientists working on a single solid malignancy. Ludwig Melbourne-Parkville researchers further consolidated their success by securing highly competitive funding from Australia's primary medical research funding body, the National Health and Medical Research Council, at a ratio among the top in the country.

Ludwig contributions to basic and translational research continue to be recognized at the highest international levels, with publications in *Proceedings of the National Academy of Sciences*, the *Journal of Immunology*, *Public Library of Science Genetics*, *Nature Reviews Cancer* and other journals. At the same time, our junior and senior staff have received prestigious national and international awards as testimony to the outstanding quality of their basic and translational research.

Matthias Ernst

Colon molecular and cell biology

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

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

The major focus of the laboratory is to dissect the molecular mechanisms that link inflammation to tumor promotion, on the premise that tumor promotion is often facilitated though interactions between neoplastic cells and the microenvironment is characterized by many inflammatory mediators. Although the molecular mechanisms linking inflammation and cancer may vary between different malignancies, ultimately most soluble mediators converge on nuclear factor κB and Stat3 in tumor cells to enhance survival and, in the case of Stat3, to promote proliferation and invasion and mediate an angiogenic switch.

The interleukin-6 (IL-6) family member IL-11 is readily detected in various inflammation-associated pathologies, including colorectal and gastric cancers. IL-11 mediates its pleiotropic activities through the ubiquitously expressed gp130 receptor. Using various combinations of loss- and gain-of-function alleles in mouse models of inflammation-associated gastric and colitis-associated colon cancer, we have established a novel link among IL-11, the gp130/Stat3 signaling axis and tumor progression. Accordingly, gastric cancer and colitis-associated colon cancer lesions are exaggerated in mice, where ligand-dependent Stat3 hyperactivation mimics the smoldering inflammation observed in corresponding human cancers. Meanwhile, tumor maintenance is susceptible to systemic administration of Stat3 or IL-11 antagonists, and the physical proximity within the tumor microenvironment between IL-11-producing and IL-11-responsive neoplastic epithelium suggests an attractive target for novel therapeutic targets in inflammation-associated (gastrointestinal) cancers. The laboratory has been exploring this notion in a collaborative research arrangement with the Australian biotechnology company CSL Limited.

Recent findings from the laboratory also indicate that the aforementioned gp130-cytokine/Stat3-addicted tumors become susceptible to orthogonal treatment intervention based on a striking coactivation of mTor complex 1 (mTorc1) and Stat3. Surprisingly, mTorc1 inhibitors suppressed initiation and progression of inflammation-associated gastric cancer and colitis-associated colon cancer in mice without inhibiting Stat3 activation. We

used genetic complementation experiments to identify the molecular mechanism underpinning gp130 receptor–dependent PI3K/mTorc1 activation to uncover an unsuspected gatekeeper role for this signaling cascade. Because long-term therapeutic administration of mTorc1 inhibitors impaired tumor proliferation and vascularization despite excessive Stat3 activity, clinically approved "rapalogues" and/or related PI3K inhibitors may afford readily testable therapeutic opportunities for inflammation-associated malignancies.

Given that aberrant activation of Wnt/β-catenin signaling is a unifying finding in sporadic colorectal cancer in humans, the laboratory also investigates the mechanisms underpinning cross-talk between the Stat3 and Wnt pathways. For instance Wnt-mediated regulation of intestinal stem cells is critical for epithelial regeneration and tumorigenesis, whereas excessive Stat3 signaling is a recurrent feature in intestinal cancer and promotes stem cell self-renewal. Surprisingly, we found that excessive, cell-autonomous gp130/Stat3 signaling promotes Wnt-induced intestinal regeneration after γ-irradiation. Similarly, excessive Stat3 activity exacerbates intestinal tumorigenesis in Apc^{Min} mice, which, akin to familial adenomatous polyposis patients, carry a heterozygous impairment mutation in the negative regulator of canonical Wnt signaling, *Apc*. In these mice, Stat3 enhances only tumor initiation, and not tumor growth, while promoting both processes in situations of chronic inflammation and colitis. Conversely, genetic restriction of Stat3 expression reduces tumor incidence in Apc^{Min} mice and suggests that Stat3 is rate limiting for Wnt-induced intestinal tumorigenesis. We observe reduced colony formation of human colonic cancer cells that express mutant (that is, defective) APC, but not in their isogenic counterpart with restored full-length APC expression. We reconcile these observations on a molecular level with the finding that excessive gp130/Stat3 signaling induced expression of antiapoptotic expression programs to confer a survival advantage for the intestinal stem cell compartment in situations of high Wnt signaling that occur during regeneration and tumorigenesis. These findings establish a novel role for gp130/Stat3 signaling that can be therapeutically exploited in regenerative medicine and treatment of Wnt-dependent tumorigenesis.

In the laboratory led by Joan Heath, we have focused on characterizing the collection of zebrafish intestinal mutants we identified in collaboration with Didier Stainier at University of California, San Francisco. Through our analysis of the *caliban* mutant, which harbors a point mutation in a gene (*rnpc3*) encoding a protein that is a unique component of the minor-class spliceosome, the role of minor-class mRNA splicing in gene expression has captured our attention. This process is required specifically to remove a small set of introns (<0.5% of all introns) that are distributed throughout the genome. In our *caliban* mutant, aberrant minor-class splicing leads to intron retention in transcripts harboring minor-class introns and causes abnormal patterns of gene expression that are incompatible with life. The significance of this observation was underscored recently in humans: the genetic basis of a severe developmental disorder (TALS/MOPD) was found to reside in a recessive mutation in another unique component of the minor-class

spliceosome. This provided the first evidence that loss of minor-class splicing causes human disease. Interestingly, some prominent oncogenes and tumor suppressor genes contain minor-class introns and *RNPC3* is frequently downregulated in colon cancer. To test our hypothesis that aberrant splicing of transcripts containing minor-class introns may contribute to colon tumorigenesis, we have recently generated conditional mouse models of Rnpc3 deficiency on tumor-prone backgrounds. In the coming months, we will be evaluating the propensity of these animals to develop cancer.

In collaboration with Frank Caruso, a researcher in the Department of Chemical and Biomolecular Engineering at the University of Melbourne, we recently demonstrated the capacity of submicron-sized layer-by-layer capsules functionalized with the intestine-specific A33 antibody to target colorectal cancer cells *in vitro*. These results support the use of tissue-specific antibodies to functionalize the surface of drug delivery vehicles. We also investigated the influence of thiols and disulfide linkages of layer-by-layer-assembled poly(methacrylic acid) capsules on cell entry and cargo release. Thiolated poly(methacrylic acid) was used to prepare single-component polymer capsules stabilized by disulfide bonds. These disulfide-bonded capsules were stable under oxidizing conditions, such as the circulating blood, but collapsed in the reducing environment of the cell cytoplasm. We found that disulfide bonds in the thiolated capsules were crucial for the effective release of small hydrophobic cargo inside the cell. Futhermore, the presence of free thiols on the surface of the capsules enhanced their adhesion to the cell surface, thereby facilitating cell membrane wrapping, internalization of the capsules and cargo release. We hope that these findings will contribute to the rational design of thiol-disulfide-containing delivery systems for biomedical applications.

Epithelial biochemistry

Tony Burgess, Ph.D.

Our laboratory is focused on understanding the biochemistry and biology of normal and cancerous colonic cells. We are studying the role of Apc in the β-catenin destruction complex and comparing this with potential roles for Apc in cell adhesion and migration. We are particularly interested in the roles of phospho-β-catenin in activation of transcription, cell adhesion and migration; the synergy between Myb and β-catenin regulation of transcription in crypt stem cells during Wnt signaling; and the effects of growth factor regulatory systems on crypt morphogenesis, stem cell localization and crypt fission.

Leucine-rich repeat–containing G protein–coupled receptor 5 (LGR5) is the most established marker for intestinal stem cells, however very little is known about LGR5 function or its contribution to the stem cell phenotype and to colorectal cancer. We modulated the expression of LGR5 by RNA interference or overexpression in colorectal cancer cell lines. Paradoxically, ablation of LGR5 induces increased invasion and anchorage-independent growth and enhances tumorigenicity in xenograft experiments. Conversely, overexpression of LGR5 augments cell adhesion, reduces clonogenicity and attenuates tumorigenicity. Our results indicate that cell surface LGR5 is important for restricting stem cells to their niche and that loss of LGR5 may contribute to the invasive phenotype of colorectal carcinomas.

β-catenin is a signaling protein with diverse functions in cell adhesion and Wnt signaling. Although β-catenin participates in many protein-protein interactions, it has not been clear which combinations of β-catenin-interacting proteins form discrete complexes. We generated a novel antibody (4B3) that recognizes a small subset of total cellular β-catenin. Affinity proteomics using 4B3, in combination with subcellular fractionation, has allowed us to define a discrete trimeric complex of β-catenin, α-catenin and the tumor suppressor APC, which forms in the cytoplasm in response to Wnt signaling. Wnt signaling has also been associated with stabilization and nuclear accumulation of β-catenin and consequential upregulation of β-catenin/TCF gene transcription. As a basis for understanding Wnt signaling in mammalian cells, we have developed a confocal live-cell imaging measurement technique to measure the cell and nuclear volumes and the concentrations of β-catenin, Axin, APC, GSK3b and E-cadherin in colon cells. These parameters provide the basis for formulating Wnt signaling models for both normal and cancerous intestinal cells.

Colonic cells are also influenced by epidermal growth factor receptor (EGFR) and Notch signaling. We have continued to investigate the

mechanism of activation of EGFR. Our previous structural studies have revealed two distinct conformations of the ectodomain of EGFR: a compact, tethered conformation and an untethered, extended conformation. However, biophysical studies in conjunction with Andrew Clayton (Swinburne University) indicate that EGF-induced activation occurs within or between preformed and extended dimers with very little change in extension of the N termini from the membrane surface. Ligand or erbB2 binding to cell surface EGFR dimers seems to induce conformational changes. Interestingly, the mAb806-binding site on wild-type EGFR is not exposed when the receptor is expressed on the cell surface at low levels. However, in the presence of erbB2, mAb806 binding to EGFR increases significantly, whereas in the presence of ligand the mAb806 epitope is hidden. Our results indicate that ligand induces EGFR dimers to form tetramers where activation of the intracellular tyrosine kinase occurs via an asymmetric interaction between EGFR dimers.

We have developed a Notch-m-cherry epithelial cell line and have determined the rate of Notch intracellular domain production in response to delta1-Fc. We are now investigating the interactions among EGFR, Notch and Wnt signaling in epithelial cells. Our Notch modeling studies include the modulation of Notch signaling by intracellular regulators such as numb and musashi.

Joint protein research

Richard Simpson, Ph.D.

The year 2011 was one of change for the Joint Protein Research Laboratory. We established new laboratories at La Trobe University, Melbourne, in July. We continue to focus on remaining at the forefront of the field of analytical protein chemistry and innovative proteomic analysis systems, as well as instrumentation and software for mass spectrometry. The techniques and methods we have developed are broadly applicable to many areas of research.

The stem cell niche comprises stem cells, stromal cells, soluble factors, extracellular matrix constituents and vascular networks. The ability to identify signals that regulate stem cell self renewal and differentiation is confounded by the difficulty in isolating pure stem cell niche components in sufficient quantities to enable their biochemical characterization. We have analyzed extracellular (secretome) and adherent plasma membrane proteomes of three distinct epithelial cell subpopulations isolated and immortalized from the mouse mammary gland: basal and mammary stem cell (basal/MaSC), luminal progenitor and mature luminal cell lines. Proteomic profiling revealed a distinct switch in components modulating Wnt and ephrin signaling and integrin-mediated interactions among the three cell subpopulations. Conspicuously, Wnt10a was uniquely detected in basal/MaSCs, and may modulate the canonical Wnt signaling pathway to maintain basal/MaSC activity. In contrast, noncanonical Wnt signaling might be elevated in mature luminal cells, as evidenced by the high expression levels of Wnt5a, Wnt5b and the transmembrane tyrosine kinase Ror2.

Infection by some parasites can cause severe disease and death. The reduced effectiveness of available drugs is largely due to acquired drug resistance, and new drugs to novel targets are always needed. Through one of our collaborative projects, we identified and obtained the crystal structure of a mitochondrial membrane–anchored protein (MIX) that occurs exclusively in *Leishmania* and *Trypanosoma* and is essential for virulence. MIX forms an all-a-helical fold comprising seven such helices that fold into a single domain. The distribution of helices is similar to that in several scaffold proteins, suggesting that MIX mediates protein-protein interactions. We identified several potential protein partners. As it is parasite specific, MIX is a promising new drug target.

Plasma membrane proteins are also attractive therapeutic targets because of their accessibility to drugs. Although genes encoding these proteins represent 20–30% of eukaryotic genomes, a detailed characterization of their encoded proteins is underrepresented owing to low copy number

and inherent difficulties in isolation and purification because of their high hydrophobicity. We have developed a two-step method to isolate and purify these proteins from Madin-Darby canine kidney cells. In this method, we first used cationic colloidal silica to isolate adherent and nonadherent fractions, and then subjected each fraction to Triton X-114 phase partitioning to further enrich for hydrophobic proteins. These studies reveal that this technique is powerful for isolating low-abundance proteins and a useful adjunct for in-depth cell surface proteome analyses.

Microsomes are small lipid vesicles that are rich in plasma membrane proteins and that form spontaneously when cells are mechanically disrupted. We have used these characteristics to isolate and analyze mouse liver microsomes by differential centrifugation, purification and GeLC–MS/MS analysis. A total of 1,124 proteins were identified, with 47% predicted to contain at least one transmembrane domain. We evaluated phase partitioning using the detergent Triton X-114 to further enrich for membrane proteins. Evaluation of biological processes showed enrichment of proteins involved in fatty acid biosynthesis and elongation as well as steroid synthesis. We also identified transport proteins including 24 members of the Rab family of GTPases. Comparison of this data set with the current mouse liver microsome proteome contributes an additional 648 protein identifications, of which 50% contain at least one transmembrane domain.

Ludwig colon cancer initiative

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

Colorectal cancer development is driven by genetic, epigenetic and transcriptomic changes, and these profiles seem to define distinct molecular subtypes of the disease associated with different patient clinical features and outcomes. Colorectal cancer prognosis may further be influenced by various lifestyle factors and comorbidities. Our laboratory focuses on identifying key molecular and epidemiologic markers of disease outcome using population-based studies to improve prognostication and patient management. Through work in cell line and animal model systems, we aim to understand the role of molecular markers in influencing tumor behavior.

The initiative has completed extensive molecular profiling studies on colorectal cancers from 1,000 patients, including genome-wide assessment of inherited common variants using single-nucleotide polymorphism microarrays, mutation screening of key colon cancer genes, genome-wide assessment of tumor-acquired DNA copy-number changes and microsatellite instability status analysis. So far, this work has defined a set of patient clinicopathological features associated with presence of tumor BRAF(V600E) mutation, providing information on the optimal design for clinical trials of agents targeting this oncoprotein. Further studies have revealed the impact of *BRAF* mutation and microsatellite instability on the pattern of metastatic spread and prognosis in metastatic colorectal cancer. Similarly, presence of *KRAS* mutation has been found to be associated with particular patterns of relapse for patients with curatively resected colorectal cancer. In particular, the presence of *KRAS* mutation was shown to modify the risk of lung but not liver relapse. These data have direct clinical implications, highlighting the potential of somatic mutations to inform 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.

In collaboration with the international COGENT consortium, we have further contributed to genome-wide association studies aiming to identify common inherited variants influencing susceptibility to colorectal cancer. These studies have led to the discovery of multiple common susceptibility variants near the BMP pathway loci GREM1, BMP4 and BMP2 and further refined the known associations between risk of colorectal cancer and polymorphisms on chromosomes 1q41 and 12q13.13.

Using a combination of human, cell line and animal models, we have identified a novel marker of putative epithelial stem cells of the human intestinal tract, PHLDA1. In the human small and large 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. The use of clinical data for research and various biomarker collaborations with external groups has continued to expand during 2011.

In 2011, we studied the value of circulating tumor DNA as a biomarker of minimal residual disease and/or treatment response in a variety of colorectal cancer contexts. We initiated studies in patients with resectable liver metastases (funded by Ludwig Institute and the Conrad N. Hilton Foundation) and stage II colon cancer (funded by the Victorian Cancer Agency). A successful National Health and Medical Research Council application will support a study of 250 patients with locally advanced colorectal cancer. We have initiated additional collaborations with academic partners, including studies of a novel biomarker of irinotecan response in patients with metastatic disease and studies of inflammatory response in primary tumors to predict outcome for early-stage colon cancer (Immunoscore international consortium, Jerome Falon).

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Director's message

Detailed serological analysis of human cancer has been a major objective of the Ludwig 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 emerging. Ludwig New York is pursuing three targeted approaches to cancer: therapeutic antibodies, antigen-specific vaccines and enzyme depletion therapy. These approaches are connected by a common theme of high specificity for cancer, and reflect our 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 (mAbs). Thirteen of our mAbs have been licensed to commercial partners for further clinical development. We are currently focusing on developing antibodies against targets on cancer stem cells (CSCs), selected intracellular targets and molecules involved in immunosuppression in tumors.

The cancer/testis (CT) antigen NY-ESO-1, which was discovered by Ludwig scientists in New York, has been chosen as the prototype CT antigen for vaccine development in the Cancer Vaccine Collaborative, a coordinated network of clinical and laboratory investigators. To select the most immunogenic vaccine for therapeutic trials, it is key to monitor the immune response elicited by NY-ESO-1 vaccines. Ludwig New is the central NY-ESO-1 reference laboratory for the Cancer Vaccine Collaborative.
A major challenge for cancer vaccine development is the profound immunosuppressive environment of the tumor mediated by regulatory T (Treg) cells and other immunological factors. To overcome this obstacle, we are integrating modulators of immunosuppression, such as cytotoxic T lymphocyte antigen 4 (CTLA-4)- and PD1-blocking antibodies and GITR agonist antibody, into our vaccine strategies. We are evaluating additional vaccine targets (CT and non-CT antigens) 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 clinical use of l-asparaginase, which is now part of standard therapy for acute childhood leukemia. Certain cancers lack the enzyme argininosuccinate synthetase (ASS), which is necessary to convert citrulline to arginine. We have 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 2 clinical trial of ADI in patients with SCLC has now been initiated.

Ludwig New York mourns the loss of Lloyd J. Old, who died of prostate cancer on November 28, 2011. Old was the director of the New York Branch from its inception in 1991 until his death. In his honor, a memorial online forum has been established by *Cancer Immunity*, the journal he founded and edited (http://cancerimmunity.org/lloyd-old-2/). In addition, a special issue of *Cancer Immunity* dedicated to him features a collection of reflections about him and his work covering his entire scientific career (http://cancerimmunity.org/volume-12-issue-1/). His work and vision—to find and creatively develop effective therapies for cancer—is being carried forward.

Gerd Ritter

Cancer cell surface antigens

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

A major goal of Ludwig New York is to identify and validate antigenic surface targets expressed on cancer cells that are suitable for antibody-based immunotherapy of cancer. To pursue this goal, we have used several target-tailored approaches. These include (i) immunization of mice with selected tumor cell preparations, generation and screening of hybridomas for novel mAbs with selective cell surface binding to human cancer cells and subsequent molecular elucidation of the cell surface target antigen and (ii) use of bioinformatics 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 mAbs. Thirteen of our mAbs have been licensed to commercial partners for further clinical development and more then ten of these antibodies have entered into clinical trials at Memorial Sloan-Kettering Cancer Center (MSKCC). The therapeutic effects of mAbs targeting cancer cell surface antigens are mediated by various distinct functions including antibody-dependent cellular cytotoxicity, complement-mediated cytotoxicity, signal agonism or antagonism, and delivery of a "payload" such as toxic small molecules, short interfering RNA, 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 CSCs, which initiate cancer and provide a continued source of self-renewing cancer cells. The failure of most current therapies to cure cancers is thought to be caused by the relative resistance of CSCs to chemotherapy and radiation. Because few CSCs exist in cancers, it has been extremely difficult to obtain enough of these cells for analysis and characterization. This is a major barrier to developing effective CSC-targeted cancer therapeutics. We hypothesize that CSCs can be distinguished from more differentiated tumor cells and normal cells by a different antigenic cell surface profile, and that these surface antigens can be recognized by mAbs. The work of Malcolm Moore at MSKCC has provided a new opportunity to define cell surface targets on CSCs. He and his colleagues have developed a novel method to expand CSCs in tissue culture, making it possible to obtain for the first time a continued source of CSCs from ovarian cancer. This major finding enabled us to obtain sufficient quantities of CSCs for immunization and screening to generate and develop therapeutic antibodies against ovarian CSCs. In collaboration with Moore's team, we now have begun generating and characterizing novel mAbs against

ovarian CSCs. Screening assays, which include mixed hemadsorption assays, fluorescence-activated cell sorting, cytotoxicity assays and immunohistochemistry have 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 complement-mediated cytotoxicity, antibody-dependent cellular cytotoxicity, effects on CSC vitality and growth *in vitro* and *in vivo*, interference with the resistance of ovarian CSCs to current chemotherapeutic drugs, and internalization characteristics. The antigens recognized by these antibodies are also being biochemically identified. Antibodies that selectively recognize CSCs 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 CSCs rather than on cancer cells with lower tumorigenic or metastatic potential. We also plan to develop these antibodies and the target antigens 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.

Grand serology and seromics

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

We pioneered high-throughput serological monitoring using a semiautomated ELISA platform for serological responses against a series of tumor antigens, and used protein microarrays for antibody responses to ~9,000 antigens simultaneously. We also developed analytical tools to interpret and validate these antibody responses and introduced new standards for the field of "seromics," that is, the scope of antigens defined by serological profiling of protein microarrays. Such analyses should help us evaluate the inherent immunogenicity of different cancers, identify the most frequently immunogenic tumor antigens, establish correlations with antigen expression and with cellular immunity to the antigen, identify a potential functional role for antibody responses, define biomarkers of cancer for diagnostic or prognostic purposes, and 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 have established the frequency of spontaneous antibody responses to NY-ESO-1, using it as a model immunogenic tumor antigen in various cancer types (>2,800 cancer patients and >250 healthy donors tested). The frequency of antibodies against NY-ESO-1 by ELISA ranged from 0.5% to ~20%, depending on the cancer type (low in pancreatic cancer and healthy donors, and high in ovarian cancer and melanoma). Antibody responses were also correlated with stage of disease and other clinical parameters, and they ranged from low titers (1/100 to 1/1,000) up to titers reaching more than 1/1,000,000. NY-ESO-1 antibodies were associated with naturally occurring CD8 and CD4 T cell responses in cancer patients. We determined the correlation of NY-ESO-1 serum antibodies with clinical events and with overall survival in non-SCLC and prostate cancer patients, in parallel with data on antigen expression in the tumor. We also expanded our ELISA screenings to a series of >30 known tumor antigens, mostly representing CT family members such as MAGE and SSX proteins, as well as differentiation antigens (Melan-A), mutational antigens (P53) and stem cell/embryonic antigens (SOX2). We ranked and compared antigens in large subsets of patient cohorts representing various tumor types (n > 2,000 individual sera). We found that some cancers were more immunogenic than others, and that some antigens were rarely immunogenic whereas others were more common. Possible factors explaining these differences include differential antigen expression, aggressiveness of tumor and local suppressive environment. We identified some interesting targets for vaccine

development with sporadic high spontaneous immunogenicity. Among them, p53 is one of the antigens with the most frequent immunogenicity in various cancers based on serum antibody responses. We therefore decided to compare this antigen to NY-ESO-1 using our standardized methods for CD8 and CD4 T cell monitoring. We found that 21% (64 of 298) of ovarian cancer patients but no healthy donors showed specific IgG responses against wild-type p53 protein. Although none of 12 patients with high-titer p53 antibody showed spontaneous p53-specific CD8+ T cell responses after a single *in vitro* sensitization, significant p53-specific interferon-γ-producing CD4+ T cells were detected in 6 patients. Surprisingly, similar levels of p53-specific CD4+ T cells but not CD8+ T cells were also detected in 5 of 10 seronegative cancer patients and 9 of 12 healthy donors. These results raise the possibility that p53-specific CD4+ T cells reflect abnormalities in p53 occurring in normal individuals, and that they may play a role in processes of immunosurveillance or immunoregulation of p53-related neoplastic events. This discrepancy between CD8 and CD4 T cell responses was named "split T cell tolerance" and will be important to assess when considering new antigens for immunotherapy.

We analyzed >180 sera from prostate cancer patients with different stages of disease and looked for immune response to CT antigens and 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 were analyzed in seromics for association of seroreactivity to human endogenous retroviral antigens with other antigens. Another major application of seromics and grand serology is to measure changes in antibody profiles after immunotherapy; this is described in detail in the Humoral and Cellular Immunity section of this report. We have initiated multiple projects in this regard, starting with the immunological effects of CTLA-4 blockade (ipilimumab). We have looked first for antibody and T cell responses to NY-ESO-1, and found evidence of NY-ESO-1 immunity in patients with clinical benefit. Based on these results we have further expanded our seromic profiling to search for additional immunogenic antigens potentially associated with clinical benefit to treatment with anti-CTLA-4 and anti-PD1 in patients with melanoma, prostate cancer and colon cancer. We are also monitoring patients with breast cancer who received neoadjuvant radio- and chemotherapy (before surgery) to study the abscopal effects of radiotherapy on immunogenicity (in collaboration with Silvia Formenti and Sylvia Adams, New York University).

Cancer/testis antigens

Achim Jungbluth, M.D., Yao Chen, M.D., Ph.D.
and Lloyd Old, M.D.

CT antigens are encoded by genes that are normally expressed only in the human germ line but that are activated in various malignancies. CT antigens are frequently immunogenic in cancer patients and their expression is highly restricted to tumors; therefore they constitute important targets for anticancer immunotherapy. There are now about 130 CT gene families, and many of them are promising vaccine candidates.

With Ludwig scientists Otavia Caballero and Andrew Simpson and others, we previously 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 in the database our data on RT-PCR expression analysis of a large series of new CTs in normal and cancer tissues and cell lines.

Several CT antigens are useful biomarkers and promising targets for therapeutic cancer vaccines. To further identify tumor types or tumor subtypes that may be suitable for immunotherapy with CT cancer vaccines, we have analyzed CT antigen expression in a series of tumor types. When data were available, we examined the correlation of CT antigen expression with tumor stage and disease prognosis. In collaboration with Munro Neville (London) and A. Goldhirsch and colleagues (Milan) we expanded our initial study in breast cancer and analyzed by immunohistochemistry 100 invasive breast cancers, including 50 estrogen receptor $(ER)^+/HER2^-$ and 50 triple-negative (TN) breast cancers. We found significantly higher expression of MAGE-A and NY-ESO-1 in TN breast cancers compared with ER^+ tumors ($P = 0.04$). MAGE-A expression was detected in 13 (26%) TN cancers compared with 5 (10%) ER^+ tumors ($P = 0.07$). NY-ESO-1 expression was confirmed in nine (18%) TN tumor samples compared with two (4%) ER^+ 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 $ER^-/PR^-/HER2^-$ (TN) carcinomas. We found significantly more frequent expression of all eight CT antigens in ER^- cancers, and five of them—MAGEA, CT7, NY-ESO-1, CT10 and CT45—were expressed in 12–24% of ER^- cancers, versus 2–6% of ER^+ cancers ($P < 0.001$–0.003). In comparison, GAGE, SAGE1 and NXF2 were expressed only in 3–5% of ER^- and 0–2% of ER^+ cancers. More frequent CT expression was also found in tumors with higher nuclear grade ($P < 0.001$ to $P = 0.01$) and larger 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 TN breast cancer, the potential of CT-based immunotherapy should be explored.

In a subsequent study we also analyzed the presence of CT antigens in BRCA-associated breast cancers. We showed increased expression of these antigens in BRCA$^+$ carcinomas. Interestingly, they are not expressed in healthy BRCA mutation carriers, suggesting a role for CT antigens as potential vaccine targets in BRCA-mutation-positive breast cancer. Analysis of CT antigen expression was expanded to tumors of the head and neck, for which knowledge about tumor-associated antigens is still limited. In an extended study in thyroid carcinomas, we found little expression of MAGE-A4 and CT7, thus confirming the low prevalence of CT antigens in this type of malignancy.

Interestingly, a large analysis of another malignant head and neck tumor showed a very high prevalence of CT antigens in neoplasms of the larynx. In carcinomas of the oral squamous mucosa we demonstrated a high prevalence of MAGE-A4 and CT7. The presence of these two antigens had no impact on clinical parameters such as survival in this type of cancer.

Other studies were undertaken in myeloma in collaboration with Hearn Cho (New York University and Mount Sinai Hospital, New York). We found that MAGE-A interacted with Bax and survivin and inhibited apoptosis in myeloma cells. Myeloma is also the focus of a collaboration with Guenther Koehne at MSKCC. In this study, the role of CT7 is being analyzed in patients with myeloma undergoing allogeneic bone marrow transplantation with consecutive donor lymphocyte infusion. Early results indicate a possible role of CT7 in potential recurrence of disease after transplantation. We have shown previously that the CT 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 of 72, or 58%) of CT45 expression among all malignancies tested so far; the frequency of CT45 expression in diffuse large B cell lymphomas 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.

In a collaboration with Annette Mueller (University of Bonn), we are looking at various tissues and organs in fetuses. We are analyzing the potential expression of CT antigens in tissues other than gonads. Interestingly, there seems to be focal presence of CT antigens in cells outside germ cells, as CT antigen–expressing cells were found in certain areas of the thymus.

Cancer vaccines and immunotherapies

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

A series of clinical trials with various NY-ESO-1 vaccines was conducted as part of the Cancer Vaccine Collaborative clinical trials network to optimize immunological responses and to learn about clinical effects.

We have been conducting at MSKCC a clinical trial of ovarian cancer patients vaccinated with NY-ESO-1 overlapping long peptides (OLPs) with or without Montanide and poly-ICLC (principal investigator, Paul Sabbatini). This trial is testing for the first time the use of OLPs 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 on the T cell and antibody level. The majority of patients developed both CD8 and CD4 T cell responses against NY-ESO-1 during vaccination with OLP plus Montanide, compared to none in the group without Montanide. Antibody responses generally appeared later than T cell responses, around week 13 on average after vaccination for antibodies versus as early as week 4 for CD8 and/or CD4 T cells. Compared with other NY-ESO-1 protein or short peptide vaccines, OLPs in combination with Montanide seem more consistently immunogenic for generating integrated immune responses (CD4, CD8 and antibodies), and T cell immunity develops faster. Notably, when poly-ICLC was added to the vaccine, patients developed far greater levels of both humoral and cellular immune responses. Antibodies were detected in nearly all patients as early as week 7. T cell responses to both human leukocyte antigen 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 T_{reg} cells and the avidity of effector cells. The combination of OLPs with Montanide and poly-ICLC seems superior in immunogenicity to most other vaccination methods attempted for NY-ESO-1 so far using either peptides or protein.

In collaboration with Elke Jaeger (Krankenhaus Nordwest) and Alexander Knuth (University of Zurich) we have conducted a phase 1 clinical trial in patients with metastatic prostate cancer evaluating immunological and clinical effects of vaccination with recombinant NY-ESO-1 protein combined with CpG as adjuvant. Our data show that NY-ESO-1 protein/CpG vaccine can induce integrated antigen-specific immune responses *in vivo* and

efficiently prime CD8+ T cell responses in NY-ESO-1 antigen–negative patients. Our results may also support further clinical vaccination protocols with NY-ESO-1 protein that focus not only on the treatment of existing cancer but also on preventing further development of NY-ESO-1+ cancers *in vivo*.

In collaboration with Eiichi Nakayama (Okayama, Japan) and his colleagues, we conducted a phase 1 clinical trial of a cancer vaccine using a 20-mer NY-ESO-1f peptide (NY-ESO-1 91-110) that includes multiple epitopes recognized by antibodies and CD4 and CD8 T cells. Ten patients were immunized with 600 μg NY-ESO-1f peptide mixed with 0.2 KE Picibanil OK-432 and 1.25 ml Montanide ISA-51. Primary end points of the study were safety and immune response. Subcutaneous injection of the NY-ESO-1f peptide vaccine was well tolerated. Vaccination with the NY-ESO-1f peptide resulted in an increase or induction of NY-ESO-1 antibody responses in nine of ten patients. The sera reacted with recombinant NY-ESO-1 whole protein and the NY-ESO-1f peptide. An increase in CD4 and CD8 T cell responses was observed in nine of ten patients. Of ten patients, two with lung cancer and one with esophageal cancer showed stable disease.

In collaboration with Kunle Odunsi (Roswell Park Cancer Institute) and Elke Jaeger, recombinant poxviruses (vaccinia and fowlpox) expressing tumor-associated antigens were evaluated in clinical trials as cancer vaccines to test whether a diversified prime-and-boost regimen targeting NY-ESO-1 has clinical benefit. We conducted two parallel phase 2 clinical trials of recombinant vaccinia-NY-ESO-1, followed by booster vaccinations with recombinant fowlpox-NY-ESO-1 in 25 melanoma and 22 epithelial ovarian cancer (EOC) patients with advanced disease who were at high risk for recurrence/progression. Integrated NY-ESO-1-specific antibody and CD4+ and CD8+ T cells were induced in a high proportion of melanoma and EOC patients. In melanoma patients, objective response rate (complete and partial response) was 14%, mixed response was 5% and disease stabilization was 52%, amounting to a clinical benefit rate of 72% in melanoma patients. Median progression-free survival in the melanoma patients was 9 months (range of 0–84 months) and median overall survival was 48 months (range of 3–106 months). In EOC patients, median progression-free survival was 21 months (95% confidence interval, 16–29 months), and median overall survival was 48 months (confidence interval not estimable). CD8+ T cells derived from vaccinated patients lysed NY-ESO-1-expressing tumor targets. These data provide preliminary evidence of clinically meaningful benefit for diversified prime-and-boost recombinant poxvirus-based vaccines in melanoma and ovarian cancer, and support further evaluation of this approach in these patient populations.

CTLA-4, a molecule expressed by T lymphocytes, acts as a natural break to immune responses when stimulated and prevents them from going into overdrive in response to an infection or foreign body. mAbs developed by 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 immune responses are unleashed against tumor cells in patients treated with CTLA-4 blockade immunotherapy. Jedd Wolchok (Ludwig Center and Ludwig New York Branch at MSKCC) has been a leader in the clinical development of CTLA-4 blockade immunotherapy. He and his colleagues recently reported results from a double-blind phase 2 trial showing that anti-CTLA-4 antibody ipilimumab elicited a dose-dependent effect on efficacy and safety measures in pretreated patients with advanced melanoma. They also reported results from a phase 3 study showing that ipilimumab improved overall survival in patients with previously pretreated metastatic melanoma. These results led to approval of the drug by the US Food and Drug Adminstration in March 2011. In addition, in a phase 3 study, ipilimumab (at a dose of 10 mg kg^{-1}) in combination with dacarbazine, as compared with dacarbazine plus placebo, improved overall survival in patients with previously untreated metastatic melanoma. The types of adverse events were consistent with those seen in prior studies of ipilimumab; however, rates of elevated liver-function values were higher and rates of gastrointestinal events were lower than expected on the basis of prior studies.

We also developed in the laboratory and in preclinical studies novel approaches for cancer vaccines. Targeting of protein antigens to cell surface receptors on dendritic cells such as DEC-205 or mannose receptor (MR) is considered to enhance induction of antigen-specific immune responses. In collaboration with Celldex, we have investigated whether this concept is applicable to NY-ESO-1, a CT antigen widely used in clinical cancer vaccine trials. We constructed two novel targeting proteins comprising full-length NY-ESO-1 fused to the C terminus of two human mAbs against human MR and DEC-205 and analyzed them *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 peripheral blood lymphocytes of cancer patients. Receptor-specific delivery of NY-ESO-1 to antigen-presenting cells seems to be a promising vaccination strategy for efficiently generating integrated and broad antigen-specific immune responses against NY-ESO-1 in cancer patients. The DEC205-NY-ESO-1 construct is now being explored in phase 1/2 clinical trials.

In collaboration with Ricardo Gazzinelli and colleagues (Belo Horizonte, Brazil) we addressed one of the main challenges in cancer vaccine research, the development of vaccines that induce effective and long-lived protective immunity against tumors. Using a recombinant nonpathogenic clone of *Trypanosoma cruzi* as a vaccine vector, we induced vigorous and long-term T cell–mediated immunity. The rationale for using the highly attenuated *T. cruzi* clone was (i) the ability of the parasite to persist in host tissues and therefore to induce a long-term antigen-specific immune response; (ii) the existence of intrinsic parasite agonists for Toll-like receptors and consequent induction of highly polarized T helper cell type 1 responses and (iii) parasite replication in the host cell cytoplasm, leading to direct antigen presentation through the endogenous pathway and consequent induction of antigen-

specific $CD8^+$ T cells. Importantly, we found that parasites expressing a CT antigen (NY-ESO-1) elicited human antigen–specific T cell responses *in vitro* and solid protection against melanoma in a mouse model. Furthermore, in a therapeutic protocol, parasites expressing NY-ESO-1 delayed the rate of tumor development in mice. We conclude that the *T. cruzi* vector is highly efficient in inducing T cell–mediated immunity and protection against cancer cells. More broadly, this strategy could be used to elicit long-term T cell–mediated immunity and for prophylaxis or therapy of chronic infectious diseases.

Humoral and cellular immunity

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

With proper immunotherapeutic strategies, especially the use of modulators of immune suppression, patients may experience dramatic clinical benefit in an immunologically mediated fashion. We therefore asked whether patients responding to immunotherapy, especially CTLA-4 blockade, may have better odds of responding to treatment if they have underlying measurable immune responses against their tumor. We also looked for general immunological markers that could help predict or explain response to CTLA-4 blockade. Ipilimumab, a mAb against CTLA-4, improves survival in patients with advanced metastatic melanoma. It also enhances immunity to NY-ESO-1. In collaborations with Jim Allison and Jianda Yuan (Ludwig Center at MSKCC), and Ruth Halaban and Mario Sznol (Yale University), we began characterizing the association between immune response and clinical outcome. We first analyzed NY-ESO-1 serum antibody by ELISA in 144 ipilimumab-treated patients with melanoma and found 22 of 140 (16%) seropositive at baseline and 31 of 144 (22%) seropositive after 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; relative risk = 1.8; two-tailed Fisher test). To understand why some patients with NY-ESO-1 antibody did not 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 of 13; 77%) than those with undetectable $CD8^+$ T cell response (1 of 7; 14%; P = 0.02; relative risk = 5.4; two-tailed Fisher test) and significant survival advantage (P = 0.01; hazard ratio = 0.2; time-dependent Cox model). Together, our data suggest that integrated NY-ESO-1 immune responses may 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 clinical use of modulators of immunosuppression with concurrent approaches to favor tumor antigen–specific immune responses, such as vaccines or adoptive transfer, in patients with cancer. Other experimental evidence for the strong influence of intratumoral immune suppression in cancer comes from our collaborative studies with Kunle Odunsi. We analyzed 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, Germany) we also analyzed T cell responses

to the tumor antigen NY-CO-58/KIF2C, which was originally defined by serological analysis of recombinant cDNA expression libraries, and found that NY-CO-58/KIF2C induced spontaneous $CD4^+$ T cell responses of the Th1 type that were tightly controlled by peripheral T_{reg} cells.

We also analyzed the immunological correlates of a clinical response observed in a melanoma patient treated with ipilimumab and concurrent radiotherapy. We reported an abscopal effect in this patient, a phenomenon in which local radiotherapy is associated with regression of metastatic cancer at a distance from the irradiated site. We noted temporal associations including tumor shrinkage with antibody responses to the CT antigen NY-ESO-1, changes in peripheral-blood immune cells and increases in antibody responses to other antigens after radiotherapy. We followed antibody responses to various epitopes within NY-ESO-1. We used protein microarrays containing >9,000 distinct proteins to detect serum autoantibodies specifically binding to a subset of these proteins before and after radiation treatment. This is the first report of an abscopal effect in melanoma, and our understanding of immune responses may help make this observation more common in the future.

Monoclonal antibody trials

Gerd Ritter, Ph.D., Achim Jungbluth, M.D.,
Sacha Gnjatic, Ph.D., Jedd Wolchok, M.D., Ph.D.
and 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 mAbs. Thirteen of our mAbs have been licensed to commercial partners for further clinical development and more than ten of these antibodies have entered into clinical trials at MSKCC. Current clinical trials at MSKCC in collaboration with Steve Larson and colleagues (Ludwig Center at MSKCC) investigate the ability of mAbs to selectively target human cancers. They include studies with ^{90}Y-cG250, ^{111}In-cG250 and ^{177}Lu-cG250 in renal cancer, ^{124}I-cG250 (phase 3 for differential diagnosis of renal cancer), and ^{124}I-huA33 in colon cancer for positron emission tomography (PET) imaging. In a study with huA33 we evaluated ^{124}I-huA33 targeting, biodistribution and safety in patients with colorectal cancer. We also determined the biodistribution of ^{124}I-huA33 when a large dose of human intravenous IgG was administered to manipulate the Fc receptor or when ^{124}I-huA33 was given via hepatic arterial infusion. Our results showed good localization of ^{124}I-huA33 in colorectal cancer with no significant toxicity. PET-derived ^{124}I concentrations agreed well with those obtained by well counting of surgically resected tissue and blood, confirming the quantitative accuracy of ^{124}I-huA33 PET. The hepatic arterial infusion route had no advantage over the intravenous route. No clinically significant changes in blood clearance were induced by human intravenous IgG. In collaboration with Chaitan Divgi and colleagues at the University of Pennsylvania and MSKCC we studied whether there is a correlation between quantification of radiolabeled macromolecular uptake in tumors determined *in vivo* using PET/CT and *in vitro* using autoradiography and gamma-counting of tumor tissues using ^{124}I-labeled cG250. We found that PET/CT can be reliably used in cancer patients to quantify radioisotope-labeled marcomolecular uptake *in vivo*; this has important implications for quantitative pharmacokinetic estimates of macromolecular biodistribution. Further, a phase 1 clinical trial was conducted with farletuzumab (LK26), a humanized mAb against folate receptor-α, in EOC (in collaboration with Paul Sabbatini).

In our pursuit to improve antibody efficacy in destroying tumor cells and to identify novel mechanisms 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, the Netherlands) and Andrew Scott and colleagues at Ludwig Melbourne-Austin, 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 toward more aggressive forms of the disease. The new Fab antibody MSC8 and its corresponding full-length IgG inhibited CAIX activity by up to 57% and 76%, with half-maximal inhibition at 0.3 $\mu g\ ml^{-1}$. 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.

We are also pursuing the use of mAbs to react with cell surface targets on immune cells critically involved in controlling the immune suppressive environment in tumors. We aim 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 CTLA-4 and GITR, two key molecules in immunomodulation (for our anti-CTLA-4-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 T_{reg} cell–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 mAb 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 costimulatory agents may lead to global T cell activation and potentially autoimmunity, we are developing more targeted strategies for these reagents.

Many tumor antigens are intracellular molecules that generally would not be considered suitable targets for mAb therapy. In collaboration with H. Nishikawa (Osaka, Japan) and H. Shiku (Mie, Japan) we have now provided the first experimental evidence challenging this view. We show therapeutic efficacy of mAbs directed against NY-ESO-1, a widely expressed immunogen in human tumors that is expressed intracellularly but not on the surface of cells. On their own, NY-ESO-1 mAbs could neither augment antigen-specific $CD8^+$ T cell induction nor cause tumor eradication. To facilitate mAb access to intracellular target molecules, we combined anti-NY-ESO-1 mAb with anticancer drugs to accentuate the release of intracellular NY-ESO-1 from dying tumor cells. Strikingly, combination therapy induced a strong antitumor effect that was accompanied by the development of NY-ESO-1-specific effector/memory $CD8^+$ T cells that were not elicited by single treatments alone. The combinatorial effect was also associated with upregulation of maturation markers on dendritic cells, consistent with the organization of an effective antitumor T cell response. Administration of Fc-depleted F(ab) mAb or combination treatment in Fcγ receptor–deficient host mice abolished the therapeutic effect. Together, our findings show that intracellular tumor antigens can be captured by mAbs and engaged in efficient induction of CD8+ T cell responses, greatly expanding the possible use of mAbs for passive cancer immunotherapy.

Targeted enzyme-amino acid deprivation therapy

Lloyd Old, M.D., Achim Jungbluth, M.D.,
Jedd Wolchok, M.D., Ph.D. and 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, which is now a part of the standard therapy for acute childhood leukemia. Arginine, like asparagine, is considered a nonessential acid. But certain cancer cells, especially melanoma and hepatocellular cancer, lack the enzyme ASS, which is necessary to convert citrulline to arginine, thus making these tumors auxotrophic for arginine. The Mycoplasma-derived enzyme 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 antitumor activity *in vitro* and *in vivo* in mouse models. Ludwig New York has played a critical role in elucidating the mechanism and in developing ADI for the clinic. In collaboration with clinical investigators at MSKCC, the Ludwig New York has initiated and conducted a phase 1/2 study studying the efficacy of ADI treatment in patients with ASS$^-$ melanomas. To better understand the mechanism of this new form of deprivation therapy in melanoma, microPET studies in mice were conducted in collaboration with Steve Larson and his nuclear medicine team at the Ludwig Center at MSKCC to quantify tumor responses to arginine depletion after treatment with ADI. The antitumor effects of ADI-PEG20 in mouse melanoma models seemed moderate and may be explained by adapted intrinsic resistance of melanoma cells to ADI-PEG20 treatment. Thus, there is a need to identify tumors that are more sensitive to ADI-PEG20 treatment and remain that way.

We investigated ASS expression in human SCLC tumor tissues by immunohistochemistry and found that ~45% of SCLC tumors were negative for ASS. We also screened a panel of ten available human SCLC cell lines for ASS mRNA and protein expression by quantitative RT-PCR and western immunoblotting analysis, and we detected little or no ASS in five of ten 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$^-$ 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 begun at MSKCC and Duke University. The eligibility criteria for patient enrollment includes lack or focal expression of ASS in SCLC.

This is an example of how the long-standing expertise in clinical discovery research in New York and the coordinated team effort of Ludwig investigators and external collaborators have allowed us to rapidly follow up on our emerging laboratory discoveries by expediting investigations in animal models and then directly translating these findings into an early-phase clinical trial in patients with SCLCs.

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Oxford

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Director's message

The Ludwig Oxford Branch has continued to build on its strengths since opening in 2007. We have continued to pursue our overarching research aims in nuclear reprogramming and cancer heterogeneity, and we are committed to translating our basic science discoveries into clinical applications for patient benefit. Our newest members, Sarah De Val and Skirmantas Kriaucionis, joined us in 2010 and have expanded their research groups over the course of 2011, going from strength to strength.

Ludwig Oxford continues to maintain a coherent research strategy while retaining sufficient diversity for the beneficial exchange of ideas and methodologies. Transcription factors are molecular switches of cell fate, and their target selectivity lies at the heart of cell fate determination. One of the main research focuses of Ludwig Oxford is, therefore, the identification of molecular switches involved in cell growth or death (Xin Lu), stem cells and differentiation (Colin Goding), and tumor vascularization (Sarah De Val). This is complemented by research focuses of the other groups, including Skirmantas Kriaucionis' work on 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. John Christianson's research is focused on post-translational regulation of molecular switches. In addition, the work of Gareth Bond aims to identify molecular genetic signatures of cancer predisposition, progression and response to therapy. We have continued to publish our findings in high-impact journals during 2011, with more publications pending and planned for the year ahead.

We hosted a Ludwig-wide Cancer Biology Meeting in September, and we also organized and ran a very successful international workshop on Cell Polarity in Health and Disease in November. These events raise our profile within the Oxford community and elsewhere, and generate opportunities for collaboration. Ludwig Oxford continues to develop collaborations and contacts with clinical and biomedical colleagues in Oxford, the UK and internationally, and we anticipate many more successful partnerships in future.

Looking to the future, we are actively recruiting research staff. This will include a strong presence in Oxford University's new Target Discovery Institute, which is expected to open by the end of March 2013. With continued support, we look forward to building a dynamic and world-class multidisciplinary research environment where we will fulfill our mission to undertake excellent basic research and translate it for the benefit of patients.

Xin Lu

Tumor suppression

Xin Lu, Ph.D.

The tumor suppressor p53 is a master sensor of stress and a key player in the mediation of cancer therapeutic agents. Although p53 is mutated in ~50% of human tumors, its tumor suppressive function is inactivated in the remaining 50%. As there are ~22 million cancer patients worldwide, ~11 million may therefore carry wild-type p53 that has lost its tumor suppressive function. Thus, a long-term research focus of the group has been to identify ways in which the tumor suppressive function of p53 can be selectively stimulated. p53 is a transcription factor and it controls many biological functions, including implantation, metabolism, cell cycle arrest, apoptosis and autophagy, by transactivating or transrepressing different target genes. The identification of switching molecules that can selectively enhance the apoptotic function of p53 may therefore enable the sensitization of cancer cells to therapy. Our identification of the ankyrin repeats, Src homology 3 domain and proline rich region–containing protein (ASPP) family of proteins showed how alteration of promoter selectivity of p53 can be used to sensitize cancer cells to therapy. The three family members, ASPP1, ASPP2 and the inhibitory member of the family (iASPP), all bind to the DNA-binding domain of p53, a region that is evolutionarily conserved and most frequently mutated in human cancers. ASPP1 and ASPP2 selectively stimulate p53's apoptotic effects and the transcriptional activation of proapoptotic genes, whereas iASPP inhibits them. This regulation of p53 by the ASPP family of proteins is evolutionarily conserved from *Caenorhabditis elegans* to humans, and ASPP proteins are also common regulators of p53 family members p63 and p73. Recently we showed that the ASPP proteins can bind and cooperate with p300, a well-defined cofactor of p53, to selectively regulate p53's transcriptional activity on promoters such as p53-inducible gene 3 but not on p21waf1. This is the first demonstration that the ASPPs can function together with p300 in regulating the transcriptional activity of p53.

In 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*. We have also generated several model systems for studying the roles of ASPP family members in normal development and disease. We showed previously that ASPP2 is a haploinsufficient tumor suppressor that shares overlapping function(s) with p53 in mouse development and tumor suppression. We recently identified ASPP2 as a novel regulator of Par-3 and a key player in controlling cell proliferation, polarity and tissue organization during development of the central nervous system. ASPP2 is recruited with Par-3 to cell-cell junctions to maintain the integrity of tight/adherens junctions, and its deficiency results

in loss of cell polarity and formation of neuroblastic rosettes that resemble primitive neuroepithelial tumors. Mechanistically, ASPP2 suppresses cell proliferation through a novel pathway that is independent of p53/p19(Arf) and p21(waf1/cip1). ASPP2 suppresses the Ras-induced small ubiquitin-like modifier (SUMO)-modified nuclear cyclin D1 and inhibits retinoblastoma protein phosphorylation. Nuclear cyclin D1 is also far more potent than wild type in bypassing Ras-induced senescence. Thus, we have shown that SUMO modification positively regulates nuclear cyclin D1 and revealed a new way in which cell cycle entry and senescence are regulated. These new findings led us to propose that epithelial cell polarity might be a major gatekeeper against cancer. We argued that loss of epithelial cell polarity may have an important role both in the initiation of tumorigenesis and in later stages of tumor development, favoring the progression of tumors from benign to malignant.

We also identified iASPP as an inhibitor of senescence and a key player in controlling epithelial stratification. We observed that iASPP inhibits premature cellular senescence of mouse embryo fibroblasts and keratinocyte differentiation *in vitro*. *In vivo*, nuclear iASPP often colocalized with p63 in the nuclei of basal keratinocytes. Consistent with this, iASPP bound p63 and inhibited the transcriptional activity of both TAp63-α and ΔNp63-α *in vitro* and influenced the expression level of p63-regulated genes such as loricrin and involucrin *in vivo*. Thus, iASPP is likely to control epithelial stratification by regulating p63's transcriptional activity. Biochemically we also identified iASPP as a novel binding partner of protein phosphatase 1 (PP1). We show that all ASPP family members can bind PP1 via two distinct interacting motifs. ASPP2 interacts with PP1 through an RVXF PP1 binding motif, whereas iASPP interacts with PP1 via a noncanonical motif (RNYF) located within its Src homology 3 domain. Phe815 is crucial in mediating iASPP-PP1 interaction, and iASPP(F815A) does not inhibit the transcriptional and apoptotic function of p53. Thus we identified iASPP as a new binding partner of PP1 that interacts through a noncanonical PP1-binding motif.

Detailed investigation of the biological functions of ASPP2 and iASPP *in vivo* has provided us with unique opportunities to address important questions in cancer biology. Over the next few years, we will focus our efforts on elucidating (i) the biological importance and molecular mechanisms of cell polarity in tumor suppression and (ii) how signals from the cell surface are integrated into transcription and cell fate determination.

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 lab is focused on understanding how this is achieved at the global level via networks of transcription factors and signaling pathways using the melanocyte/melanoma system, while addressing more mechanistic questions about gene regulation using *Saccharomyces cerevisiae*.

In the melanocyte/melanoma system we uncovered a key transcription factor cascade that coordinates cell survival, proliferation, differentiation, invasion and senescence and that is deregulated in melanoma. This network is regulated dynamically by the tumor microenvironment, which not only regulates its expression but also imposes a complex series of post-translational modifications on regulatory transcription factors such that they can switch from activators to repressors, shuttle from the nucleus to the cytoplasm, and be redirected from one set of target genes to another. As a consequence, melanoma cells *in vivo* can transiently adopt different phenotypes ranging from invasive and stem-cell-like to proliferating or differentiated. We are currently working to understand the interplay between these transcription factors and precisely how each post-translational modification affects their capacity to regulates genes, repair DNA and provide a distinct biological outcome.

A major conclusion from our work is that cells can switch phenotypes depending on the levels and activity of the microphthalmia-associated transcription factor MITF. We predicted that manipulating MITF activity may enable therapeutically resistant melanoma subpopulations to be switched to a sensitive phenotype. We have now begun to explore this therapeutic option, and in collaboration with colleagues in Spain we have identified ways to eradicate invasive stem-like cells in cultured melanoma cell lines and combine this with a novel prodrug designed to kill non-stem-like cells in a highly specific way. Over the next year we aim to take this novel therapy toward clinical trials.

In the past few years senescence has been identified as a major barrier to cancer initiation. In melanoma and other cancers, we identified two members of the T-box family of transcription factors, Tbx2 and Tbx3, as key antisenescence factors that act to prevent p53-driven activation of the p21 cyclin-dependent kinase inhibitor. Crucially, our work on Tbx2 revealed that melanomas contain an intact but suppressed senescence pathway. We have consequently been working toward identifying signaling pathways that are upregulated in melanoma but that provide an antisenescence function, for

example via upregulation of Tbx2 or Tbx3. We have shown that in some cell lines inhibition of one signaling pathway leads to DNA damage, a hallmark of senescence. We have also identified a small molecule that strongly upregulates MAPK signaling in melanomas that have activating mutations in the MAPK pathway, but not in cell lines without such mutations. We are now analyzing the mechanism underlying effects on DNA damage and will soon undertake a synthetic lethal screen for drugs that eradicate cells with activating MAPK mutations. This may be of therapeutic use in both melanoma and other cancers.

Whereas our work on melanoma explores the effect of signaling pathways on gene regulation, we use *S. cerevisiae* to examine the mechanistic aspects of how gene expression is regulated. Our recent findings have determined that transcription activators may depend on the prior activity of a class of proteins we term "promoter education factors" (PEFs), which can recognize the same elements as classical activators. In the highly inducible *PHO5* gene, the Cbf1 bHLH-LZ factor, which has no intrinsic transcription activation capacity, uses a chromatin-remodelling complex to facilitate stable recruitment of the Pho4 transcription activator and the basal transcription machinery. Moreover, Cbf1 enables *PHO5* to be redistributed to a specific nuclear compartment, most likely in cooperation with processing of a noncoding antisense RNA. The results have major implications for our work in mammalian cells, because Mitf-binding sites are also recognized by a ubiquitous factor that may play a role as a mammalian PEF. Understanding how PEFs function and how their deregulation might impact cancer will be a key future goal.

Human cancer genetics

Gareth Bond, Ph.D.

Since publication of the first human genomes, there has been much excitement about the potential of genomics to help researchers better understand the origins, progression and treatment of human diseases. However, progress toward identifying the common causal genetic variations underlying many human conditions like cancer, and their incorporation into clinical practice, has been more complicated than anticipated. Our research program integrates computational, molecular, cellular and human genetic approaches with the aim of identifying and characterizing common variations in the human genetic code. These variations affect cancer risk, progression and survival, and we strive to understand the molecular mechanisms behind the variants and their effects on cellular signaling and behaviors. The long-term goal of this work is to gain a better understanding of the contribution of germline genetics to both susceptibility to and progression of cancer, and to expose potential nodes of intervention that could prove valuable in both 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 between individuals. The most common approach to overcome this is a very large case-control study, which searches genome-wide for association of genetic variants with cancer in thousands of patients to reduce false-positive rates (genome-wide association study, GWAS). This approach has been very successful in the identification of >150 regions associated with altered risk in two dozen cancers. However, the GWAS approach has limitations. For example, extremely few causal variants from these regions have subsequently been successfully identified, validated and modeled in experimental systems. Low-incidence cancers are underrepresented, as are outcomes like cancer progression and survival. Our group aims to address these limitations by focusing on the identification of candidate single-nucleotide polymorphisms (SNPs) that (i) are associated with time-to-event phenotypes such as survival; (ii) can be more rapidly validated and explored in experimental models and (iii) can be more rapidly placed into well-studied cancer signaling pathways and targeted in cancer prevention and treatment strategies.

During the past year, we have made great strides in developing an approach to identify SNPs in high-dimensional data sets, in which the number of SNPs tested is much larger than the sample size, while retaining an acceptable false discovery rate. We use a combination of two nonparametric survival methods, the log-rank test and the random survival forest, both of which are model-free and therefore do not require diagnostic tests. We have used

simulations to examine whether the variable selection method of the random survival forest improves the power of the log-rank test to identify genetic variants associated with survival phenotypes, and to test the power of our methodology. These studies have resulted in the identification and validation of a high-frequency SNP in the obesity gene that associates with progression of B cell chronic lymphocytic leukemia. This is a common leukemia for which both biomarkers and a deeper understanding of progression are greatly needed. We have also applied our model to the identification of SNPs that affect metastatic progression of melanoma. This work has been a close collaboration with Colin Goding and with other researchers and clinicians in Oxford, Southampton and Bournemouth, UK.

We have also progressed in our efforts to identify and characterize the causal SNPs in regions that associate with cancer. As most SNPs in cancer-associated regions are found in noncoding loci, we focus on regulatory processes that could be significantly affected by single-base pair changes, such as transcription, splicing and microRNA regulation. We and others have shown that SNPs in transcription factor–binding sites can affect gene regulation and human cancer. We therefore reasoned that directly scanning for SNPs that alter transcription factor–binding sites could be an efficient method for the identification of causal SNPs in regions associated with cancer. To do this, we have developed a methodology that harnesses publicly available data, DNA-protein binding and transcription assays to screen for SNPs identified by GWASs to be associated with increased cancer risk. This work represents a close collaboration with Ludwig Oxford scientists Colin Goding and Sarah De Val.

Protein quality control

John Christianson, Ph.D.

A third of the human genome encodes proteins whose biogenesis occurs in the secretory pathway. The cell surface and secreted proteins and complexes maturing there represent important effectors of cellular homeostasis, growth, signaling and motility. While these proteins and complexes are in the endoplasmic reticulum (ER), their integrity and abundance are scrutinized by quality control (QC) mechanisms in the organelle. ER-associated degradation (ERAD) is an integral facet of the QC mechanism that ensures fidelity and regulates expression of secreted and integral membrane proteins through rapid and selective culling. It is an essential surveillance checkpoint for protein expression in all eukaryotic cells. Our research program aims to understand what role protein QC in the early secretory pathway plays in the initiation and progression of cancer, with a specific focus on how QC shapes the cell surface proteome in tumor and metastatic cells. Our group uses a systems biology strategy that integrates cell and molecular biological, biochemical and proteomic techniques to characterize the ERAD mechanism, its constituents and its substrates. The long-term goal of our work is to exploit QC mechanisms as a novel point of intervention for cancer therapies.

The complexity, adaptability and diversity of the ERAD mechanism are only just being appreciated. 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 to degrade the diverse range of proteins that challenge it. We have been at the forefront of identifying novel metazoan-specific components and mapping basic functional interactions mediating ERAD. In work submitted for publication in 2011 from our group, we integrated proteomic and functional genomic strategies to uncover >70 additional proteins of interest that interact with known components of the ERAD network. This study represents the most comprehensive mapping of the mammalian ERAD network so far and is proving an invaluable resource for our group to further elucidate the mechanisms underlying this process.

Building on insight gained from systematic studies, the lab is pursuing questions related to the underlying mechanisms of ERAD. The ER-resident ubiquitin ligase Hrd1 is a core ERAD component. Although it is not essential for degradation of all substrates, current studies suggest that Hrd1 has a robust, adaptive ability to identify and ubiquitinate substrates with disparate topologies and features. Hrd1 uses accessory proteins such as SEL1L and FAM8A1 to gain access to misfolded substrates, and we are working to uncover the molecular interactions that endow this complex with its

degradation capacity. Additionally, the lab has developed a tandem affinity purification strategy to isolate and identify novel ERAD substrates. We are using this technique to identify cell surface tumor suppressors and oncogenes whose expression is modulated by ERAD.

In the ER, the fate of nascent polypeptides is decided: they either fold properly or are degraded. We have previously reported an interaction between the ERAD component and ER lectin OS-9 and the ER-resident, Hsp90 chaperone homolog GRP94. This interaction represents the interface between protein folding and degradation machinery, acting as a platform for molecular "triage" decisions. The region of OS-9 we identified as crucial for GRP94 interaction was also found to harbor several cancer-related mutations, which are currently being investigated.

Regulation of vascular growth

Sarah De Val, Ph.D.

The group was established in early 2010 and currently focuses on the transcriptional regulation of vascular establishment and differentiation, with a particular interest in transcriptional pathways involved during angiogenesis and arteriovenous differentiation. The establishment of an adequate blood supply is a necessary step in 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 dysregulated and aberrant structure of the vessels within these tumors makes them poor conduits for drug delivery, and results in pockets of cancer cells that are inaccessible to blood-borne drugs. Consequently, the identification of targets for blocking or modulating vessel growth is an important therapeutic goal for cancer treatment.

Although many studies have examined the signaling molecules involved in vasculogenesis and angiogenesis, the transcriptional mechanisms that regulate the expression of genes within and downstream of these pathways remain important questions in vascular biology. To investigate them, 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. We are addressing questions about 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 multipronged approach to regulatory element identification, using *in silico* motif searches, publicly available data detailing histone marks, DNaseI hypersensitivity and ChIP data and phylogenetic footprinting. Regulatory regions are confirmed through transgenesis, and we have successfully developed a moderate-throughput screen for functional vascular enhancers using Tol2-transposase-induced mosaic transgenic zebrafish. We have also developed a system to generate single-copy, controlled-integration-site mouse transgenic lines in which to confirm, compare and study vascular enhancer expression patterns during both developmental and pathological conditions. By using these two techniques in combination with 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 knockdown, knockout and overexpression models, we have begun to delineate some of the transcriptional pathways involved in angiogenesis, particularly those involved in the Notch-Dll4 signaling cascade.

The group is also investigating the unsuspected or poorly studied roles of transcription factors during vascular differentiation and at the angiogenic front, particularly those in the Tbox and Ebox families of transcription factors. We are using a combination of gene expression studies and phenotypic analysis of vascular development after gene alteration (including knockout, knockdown through morpholino or short interfering RNA, and overexpression) 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. This includes both histological analysis of spontaneous human and mouse tumors, and implantation of tumors into mouse models with transgenic or altered gene expression.

Epigenetic mechanisms

Skirmantas Kriaucionis, Ph.D.

The laboratory focuses on the function of 5-hydroxymethylcytosine (5hmC) in normal tissue homeostasis and during transformation in cancer. Both the structural relation of 5hmC with 5-methylcytosine and recent data on the pathways of demethylation suggest a link between 5hmC and hypermethylation of CpG islands in cancer. Genetic evidence shows that TET2 protein, which catalyzes 5hmC deposition, is frequently mutated in myelodysplastic syndromes and acute myelogenous leukemia. Finally, cancer cells show a greater than ten-fold reduction in 5hmC in comparison to normal tissues. This indicates that TET2-catalyzed 5hmC deposition is important for initiation or clonal evolution of cancer cells, but the molecular details of these events are unknown. Additional emphasis on the importance of TET2-5hmC in cancer comes from data demonstrating that TET2 and IDH1/2 mutations are mutually exclusive in acute myelogenous leukemia. Mutant IDH enzymes produce 2-hydroxyglutarate instead of 2-oxoglutarate, and 2-hydroxyglutarate was shown to inhibit the function of TET enzymes, which use 2-oxoglutarate as a cofactor.

We have been adapting existing techniques and developing some new methods for localizing 5hmC and 5-methylcytosine in the genome; these methods will be useful for asking questions about DNA (un)methylation maintenance and fidelity. We plan to use these techniques on selected cell lines that model certain hematopoietic differentiation steps, with the aim of characterizing changes in 5hmC deposition in hematopoietic lineages *in vivo*. Fu rther, mapping of TET2 localization will reveal sites subjected to active turnover of DNA modifications. We are analyzing how DNA modification signatures differ between terminally differentiated cells and cells actively participating in the cell cycle. Because the majority of epigenetic mechanisms are involved in faithful re-establishment of gene expression and chromatin states after cell division, exit from the cell cycle may be followed by unique chromatin adaptations for the efficient function of a nucleus.

We are working to identify protein interaction partners of TET proteins, as these can reveal regulatory pathways that converge on 5hmC deposition and target modification activity in the genome. We are using several techniques to identify interactions in both common and rare cell types.

The third direction involves using of small molecules for targeting DNA modification pathways. 5-azacytidine induces DNA hypomethylation and was shown to have positive effects when administered to patients with myelodysplastic syndromes. However, there are many stability, toxicity and resistance issues that restrict 5-azacytidine use, highlighting the need for alternative demethylating agents.

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San Diego

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Director's message

The eight research groups at the Ludwig San Diego Branch focus mainly on cancer genetics, cell signaling, gene regulation and mechanisms of cell division. Ludwig researchers in San Diego have made important discoveries about how malignant cells slacken their genomic integrity, how these cells 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.

The relationship between Ludwig San Diego and the University of California, San Diego (UCSD) has gained strength through the efforts of Institute member Don Cleveland, chair of the UCSD School of Medicine's Department of Cellular and Molecular Medicine, and by our successful joint efforts in recruiting the next director of the UCSD Moores Cancer Center, Scott Lippman, of the University of Texas MD Anderson Cancer Center. Many postdoctoral fellows and graduate students move on to independent positions in academia or industry after finishing their training with Ludwig in San Diego, and we are an increasingly vibrant place for advanced training. We also have a continuously growing number of publications resulting from collaborative efforts among our laboratories. We continue to be very successful at garnering competitive grant support, despite increasingly difficult national circumstances.

In our last review, it was suggested that we develop more power in translational research. We have addressed this vigorously as we believe that applying first-class basic studies is an important part of our mission. We first developed our Small Molecule Development Laboratory, headed by Andy Shiau and Tim Gahman, which is now fully functioning and providing enormous value to us and the whole Ludwig Institute. We then enhanced our capabilities in molecular structure. In July, Kevin Corbett joined us from Harvard Medical School. He is a wonderful structural biologist who has rapidly developed a fully functional laboratory with several ongoing collaborations. We are now in the final stages of recruiting an internationally renowned translational cancer biologist to complement these actions, and I expect to report on that next year.

The quality, accomplishment and breadth of expertise of the Ludwig San Diego staff has been praised and welcomed by UCSD Chancellor Marye Anne Fox and Dean of UCSD School of Medicine David Brenner. The recent decision by the Institute's Board of Directors to establish San Diego as one of the few flagship sites reinforces our confidence in our path and gives us great hope and expectation for the future. We greatly appreciate this vote of confidence; it will drive us to ever further discovery in the future.

Web Cavenee

Tumor biology

Webster K. Cavenee, Ph.D.

Headed by Web Cavenee, this laboratory now includes the Section of Human Carcinogenesis led by Frank Furnari, and focuses on genetic alterations that drive the malignancy of grade IV high-grade gliomas (glioblastoma multiforme; GBM). We have three major objectives: (i) to determine how expression of the commonly amplified and truncated epidermal growth factor receptor gene (known as Δ*EGFR* or *EGFRvIII*) potentiates tumor heterogeneity and aggressiveness; (ii) to determine the role of this receptor in tumor maintenance and (iii) to determine how *PTEN/PI3K* signaling modulators influence the effectiveness of ΔEGFR/EGFR receptor-directed therapeutics.

ΔEGFR and tumor aggressiveness

Although guanylate binding protein 1 (GBP1) was among the first interferon-inducible proteins identified, its function is still largely unknown. This year, we showed that EGFR activation promoted GBP1 expression in GBM cells through a signaling pathway involving Src and p38 MAPK. Yin Yang 1 was identified as the downstream transcriptional regulator of EGFR-driven GBP1 expression. GBP1 was required for EGFR-mediated matrix metalloproteinase-1 (MMP1) expression and glioma cell invasion *in vitro*. Although deregulation of GBP1 expression did not affect glioma cell proliferation, overexpression of GBP1 enhanced invasion through MMP1 induction, which required its C-terminal helical domain and was independent of its GTPase activity. GBP1 expression was high and positively correlated with EGFR expression in human GBM tumors and cell lines. Together, these findings establish GPB1 as a previously unknown link between EGFR activity and MMP1 expression, and nominate it as a novel potential therapeutic target for inhibiting GBM invasion.

ΔEGFR and glioma maintenance

A hallmark feature of high-grade glioma is extensive angiogenesis. So far, targeting this tumor feature with inhibitors or antibodies that block a major angiogenesis driver, vascular endothelial growth factor (VEGF), and its signaling pathway has been only marginally effective therapeutically. This year we examined the function of ΔEGFR in promoting glioma angiogenesis and found that it promotes vessel formation not through VEGF production but through robust upregulation of IL-8. We found this link between IL-8 expression and ΔEGFR in glioma cell lines expressing ΔEGFR and validated it in human glioma specimens and primary glioma stem cells. Furthermore, selective short interfering RNA knockdown of IL-8 or the nuclear factor κB (NF-κB) subunit p65, a transcription factor required downstream of ΔEGFR for IL-8 expression, resulted in attenuated xenografted tumor growth and a

reduction in vessel formation. Conversely, forced IL-8 expression in ΔEGFR-negative glioma cells resulted in enhanced tumorigenicity and associated angiogenesis. Thu s, these findings suggest that targeting ΔEGFR/NF-κB/IL-8 signaling in glioma may be an alternative way to attenuate tumor growth through ablation of blood vessel formation.

Effectiveness of ΔEGFR/EGFR receptor–directed therapeutics

Extensive bidirectional cross-talk occurs in cell-signaling pathways downstream of EGFR and the urokinase-type plasminogen activator receptor (uPAR); however, direct cross-talk between ΔEGFR and uPAR has not been examined. In a collaborative effort with Steve Gonias, chair of the Department of Pathology at UCSD, uPAR was shown not to regulate ERK activation in ΔEGFR-expressing GBM cells. However, in GBM cells isolated from four separate xenografts in which ΔEGFR expression was downregulated *in vivo*, uPAR assumed the major role in sustaining ERK activation. These studies identified distinct cell-signaling activities for uPAR in GBM cells that express ΔEGFR and in cells released from dormancy when ΔEGFR is neutralized. From this, uPAR and its cross-talk pathways with ΔEGFR emerge as possible targets for therapeutic development in GBM.

Cancer genetics

Richard D. Kolodner, Ph.D.

The laboratory, which includes the Section of Structure and Computational Genetics headed by Christopher D. Putnam, works on two research projects using the yeast *Saccharomyces cerevisiae*. The first is elucidating mechanisms of DNA mismatch repair (MMR) pathways that prevent mutations due to errors during DNA replication. The second is defining pathways that prevent genome rearrangements such as translocations. The long-term goals are to understand how mutations and genome rearrangements arise in cancer cells and whether the presence of genome instability can be exploited for therapeutic means. Consistent with this, the laboratory has initiated studies investigating genome instability in mammalian cancer cell models.

We have focused on the biochemistry, genetics and cell biology of MMR. In collaboration with the Chromosome Biology Laboratory of Ludwig San Diego, functional fluorescent MMR proteins were visualized in living *S. cerevisiae* cells. This analysis identified two subpathways of mismatch recognition, one of which involves constitutive coupling of the Msh2-Msh6 mispair recognition complex to the replication fork. When a mispaired base is encountered, Msh2-Msh6 loads Mlh1-Pms1 onto DNA. These data also indicate that Mlh1-Pms1, a key downstream MMR factor, seems to function independently of Msh2-Msh6 once it is loaded onto the DNA. In complementary studies, the cell cycle dependence of MMR was examined by restricting expression of MMR proteins to different times in the cell cycle. These experiments showed that MMR only functions shortly after replication, suggesting that a transient DNA- or chromatin-based signal dependent on replication is required for MMR. Suppression of recombination by MMR proteins, however, can act at other times in the cell cycle. Two lines of experimentation are extending these studies. First, genetic studies are being used to identify mutations that specifically cause defects in the different MMR subpathways. Second, all known MMR proteins have been overproduced and purified and are being used to biochemically reconstitute DNA replication–coupled MMR to elucidate the mechanisms of MMR.

Identification of genes and pathways that cells use to prevent genome instability is a critical area of investigation. Most cancers are associated with increased genome instability, and the genome rearrangements that arise may play a role in driving the development and progression of cancer. The laboratory has developed multiple assays to identify and study genetic defects that cause increased rates of accumulating genome rearrangements. To extend the results of initial genetic studies, we used a systems biology–inspired bioinformatics approach to identify genes and pathways enriched for roles in maintaining genome stability. This led to the identification of

~500 candidate genome instability–suppressing genes. We are using robotic methods to examine individual and multiple mutations in these genes to determine whether these mutations cause increased genome instability alone or in combination. We aim to identify *S. cerevisiae* genetic networks that prevent genome instability. For example, these ongoing studies have identified the Mrc1 replication stress checkpoint and the Srs2 antirecombination pathway as critical pathways that interact with a diversity of repair, replication and chromatin assembly pathways to prevent genome instability. The laboratory has also been collaborating with Sandro José de Souza in the Computational Biology Laboratory, Ludwig São Paulo, to determine whether the human homologs of these genes are mutated or show aberrant expression in cancer cell lines and cancers being analyzed by cancer genomics initiatives.

Cell biology

Don Cleveland, Ph.D.

The laboratory is focused on three topics: (i) mechanisms of mammalian chromosome movement and spindle assembly during mitosis and how errors in these processes contribute to tumorigenesis; (ii) the basis for epigenetic specification of centromeres and (iii) mechanisms of neuronal growth and death, especially related to treatment of the neurodegenerative diseases amyotrophic lateral sclerosis (ALS) and Huntington's disease.

Centromeres direct chromosome inheritance, but in multicellular organisms their positions on chromosomes are primarily specified epigenetically rather than by a DNA sequence. The major candidate for the epigenetic mark is chromatin assembled with the histone H3 variant CENP-A. Recent studies offer conflicting evidence for the structure of CENP-A-containing chromatin, including the histone composition and handedness of the DNA wrapped around the histones. In the past year, we developed a model for assembly and deposition of centromeric nucleosomes that couples these processes to the cell cycle. This model reconciles divergent data for CENP-A-containing nucleosomes and provides a basis for how centromere identity is stably inherited.

Chromosome mis-segregation has long been associated with cancer. The Aurora kinases are essential for chromosome segregation and mitosis. 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 stably attach to spindle microtubules only after dephosphorylation of the major microtubule capture complexes by PP1, which rebinds to and 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.

A proportion of cases of the adult motor neuron disease ALS is caused by mutations in superoxide dismutase (SOD1). With conformation-specific antibodies, we demonstrated that misfolded mutant SOD1 binds directly to the voltage-dependent anion channel, inhibiting the conductance of

individual channels. This establishes a direct link between misfolded mutant SOD1 and mitochondrial dysfunction in inherited ALS. Moreover, by transgenically making mitochondria fluorescent, we showed that misfolded SOD1 associated with motor neuron mitochondria alters mitochondrial shape and distribution within the axonal processes.

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. In the past year, we used cross-linking and immunoprecipitation coupled with high-throughput sequencing to identify binding sites in 6,304 genes as the brain RNA targets for TDP-43. Massively parallel sequencing and splicing-sensitive junction arrays revealed that levels of 601 mRNAs were changed (including *Fus* (*Tls*), progranulin and other transcripts encoding neurodegenerative disease–associated proteins) and 965 altered splicing events were detected (including the progranulin receptor sortilin) following depletion of TDP-43 from mouse adult brain with antisense oligonucleotides. RNAs whose levels were most depleted by reduction in TDP-43 were derived from genes with very long introns and that encode proteins involved in synaptic activity. TDP-43 was demonstrated to autoregulate its own synthesis by directly binding and enhancing splicing of an intron in the 3′ untranslated region of its own transcript, thereby triggering nonsense-mediated RNA degradation. Disruption of autoregulation by cytoplasmic aggregation of TDP-43 offers an explanation for the runaway synthesis of TDP-43 in ALS and other neurodegenerative diseases.

Chromosome biology

Arshad Desai, Ph.D.

The laboratory is focused on understanding the mechanisms that accurately 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, a hallmark of cancer cells. The interface between chromosomes and spindle microtubules is also the target of antimitotic chemotherapeutic drugs, such as taxol. One major project focuses on the conserved KNL-1/Mis12 complex/Ndc80 complex (KMN) protein network, which comprises nine interacting proteins. We have previously established that KMN provides the core microtubule-binding activity of the kinetochore and acts as a scaffold for spindle checkpoint signaling. During analysis of a conserved protein family recruited by the KMN network to target the dynein motor complex to kinetochores, we observed that extended mitotic arrest in human cancer cells leads to chromosomal instability characterized by uncoordinated loss of chromatid cohesion. Subsequent efforts using multiple methods to hold cells in a metaphase-like state revealed that this uncoordinated loss of cohesion is a common outcome and is unrelated to the specific perturbation used to generate the arrest. Highly aneuploid cancer cells exhibited a higher propensity for uncoordinated loss of cohesion compared with euploid cells and, once chromosomes started losing cohesion, reactivation of the spindle checkpoint trapped them long-term in a pseudomitotic state that eventually led to cell death or exit into an aberrant interphase state. These observations reveal a new mechanism for chromosomal instability generated by extended mitotic arrest and impact current interpretations of mitotic phenotypes in cultured cancer cells, which have been used to characterize cell division proteins and antimitotic chemotherapeutic agents.

In a second project area, 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 chromatin domain containing CENP-A is proposed to be propagated by DNA replication and replenished during early G1 to epigenetically maintain centromere identity. In one project, we investigated the ability of naked DNA to form heritable chromosome-like structures in the nematode *Caenorhabditis elegans*. Heritability of introduced DNA is relevant to the design of artificial chromosome vectors, providing an alternative to viral vectors for therapeutic delivery of genetic material. This work revealed that DNA introduced into the *C. elegans* germline rapidly centromerized in a *de novo* and sequence-independent

manner. Using direct monitoring of *de novo* centromerization, we defined the kinetics and requirements of this centromerization reaction. In other work, we have shown that deposition of CENP-A occurs *de novo* following fertilization in *C. elegans* and analyzed CENP-A distribution genome-wide. This effort has revealed a relationship between CENP-A occupancy and germline transcription.

In addition to our efforts on chromosome segregation mechanisms, in a collaborative project with Richard Kolodner's group at Ludwig San Diego, we used our expertise with live-cell imaging to aid their efforts to understand the mechanism of DNA mismatch repair.

Gene regulation

Bing Ren, Ph.D.

The laboratory aims to thoroughly understand the molecular mechanisms that regulate transcription and gene expression in different mammalian cell types. To achieve this goal, we are taking a genome-wide, integrative approach. In 2011, we have made substantial progress on the following three fronts.

Identification of transcriptional regulatory sequences in genomes

Transcriptional regulatory elements, particularly long-range acting sequences such as enhancers, play critical roles in cell type–specific gene expression, but their location and function in the genome have remained elusive. Recently, we showed that enhancers are associated with characteristic and predictive chromatin modification signatures, namely presence of H3K4me1 and absence of H3K4me3. Using this chromatin signature, we have identified tens of thousands of potential enhancers in the human genome that activate transcription during differentiation of human embryonic stem cells. We showed that these enhancers are associated with dynamic chromatin state during differentiation. Analysis of these enhancers revealed potentially key transcriptional regulators of pluripotency and a chromatin signature indicative of a poised state that may confer developmental competence in human embryonic stem cells.

Since 2008, the laboratory has been part of the modENCODE consortium, generating chromatin modification maps and RNA polymerase II binding sites throughout the *Drosophila melanogaster* genome in ten development stages. In collaboration with Kevin White of the University of Chicago, we have annotated promoters, enhancers and insulator elements in the fly genome.

Epigenomic analysis of cancer cells

Together with Andy Simpson, Bob Strausberg, Otavia Caballelo, Anamaria Aranha Camargo and other Ludwig investigators, the laboratory has investigated the epigenome of a breast cancer cell line (HCC1954) to understand the potential role of epigenetic mechanisms in tumorigenesis. We produced base-resolution DNA methylomes and transcriptomes as well as several chromatin modifications in HCC1954 and primary human mammary epithelial cells. We observed large-scale, widespread DNA hypomethylation in the cancer cell genome that is closely associated with transcriptional silencing, particularly in many known tumor suppressor genes. These results suggest a potential epigenetic pathway for gene regulation in cancer cells.

Influence of epigenome on cellular responses to extracellular signaling

Signaling pathways play critical roles in cellular and organ development, homeostasis and responses to environmental changes and insults. NF-κB is a family of master transcription factors found in almost all animal cell types and plays essential roles in multiple physiological processes including inflammation and immunity. Binding and activation of κB sites depend on context and stimulus. However, it is less clear how ubiquitous NF-κB induces differential transcriptional responses in different human cell types. To address this problem, we characterized the transcriptional programs of both HeLa and THP-1 cells in response to TNF-α, a well-known inducer of NF-κB activation. We found that cell type–dependent gene induction by TNF-α is primarily governed by differential NF-κB binding, as revealed by genome-wide location analysis of NF-κB subunit p65/RelA occupancy. We also showed that lineage-specific, pre-existing chromatin modifications strongly predict differential binding of NF-κB upon TNF-α stimulation in each cell type. We further show that a group of lineage-specific transcription factors, including PU.1 and C/EBP-α in THP-1 cells, may establish these primed enhancers and confer cell specificity to ubiquitous NF-κB signaling. Our results suggest a general mechanism by which complexity and specificity of gene regulation by signaling pathways in different cell types can be achieved.

Mitotic mechanisms

Karen Oegema, Ph.D.

The laboratory uses the nematode *C. elegans* as a model system for mechanistic cell biology. We have used this system to address fundamental questions in three main areas: (i) functional genomics of cell division, (ii) molecular mechanics of cytokinesis and (iii) centriole duplication and function. Highlights in two of the three areas are summarized below.

Generating a functional gene network for the essential gene set

During the past year, we generated a functional network for the set of ~900 essential genes in a model metazoan based on high-content screening data. Genome sequencing projects have generated a comprehensive "parts" list of the genes required to build an organism, providing an inventory of cellular building blocks. The current challenge is to place these genes in functional networks to define the function of previously uncharacterized genes and provide insight into the organization and interconnectivity of cellular pathways. Generating functional maps with the power to resolve differences in gene function requires a lot of information, which is typically obtained by "high-content" screening. In a high-content screen, genes are individually inhibited and the consequences are documented by filming the behavior of individual cells. Rather than monitoring individual cells, we tested a new approach to increase information content by monitoring the effect of gene inhibitions on the structure of a complex tissue in a multicellular organism. As a test tissue, we chose the reproductive organ of *C. elegans*. We found that inhibiting different genes had a remarkably diverse and information-rich spectrum of effects on tissue structure—effectively generating a "fingerprint" for each gene that allowed us to predict its function. By developing a new method to quantitatively assess the significance of gene-gene connections, we translated the information obtained by monitoring tissue architecture into a functional gene network. In addition to predicting the function of related human genes, the success of this approach suggests that it will be broadly useful for generating functional gene networks in other organisms, including vertebrates. The manuscript describing these findings was accompanied by two online resources, a comprehensive phenotypic database and a Java-based interactive gene network.

Dissecting signaling between anaphase spindle and cortex during cytokinesis

To ensure that each daughter cell inherits a single genomic complement, contractile ring assembly and constriction are controlled by signals from the anaphase spindle. Cytokinesis is coordinately directed by the centrosomal microtubule asters and the central spindle, a set of microtubule bundles that

forms between the separating chromosomes. The central spindle promotes contractility at the cell equator, whereas the centrosomal microtubule asters suppress cortical contractility at the cell poles. The central spindle recruits cytokinesis-signaling molecules, including centralspindlin and the chromosomal passenger complex (CPC), which have an essential conserved role in cytokinesis. During the past year, we showed that inhibition of either centralspindlin or CPC in the *C. elegans* embryo leads to a three-fold reduction in constriction rate and ultimate cytokinesis failure. In each inhibition, contractile proteins are initially recruited to the equatorial cortex, but the cortex does not form a furrow with a mature contractile ring at its tip. Simultaneous inhibition of CPC and centralspindlin results in an additive defect, demonstrating that centralspindlin and CPC make independent contributions to control contractile ring assembly. We are currently focused on understanding the molecular nature of the contributions of these two complexes to contractile ring assembly.

Proteomic biology

Huilin Zhou, Ph.D.

The laboratory is focused on two areas: (i) the genetic and biochemical mechanisms of eukaryotic DNA damage checkpoint pathways and (ii) the identification and characterization of pathways controlled by the DNA damage checkpoint to suppress genome instability. We use the yeast *S. cerevisiae* as a model organism for most of our studies.

Inherited and sporadic mutations to DNA damage checkpoint genes are frequently linked to genome instabilities and cancer-prone syndromes. For example, mutation of the ATM kinase causes ataxia telangiectasia, a cancer-prone genome instability syndrome. Mutation of NBS1, a gene involved in DNA double-stranded break repair, causes Njimegen breakage syndrome. Importantly, most DNA damage checkpoint genes including ATM and NBS1 are conserved from yeast to human. Genetic studies in yeast have established the critical role of the DNA damage checkpoint in genome maintenance. However, the molecular basis is far from being completely understood. The long-term objective of our studies is to understand how DNA damage checkpoint suppresses genome instabilities.

We are pursuing two major areas of investigation. In the first area, we are studying the genetic and biochemical mechanisms of the regulation of DNA damage checkpoint kinases. We have shown that DNA replication fork protein Mrc1 functions in the DNA replication checkpoint by directly promoting Mec1/ATR kinase to phosphorylate and activate Rad53, the key effector Chk2 family kinase in the DNA damage checkpoint.

Recently, we have characterized the genetic and biochemical basis for the role of Rad9/53BP1 in DNA damage–induced Rad53/Chk2 activation. We have found that Dpb11/TopBP1, Xrs2/Nbs1 and the BRCT domain of Rad9 function in parallel pathways to control cell cycle–dependent Rad53 activation. To understand how the DNA damage checkpoint is inactivated after completion of DNA repair, we have analyzed the role of Sae2/CtIP, a DNA repair protein, in the inactivation of Rad53. Our recent study establishes a biochemical basis for how the phosphorylation of Sae2 controls Rad53 inactivation. These studies have provided new insights into the coordination between DNA repair and the activation and inactivation of the DNA damage checkpoint.

The laboratory is also studying the role of the DNA damage checkpoint in genome maintenance. We have developed and applied quantitative phosphoproteomics to identify most of the substrates of DNA damage checkpoint kinases known in yeast. We are further applying this technology

to systematically analyze how the DNA damage-signaling network is controlled by genes involved in DNA replication, repair and checkpoint. In parallel, we are using yeast genetics and protein biochemistry to study how checkpoint kinases and their substrates are involved in telomere maintenance and the suppression of chromosomal rearrangements. Together, these studies will allow us to gain new insights into how the DNA damage–signaling network is involved in genome maintenance.

Small molecule discovery

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

The drug discovery industry is facing extraordinary challenges, including the inherent difficulty and mounting costs of the research and development process and increasingly stringent regulation by the US Food and Drug Administration. To enable the Ludwig Institute to address the resulting imminent need for innovative new medicines, the Small Molecule Discovery Laboratory was formed to develop and execute an Institute-wide, systematic small molecule drug discovery process. By complementing the efforts of Ludwig Institute scientists with outsourced research capabilities, the laboratory, headed by Andrew K. Shiau and Timothy C. Gahman, seeks to rapidly identify small molecules that target proteins with important roles in cancer. In the past year, the group has leveraged its expertise in 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, to perform multiple projects in conjunction with over a dozen Institute investigators and affiliates. In particular the laboratory has made substantial progress toward generating potent and selective inhibitors of several different enzymes, including polo-like kinase 4 and maternal embryonic leucine zipper kinase.

Polo-like kinase 4

Although some cancer cells may be stably aneuploid, many exhibit some degree of chromosomal instability (CIN), an accelerated rate of chromosomal gain or loss during cell division. Recent studies have demonstrated that 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). Thus, suppression of centrosomal amplification via inhibition of PLK4 activity may represent a novel approach to blocking the malignant growth of cells driven by CIN and aneuploidy. Using high-throughput biochemical and cellular assays and molecular modeling, the laboratory has identified compounds with nanomolar affinity for PLK4 and at least 50-fold selectivity against several hundred kinases from the human kinome. In collaboration with the laboratories of Arshad Desai and Karen Oegema, the group has demonstrated that these inhibitors can block centriole duplication in certain immortalized cell lines, and can be used to generate large populations of acentrosomal cells. These cells are being used in studies to determine the roles of the centriole in cilia formation and of the centrosome in cell division. The laboratory has also discovered that PLK4 inhibitors significantly compromise the growth of multiple tumor cell lines *in*

vitro. The team will be evaluating several of these small molecules that exhibit good 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 subfamily of serine/threonine protein kinases, is largely restricted to proliferating progenitor cells. Importantly, MELK is also expressed at high levels in aggressive brain cancers and in colon, breast, ovary and lung tumors. Suppression of MELK expression inhibits proliferation of human glioblastoma and medulloblastoma cells. Hence, inhibition of MELK may be a novel approach for the treatment of central nervous system cancers, potentially through the modulation of tumor progenitor cells. In the past year, the laboratory has identified several compound classes that potently inhibit MELK in biochemical assays. In collaboration with the laboratory of Greg Riggins at the Ludwig Center at Johns Hopkins University, the group has discovered that one of these inhibitors blocks the growth of glioma neurospheres *in vitro*. This compound, which efficiently crosses the blood-brain barrier, is currently being tested in orthotopic brain cancer models.

Structural biology

Kevin Corbett, Ph.D.

The long-term goal of the laboratory is to learn the mechanisms behind chromosome segregation in meiosis. Meiosis is a two-stage cell division program that produces gametes, specialized cells with half the chromosome complement of a normal cell, which participate in sexual reproduction. The reduction in chromosome number occurs in the first stage of meiosis, termed meiosis I. In this step, two homologs, nearly identical copies of each chromosome inherited from the two parents, specifically associate with and then segregate from one another. Homolog association and segregation in meiosis is a highly regulated process, and involves a modified homologous recombination DNA repair process that physically links each pair of homologs. Our work on the molecular details of meiosis will therefore aid understanding of how aberrant recombination can contribute to both cancer and human reproductive dysfunction, including aneuploidy and infertility. Using biochemical and structural methods coupled with yeast genetics, we are studying the proteins that regulate recombination in meiosis to drive specific homolog associations, and a separate set of proteins that regulate how chromosomes attach to the meiotic spindle.

In both mitosis and meiosis, each chromosome becomes attached to the spindle apparatus through a large protein complex called the kinetochore. Uniquely, in meiosis I, each sister chromatid pair becomes aligned with its homolog. Proper attachment of homologs to opposite spindle poles (biorientation) requires that sister chromatids attach to the same pole (mono-orientation). In yeast, sister chromatids are cross-linked by the monopolin complex to enforce their mono-orientation. Corbett's previous work at Harvard Medical School outlined the architecture of the monopolin complex's structural core, a "V"-shaped assembly with kinetochore-binding patches on the ends. The laboratory is now assembling the intact four-protein complex for structural examination by both electron microscopy and X-ray crystallography. We are also studying the complex's functions using *in vitro* biochemistry and *in vivo* chromosome segregation assays.

The key event allowing reduction in chromosome number in meiosis is specific association of homologs through a controlled homologous recombination mechanism. As meiosis I initiates, the genomic DNA is broken and the resulting free DNA ends seek out and associate with their equivalent sequences on homologous chromosomes. A subset of DNA breaks is repaired as "crossovers," where the homologs are reciprocally exchanged during repair. The resulting physical links between homologs allow them to align and attach to the spindle apparatus in a bipolar manner. Our laboratory is studying two sets of proteins involved in crossover formation

and regulation. First, we are studying a pair of proteins, Hop1 and Red1, which associate with meiotic chromosomes and regulate recombination directly to favor the formation of interhomolog crossovers. Hop1 and Red1 also participate in a meiosis-specific checkpoint that monitors crossover formation on each pair of homologs. We are pursuing a combined structural and biochemical approach to learn these proteins' mechanisms, guided by the observation that Hop1 bears structural similarity to the well-studied Mad2 mitotic spindle checkpoint protein. We are testing several functional hypotheses stemming from this close structural, and presumably functional, similarity. Our other focus is on the synaptonemal complex, a ladder-like assembly that nucleates at developing crossovers and then extends along chromosomes, bringing homologs into close juxtaposition. This complex is mysterious from both a structural and a functional standpoint, and little is known about its role in meiosis. As a first step to understanding this complex, we are reconstituting the complex *in vitro* and examining its architecture and assembly mechanisms. These studies should lay the groundwork for i*n vitro* and *in vivo* functional assays in the future. Overall, our work promises to better define the molecular structures and interactions involved in meiotic chromosome segregation.

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Director's message

Over the past year, a new operating model was successfully implemented to convert the São Paulo Branch to a locally funded research effort. Our partnership with the Hospital Alemão Oswaldo Cruz reached an end and our research groups were physically relocated to different host institutions. The Molecular and Cellular Biology Group, headed by Vilma Martins, was the first to relocate, moving to the International Research and Teaching Center at the Hospital A.C. Camargo. On January 1, 2012, the research group headed by Anamaria A. Camargo moved to the newly established Molecular Oncology Center at Hospital Sírio-Libanês. The other two group leaders have also moved to new venues: Sandro J. de Souza, head of the Computational Biology Laboratory, moved to the Federal University of Rio Grande do Norte in northeast Brazil, and Luisa L. Villa and the Virology Group moved to two different host institutions, the University of São Paulo and Santa Casa de Misericórdia de São Paulo. The research groups will continue to receive Ludwig support during this transition and will also pursue local funding to underwrite their research activities. In 2011 we made discoveries of importance in the field of human papillomavirus (HPV) infections, neurobiology and cancer genomics. Collaborative interactions between our research groups and the broader Ludwig community have increased. Many investigators and postdoctoral fellows trained at the Institute have moved on to independent positions in academia. We are grateful to the Ludwig Institute for its many years of support.

Anamaria Camargo

Molecular biology and genomics

Anamaria Camargo, Ph.D.

In 2011 we enhanced our efforts to integrate genomics and functional analysis of selected cancer genes (*ADAM23* and *SIGIRR*) to identify key molecular mechanisms driving tumor progression and therapeutic response.

Our Clinical Genomics Program continues to benefit from a strong collaboration with the renowned group of surgeons led by Angelita Gama at the Hospital Alemão Oswaldo Cruz. This group of surgeons was the first to propose a nonoperative wait-and-watch approach for rectal cancer patients with complete clinical response to neoadjuvant therapy. Three collaborative projects are ongoing to help identify of patients who will benefit the most from this approach: (i) identification of a gene expression signature in the primary tumor to predict response to neoadjuvant therapy in rectal cancer patients; (ii) development of personalized biomarkers for minimal residual disease assessment after neoadjuvant therapy and (iii) identification of tumor cell subpopulations resistant to neoadjuvant therapy by comparative lesion sequencing of primary and residual tumors. In a related work to uncover the mechanisms of drug resistance in colorectal cancer, we sequenced, in collaboration with John Mariadason from Ludwig Melbourne-Austin, the exome of 12 colorectal cancer cell lines with distinct susceptibility patterns to drugs used for colon cancer treatment.

Significant progress was made characterizing the molecular mechanism behind *ADAM23* epigenetic silencing in breast cancer. We have previously demonstrated that ADAM23 promoter hypermethylation is strongly associated with metastatic disease and poor overall survival. In 2011, we focused on the biological relevance of intratumoral heterogeneity in relation to *ADAM23* gene expression. Using both *in vitro* and *in vivo* assays we demonstrated that ADAM23^{+} and ADAM23^{-} cells cooperate to enhance metastatic spread and colonization. In collaboration with Frank Furnari at Ludwig San Diego we identified key molecules involved in cross-talk between these two tumor cell subpopulations during tumor progression. We have also made progress in the characterization of SIGIRR as a new immunomodulatory molecule upregulated in erbB2^{+} tumors. *In vitro* assays suggest that SIGIRR upregulation in breast tumors may impair leukocyte recruitment and favor macrophage polarization to M2 phenotype. Tumor cell variants with SIGIRR-knockdown expression were generated during the past year and are currently being used to explore *in vivo* the molecular mechanisms behind SIGIRR upregulation in breast tumors.

As of January 1, 2012, our research group has moved to the newly established Molecular Oncology Center at Hospital Sirio-Libanês in São Paulo. The new center will conduct translational research and will be jointly funded by the Ludwig Institute and Hospital Sírio-Libanês. It will be a unique opportunity to combine our experience in conducting research in cancer genomics with the clinical oncology excellence of the Hospital Sírio-Libanês. It is a winning combination that will certainly mean clinical benefit for cancer patients. We are grateful to both institutions for their enthusiastic support.

Molecular and cell biology

Vilma Martins, Ph.D.

Neurodegenerative diseases such as prion diseases, Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and tauopathies all involve the propagation and accumulation of misfolded prion-like protein. The normal form of the prion protein, termed PrP^{C}, is a glycosylphosphatidylinositol-anchored cell surface sialoglycoprotein required for the propagation and accumulation of the misfolded protein, PrP^{Sc}. The accumulation of toxic, insoluble PrP^{Sc} is thought to cause neuronal death in transmissible spongiform encephalopathies, also known as prion diseases. Although many studies have examined the mechanisms responsible for PrP^{Sc} misfolding and accumulation, the mechanisms that trigger neuronal death, which may provide a window for early diagnosis and treatment, remain unknown. The cellular functions of PrP^{C} are unresolved and identifying its physiological roles may elucidate the neurodegenerative mechanisms underlying prion diseases.

Furthermore, the ubiquitous expression of PrP^{C} and its role in cellular signaling implicates it in multiple pathological changes. For example, PrP^{C} modulates the cleavage of the amyloid precursor protein and functions as a high-affinity receptor for toxic $\alpha\beta$-oligomers in Alzheimer's disease. The importance of PrP^{C} in tumor biology has also been explored in the literature because of its upregulation in multiple cancers and its antiapoptotic and proliferative roles in tumor cells. Understanding PrP^{C} functions in health and disease states may provide opportunities to uncover novel therapeutic approaches for multiple diseases. The overarching goals of our group are to understand the cellular mechanisms associated with PrP^{C} functions and to explore the therapeutic window offered by this molecule in prion diseases, Alzheimer's disease and cancer.

Our group demonstrated that PrP^{C} associates with several cellular proteins; this triggers diverse signaling pathways and promotes important cellular functions. Two major PrP^{C} ligands, namely laminin (LN) and the secreted form of stress-inducible protein 1 (STI1), have been described and their interaction promotes neuronal survival and plasticity and enhances learning and memory consolidation. PrP^{C} is a glycosylphosphatidylinositol-anchored protein, suggesting 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. In 2010 we demonstrated that STI1-PrP^{C}-related processes require $\alpha 7$ nicotinic acetylcholine receptor activation. During the past year we showed that LN-PrP^{C} interaction activates phospholipase C, protein kinase C and ERK1/2 and promotes Ca^{2+} mobilization from

intracellular stores. Phage display, coimmunoprecipitation and colocalization experiments have shown that the group I metabotropic glutamate receptors, mGluR1 and mGluR5, associate with PrP^C and modulate cell signaling triggered upon PrP^C-LN binding. These results are consistent with our hypothesis that PrP^C regulates trophic functions by organizing a dynamic platform for assembly of signaling modules at the cell surface. The role of PrP^C-STI1 interaction in cell fate–related mechanisms was also investigated. We used neurosphere cultures as a model to study neural progenitor/stem cell physiology. STI1-PrP^C interaction induced neurosphere formation by stimulating cell proliferation. These data suggested that the STI1-PrP^C complex plays a critical role in neural progenitor/stem self-renewal, leading to control of the stemness capacity of these cells during nervous system development.

If this is confirmed for adult neural progenitors/stem cell self-renewal, it will be possible to build a rationale for novel therapeutic strategies to treat acute brain injuries such as ischemia or trauma, and chronic neurodegenerative illness such as Alzheimer's or prion diseases. The participation of these complexes in tumor stem cells and in tumor biology is also under investigation.

Virology

Luisa L. Villa, Ph.D.

The year 2011 marked the passing of a great scientific leader who contributed significantly to the advancement of cancer research in Brazil. Ricardo Brentani, former director of the São Paulo Branch, created an ideal research environment for independent research groups that made important contributions to the fields of epidemiology, immunology, cellular biology, genetics and genomics in Brazil. In addition to his role as director, he was the head of the Hospital do Câncer, where Ludwig São Paulo was located, serving as the president of the Antônio Prudente Foundation. Upon his retirement Brentani was awarded the D.K. Ludwig Medal for his services to the Institute.

Natural history of HPV infection in men: The HIM study

This study continues to provide relevant and novel information about HPV infections in the genitals, anal canal, oral and skin from men. Together with colleagues from Mexico and the United States we are following ~4,000 men ages 18 to 44 years and 450 men ages 45 to 70 years every six months for four years. Results are accumulating on the natural history of HPV infections in the genitals and anal canal in both men having sex with women (MSW) and men having sex with men (MSM). Cumulative incidence of HPV in the anal canal was substantially higher among MSM (28.3%) than among MSW (4.8%), and only MSM had persistent HPV infections; we also found that cigarette smoking is a risk factor that contributes to HPV persistence. We continue to search for risk factors and age-specific prevalence and incidence of HPV in the oral cavity of men.

HPV and tumor microenvironment

The products of the E6 and E7 genes of HPV are pleiotropic proteins that exert different effects on the host cells, including the promotion of immune evasion. Women with cervical cancer exhibit tolerance to HPV antigens that has been associated with the presence of regulatory T cells and, to a lesser extent, of infiltrating macrophages. We sought to study in a mouse model the interactions between cells expressing HPV oncogenes and the other cellular and noncellular elements that constitute and regulate the tumor environment. HPV-associated tumors in mice induce myeloid cell proliferation and recruit monocytes to the tumor microenvironment; these monocytes in turn can recruit more monocytes. In the tumor, cells differentiate into macrophages that express IL-10, which is important for suppression of antitumor T cell responses. We are showing that cytokines involved in the triggering of Th1 T cell responses are downregulated in the spleen of tumor-bearing mice. In conclusion, HPV-positive tumors condition secondary lymphoid organs,

promoting differentiation of regulatory T cells and inhibiting antitumor responses.

Another study relates to the effect of HPV oncogenes on the expression and activity of different matrix metalloproteinases (MMPs) and their inhibitors in epithelial cells. We observed that HPV 16 E7 expression is associated with increased pro-MMP-9 activity in organotypic cultures of keratinocytes, whereas E6 and E7 oncoprotein coexpression downregulates RECK and TIMP-2. Moreover, human cervical tissues show a decrease in RECK expression levels in precancer and cancer lesions. HPV oncoproteins promote MMPs/RECK-TIMP-2 imbalance; this could contribute to tumor progression.

Molecular markers of HPV-related disease

We have shown that the protease kallikrein-7 may be a useful biomarker for high-grade cervical dysplasia and cancer. Positive results were also obtained with superoxide dismutase-2 expression in invasive cervical disease and more recently in carcinomas of the penis. These tumors spread to regional lymph nodes and often are treated surgically with high morbidity rates. Therefore, we sought to identify prognostic markers of lymph node metastasis. We observed that intense staining for superoxide dismutase-2 in primary penile carcinomas is significantly correlated with regional lymph nodal metastasis ($P < 0.03$), but not with HPV presence. Further studies will point to the relevance of these markers in disease spread.

Computational biology

Sandro J. de Souza, Ph.D.

Our group continued its efforts to develop new computational strategies for the study of cancer. We are capitalizing on the availability of next-generation sequencing technologies to deeply explore the genetic and epigenetic alterations in cancer. Large-scale studies involving genes encoding cell surface proteins (surfaceome) continued to be a target of our studies. In collaboration with Ludwig scientists at the New York Branch, a database on the human surfaceome was developed with a series of applications to fully explore this set of genes (http://www.bioinformatics-brazil.org/surfaceome).

Alternative splicing continued to be a major focus of our group. In collaboration with Diogo Meyer of the University of São Paulo, we showed that single-nucleotide polymorphisms in the human population affect the distribution of splicing regulatory sequences.

In collaboration with several groups in Brazil and abroad, we finished the genome sequencing of a breast cancer cell line (HCC1954) and a normal lymphoid cell line derived from the same patient (HCC1954-BL). Our findings confirmed that chromosomal rearrangements are more frequent in tumor cells than in normal cells. However, the number of somatic mutations in both cells was roughly equivalent in both cell lines. When we analyzed the results with a more systemic perspective, we observed that somatic mutations in the cancer cell line occur in genes whose proteins are more interconnected in the protein-protein interaction network, whereas the mutations in the normal cell line occur in a random fashion.

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Stockholm

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Director's message

The following emphasizes some of the highlights in 2011 from the Ludwig Stockholm Branch. In close collaboration with Rickard Sandberg's group, Jonas Muhr and other Ludwig Stockholm scientists used ChIP sequencing to unravel how Sox proteins influence neural development from pluripotent stem cells into differentiated neuronal cells (published in *Genes & Development*). The work was highlighted by a review article in the same issue. The group also made significant progress in work that links Sox protein function to the formation of glioblastomas. Rickard Sandberg's group also published several important papers. In collaboration with Shalini Oberdoerffer's lab, they demonstrated a novel mechanism whereby the transcriptional repressor CTCF affects pre-mRNA splicing. This work was published as a highly recognized article in *Nature*. The group also developed a method for single-cell mRNA sequencing that was successfully applied to circulating tumor cells and single cells from early embryos. Our own group published work in *Cell Stem Cell* on directed neuron differentiation from stem cells and a paper in *Genes & Development* describing a new component in DNA double-strand-break repair. Jan Stenman's group has also made significant progress in finalizing the generation of several mouse models that will help elucidate how Wnt signaling regulates the central nervous system (CNS) vasculature. Susanne Schlisio's work on how the prolyl hydroxylase EglN3 is involved in regulating apoptosis and how its malfunction can cause predisposition to certain forms of cancer has generated very interesting data during 2011. Finally, Johan Holmberg's newly established group has also made progress toward a first publication on the chromatin remodeller CHD5, which seems to be a new key regulatory factor in the generation of terminally differentiated neurons. During the year Ludwig Stockholm grew thanks to new external funding. All groups are eagerly looking forward to new and exciting results in 2012.

Thomas Perlmann

Gene expression

Thomas Perlmann, Ph.D.

The laboratory headed by Thomas Perlmann focuses on understanding the development of specific types of neurons. The lab aims to understand how a transcription factor network is influenced by signaling, 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.

The lab is particularly focused on the development of dopamine neurons, the type of cells that degenerate in patients with Parkinson's disease. Over the years the lab has identified transcription factor pathways that are critically involved in the early specification and differentiation of these cells. During 2011 the Perlmann group published a study that linked one of these factors, Nurr1, to a new and unexpected role in DNA repair that seems important in all cells (*Genes & Development*). Another study identified new roles of two critical transcription factors (Lmx1a and Lmx1b) in the development of dopamine neurons and other neuronal cell types (*Development*). The group also identified a new transcription factor that it continues to study. Strikingly, this transcription factor is selectively expressed in a subset of dopamine neurons that are particularly vulnerable in Parkinson's disease. Moreover, genetic studies in mice indicate that this factor plays a role in development to drive specific characteristics that distinguish these dopamine neurons from other types of dopamine neurons.

In regenerative medicine, there is a strong focus on the development and understanding of pluripotency of both embryonic stem (ES) cells and induced pluripotent stem 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. The group has previously identified a key transcription factor (Lmx1a) that is critical for the specification of dopamine neurons. In 2011 the lab published results extending a previous finding that Lmx1a expression in ES cell–derived neural stem cells (NSCs) can efficiently promote dopamine neuron development. The new study demonstrated that this strategy is also very robust for generation of other clinically important neuron types, including serotonergic, visceral motor and somatic motor neurons. During 2011 the group has also extended these studies to yet another important cell type that is severely affected in Parkinson's disease. The new data show that hindbrain noradrenergic neurons can be generated by forced expression of one key transcription factor in ES cell–derived NSCs. These findings raise the possibility of

using this methodology for studying disease-associated processes in these neurons.

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. In 2011 the group found that conditional knockout of two of the studied transcription factors in adult mouse dopamine neurons leads to neuron pathologies that closely resemble early phases in Parkinson's disease. These results are interesting both for the understanding of how developmental transcription factors contribute to maintenance of the dopamine neuron identity and in light of previous findings linking gene polymorphisms in these transcription factor genes to higher incidence of Parkinson's disease.

Stem cell biology

Jonas Muhr, Ph.D.

The CNS contains hundreds of billions of neurons and glia, which comprise thousands of functionally distinct subtypes. A central problem in biology is how proliferation and differentiation of NSCs are regulated to generate this abundant neuronal and glial progeny.

A key feature of NSCs is their ability to maintain a lifelong population of self-renewing stem cells while generating postmitotic neuronal and glial progeny. Thus, NSCs face the difficult task of maintaining a population of self-renewing cells while avoiding deranged proliferation and hyperplasia. Previous studies suggest that self-renewal of NSCs is controlled through cross-talk of signals from the stem cell niche, NSC regulatory transcription factors and the cell cycle machinery, but the functional connection among these regulatory components is not understood. During 2011 Jonas Muhr's group has addressed this question by examining the role of the stem cell transcription factor Sox2 in the regulation of NSC proliferation in the developing mouse cortex. These analyses have revealed that although Sox2 is expressed in all NSCs, it is expressed at high levels in slowly proliferating NSCs and at low levels in fast-proliferating ones. Using gain- and loss-of-function experiments combined with global binding analysis, we demonstrated that high levels of Sox2 cause TCF2, a transcriptional mediator of canonical Wnt signaling, to repress rather than activate key regulators of cell cycle progression. Thus, whereas high Sox2 levels maintain NSCs, slowly proliferating lower levels allow NSCs to enter a transient amplifying state. The group is now extending these studies to understand whether the expression of Sox2 also can control proliferation of stem cells present in gliomas. Together these projects promise to reveal how stem cell regulatory transcription factors interact with signals from the stem cell niche and the cell cycle machinery to regulate NSC proliferation in both the healthy and the diseased brain.

Although the presence of NSCs in discrete regions of the adult brain is potentially significant for maintenance of brain integrity, plasticity and optimal function, their presence also constitutes a potential risk as self-renewing cells are susceptible to malignant transformation. In a project aiming to explore how stem cell transcription factors act to prevent uncontrolled self-renewal of NSCs, the group has previously demonstrated that a group of Sox proteins, Sox5, Sox6 and Sox21 (Sox5/6/21), promote healthy NSCs to exit the cell cycle and commit to differentiation. These findings suggest that these proteins could function as tumor suppressors. To address this issue, we established an *in vivo* glioma mouse model that allows us to genetically delete Sox5/6/21. The results demonstrate that the loss of Sox5/6/21

dramatically increases the ability of oncogenes *H-RAS* and *AKT* to induce glioma formation and that overexpression of Sox6 or Sox21 completely blocks the ability of human glioma cells to induce secondary tumors in NOD-SCID mice. This project is promising and gives important insights into how NSC regulatory transcription factors can function as lineage-specific tumor suppressors in the adult brain.

Apart from their self-renewing capacity, a key feature of stem cells is their ability to generate many different types of mature cells. In the brain NSCs can generate many different types of neurons and glia, but how NSCs are endowed with this competence is essentially unknown. In an article published in *Genes & Development* in 2011, the group provided important insights into this question by showing that Sox2 in ES cells and NSCs prebinds a large set of silent genes that are destined to be activated by other Sox proteins in differentiating neurons.

Development and disease

Jan Stenman, Ph.D.

The Wnt family of proto-oncogenes comprises secreted glycoproteins essential for normal development of organisms ranging from fly to humans. For example, we previously reported in *Science* that Wnt/β-catenin signaling regulates both formation of blood vessels in the CNS and early differentiation of the blood-brain barrier (BBB). In adult animals and humans, alterations of Wnt signaling are key to many diseases, such as malignances. A prioritized aim for the group is to better understand the function of Wnt signaling in brain vasculature during development and ultimately in brain tumor disease. However, results obtained while generating novel reagents for these studies have also motivated us to study some of the molecular mechanisms regulating Wnt pathway activity.

Wnt in blood-brain barrier development

Formation and differentiation of CNS vasculature are very important biological processes and we are only beginning to understand how Wnt signaling regulates these events. During the year, the group has finished developing novel mouse models to (i) inhibit Wnt pathway activity; (ii) identify Wnt/β-catenin direct downstream target genes; and (iii) extract translated mRNAs by immunoprecipitation from any cell population given the availability of a suitable Cre driver line.

Transcriptional profiling using RNA sequencing of fluorescence-activated cell-sorted embryonic day 11.5 brain endothelial cells using the first mouse model has allowed the group to produce a list of Wnt-regulated genes. The bioinformatic analysis demonstrates that an endothelial cell population has been isolated and profiled with no significant neural or pericyte contamination, and that *Slc2a1*, a BBB marker, is downregulated; this was predicted based on our previous report. More important, several genes that are enriched in the adult BBB are expressed in the early brain vasculature and regulated by Wnt. These results extend the previous report and further establish the importance of Wnt/β-catenin signaling in BBB differentiation. We now aim to:

- begin addressing the function of Wnt downstream targets in brain endothelial cells. For example, the group has identified transcription factors that are positively regulated by Wnt signaling. We are currently trying to determine if these transcription factors can activate at least some aspects of the CNS endothelial cell differentiation program in non-CNS endothelial cells.

- identify direct downstream target genes by *in vivo* β-catenin ChIP-sequencing experiments.

- determine the translational profiles of brain endothelial cells throughout BBB development.

- study the specificity of interactions between Wnt7a/7b proteins and the Wnt receptors that are known to be expressed in CNS vasculature from the previous transcriptional profiling data.

- study the role of Wnt/β-catenin in brain tumor vasculature.

Molecular mechanisms of Wnt signaling

In efforts to develop novel reagents for studying the function of Wnt signaling *in vivo*, the group surprisingly discovered that the Wnt inhibitor and tumor suppressor protein Axin1 can activate Wnt/β-catenin pathway activity in *Xenopus laevis* embryos. We have extended these initial observations to show that the level of endogenous Wnt signaling activity in the embryo regulates the activator/inhibitor behavior of Axin proteins. These observations are important as they challenge the current textbook model and have implications for the use of compounds that regulate the level and stability of Axin proteins for treatment of cancer.

In addition, together with Bo Lundgren's group at the SciLifeLab Stockholm RNAi High-throughput Screening Facility, a large-scale, cell-based, endoribonuclease-prepared short interfering RNA screen has been performed to preferentially identify regulators of Wnt maturation and secretion. Preliminary analysis of the data indicates that the screen was successful, but much work remains to determine which candidate genes to select for independent verification and further analysis.

Oxygen sensing and cancer

Susanne Schlisio, Ph.D.

Oxygen sensing is mediated partly via a family of enzymes called EglN prolyl hydroxylases; this family requires molecular oxygen for enzymatic activity. Our work specifically focuses on how these enzymes execute apoptosis in neural precursors during development and how disruption of this process can lead to certain forms of nervous system tumors. Accordingly, the research is focused on (i) the molecular mechanism of how prolyl hydroxylase EglN3 executes apoptosis in neural precursors during development and (ii) how failure of developmental apoptosis mediated by EglN3 causes predisposition to certain forms of nervous system tumors.

To understand how prolyl hydroxylase EglN3 executes apoptosis, a genome-wide RNA interference screen was completed, identifying genes involved in apoptosis, transcriptional regulation and the endosomal system. Several of the genes identified in this screen reside on chromosomal loci that are frequently altered in neuroblastoma.

One identified gene of interest encodes tumor necrosis factor receptor 1 (TNFRSF1A). This protein and its associated ligand (TNFα) have a long history in the chronicles of cancer research and treatment. Both TNFα and TNFR1 contribute to the death of sympathetic neurons after nerve growth factor (NGF) starvation. Significantly, apoptotic induction by TNFa requires caspase-8, which is frequently silenced in neuroblastoma. The group demonstrated that TNFR1 and subsequently caspase-8 are required for EglN3 induced death *in vitro*, and the mechanism behind this is now being investigated.

Upon stimulation with TNFa, TNFR1 trimerizes and acts as a scaffold for several intracellular signaling complexes. Depending on cell context, this can produce a variety of either prosurvival or prodeath signals. Caspase-8 initiates apoptosis in response to TNF signaling. Silencing of caspase-8 occurs frequently in neuroblastoma and some other pediatric cancers and in neuroendocrine lung cancer. The group has now shown that caspase-8 is required for EglN3-induced apoptosis. Further, NGF-maintained sympathetic neurons are highly resistant to apoptotic stimuli, putatively owing to regulation of caspase activation by X-linked inhibitor of apoptosis (XIAP). XIAP is diminished by NGF starvation in sympathetic neurons, thus making these cells susceptible to caspase activation. Interestingly, EglN3 stimulates the expression of XAF1, an inhibitor of XIAP. The group is currently investigating whether XIAP depletion is also essential for EglN3/TNFa-induced apoptosis in other developmental contexts.

In an earlier pilot EglN3 screen, we identified a novel tumor suppressor called KIF1B-β, a kinesin motor protein. KIF1B-β is located on chromosome 1p36.2, a region of the genome that is frequently deleted in neural crest–derived tumors including neuroblastoma. The group recently demonstrated that KIF1B-β acts downstream of EglN3 and is both necessary and sufficient for neuronal apoptosis when NGF becomes limiting. The underlying mechanism behind EglN3 regulation of KIF1B-β remains unknown. Our group has now continued 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 KIF1B-β binding partners that specifically interact to the minimal region that is necessary and sufficient for apoptosis function. One such binding partner called DHX9 was identified and was further evaluated in functional assays. Importanly, DHX9 acts as a critical mediator of KIF1B-β-induced apoptosis when assayed by loss- and gain-of-function approaches. Interestingly, localization studies revealed that cytoplasmic DHX9 locates toward the nucleus upon KIF1B-β induction. Further, nuclear localization of DHX9 is necessary for KIF1B-β mediated apoptosis. The group observed a specific gene expression signature that is mediated by DHX9 in the context of KIF1B-β stimulation. We are now obtaining neuroblastoma samples from the clinics to investigate the extent to which DHX9 localization is altered in 1p36-deleted neuroblastomas.

The group is further aiming 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. We have now conditionally inactivated this gene specifically in the peripheral nervous system and have begun to investigate its phenotype.

Computational genomics

Rickard Sandberg, Ph.D.

The group's main achievement in 2011 was to establish single-cell RNA sequencing and apply it to generate preliminary transcriptome data from hundreds of individual cells of early mouse embryos. The group also finalized and published two collaborative studies and submitted a manuscript describing a new uniqueness resource called MULTo for RNA-Seq analyses. The lab recruited additional postdocs and students, initiated several new projects and upgraded the co-owned (50%) Illumina sequencer Genome Analyzer IIx to a Hiseq 2000 for higher-throughput and more cost-effective sequencing.

The group has been extensively collaborating with Gary Schroth, Shujun Luo and colleagues from Illumina on a new RNA-sequencing protocol called Smart-Seq that is applicable to single cells. Through extensive experiments on diluted RNA, we quantified the sensitivity for transcript detection and determined accuracy in estimating transcript abundances. Compared to previous single-cell RNA-Seq methods, Smart-Seq achieved superior coverage across transcripts that significantly improved splice variants and genotype calls. The group then generated single-cell transcriptomes from dozens of cancer cell line cells of different origins, and demonstrated that only a few cells per cell type are required to identify hundreds of significantly differentially expressed genes between cell types. In a pilot study with Louise Laurent's lab at University of California, San Diego, Smart-Seq was applied to individual circulating tumor cells from a melanoma. From expression profiles, the group monitored changes in cell-surface markers for new markers and insights into their biology. We are currently gearing up with Laurent's group for more complete analyses of circulating tumor cells from more patients together with single-cell sequencing of primary and secondary tumors.

Our group identified a new regulatory mechanism for pre-mRNA splicing through the demonstration that CTCF can affect pre-mRNA splicing via pausing of RNA polymerase II. Using computational analyses of global CTCF binding and RNA-Seq after CTCF knockdown, we demonstrated that CTCF binding downstream of regulated exons was associated with increased RNA polymerase II occupancy and increased inclusion levels of the regulated exon. The study was the result of a fruitful collaboration with Shalini Oberdoerffer's lab in the US National Cancer Institute (US National Institutes of Health); Oberdoerffer led this study, which was recently published in *Nature*. Further work is currently exploring roles of DNA methylation in regulation CTCF binding.

In another collaborative project with Jonas Muhr, the group finalized comparative analyses of Sox transcription factor binding in ES cells, neural

progenitors and postmitotic neurons. Interestingly, we found that Sox proteins function sequentially and bind enhancers in genes that are activated at later stages in development. These results reinforce an emerging stem cell paradigm of extensive transcription factor binding and local DNA methylation modulation at enhancers with consequences for their accessibility at later stages of differentiation.

Central nervous system tumors and development

Johan Holmberg, Ph.D.

The group has continued its efforts to explore how high-grade glioma-specific expression of transcription factors, which governs ES cell pluripotency, influences malignancy and tumor growth. To address both the functional role and the mechanisms by which these factors (Pou5F1, Nanog, Klf4 and Sox2) affect tumor cells, the group is combining *in vitro/in vivo* loss-of-function and gain-of-function experiments with genome wide ChIP-seq analysis to reveal target genes regulated by these factors in patient-derived glioma cells. Since our group is particularly focused on the possible interplay between transcription factor binding and polycomb-mediated repression of target genes, we are also currently mapping the genome-wide distribution of polycomb-deposited histone modifications in glioma cells derived from several patients. In combination with genome-wide expression analysis through RNAseq, the group is obtaining a comprehensive overview of where the Pou5f1/Nanog/Klf4/Sox2 factors are binding. Furthermore, in combination with loss- and gain-of-function experiments, we are exploring whether this binding dictates the distribution of polycomb-mediated repressive histone modifications to facilitate silencing of tumor suppressor genes.

Another project focuses on the role of the chromatin remodeler Chd5 during terminal neuronal differentiation. In a collaboration with Adrian Bracken at Trinity College in Dublin, the group has revealed that Chd5 is an important component of the molecular machinery that governs terminal neuronal differentiation in the neocortex. We have also extended efforts to elucidate whether loss of Chd5 destabilizes neuronal differentiation, thus predisposing progenitor cells for further oncogenic transformations that eventually can give rise to tumors of the neural lineage. This approach includes an *in vivo* xenograft model of neuroblastoma wherein we are assaying the contribution of Chd5 loss to tumor growth and whether the presence of Chd5 can dictate the outcome of treatment with clinically relevant agents that induce terminal neuronal differentiation. The results also indicate a role for Chd5 in adult neurogenesis, so we are now investigating whether perturbed Chd5 expression in adult NSCs can influence the generation of glioma. In a parallel project, the group has initiated experiments to understand whether Chd5 is involved in maintaining a terminally differentiated neuronal identity throughout the life span of the organism. To address the different possible roles played by Chd5 in the developing and mature nervous system as well as its function as tumor suppressor, the group has in 2011 successfully generated a conditional Chd5 knockout mouse. We are now crossbreeding Chd5 knockout mutant mice with several different cre strains to achieve tissue-specific excision of Chd5.

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Uppsala

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Director's message

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

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

The TGF-β family comprises 33 members, including TGF-β isoforms, bone morphogenetic proteins (BMPs), growth and differentiation factors and activins. They all act via heteromeric complexes of type I and type II serine/threonine kinase receptors, and have important roles during embryonal development. TGF-β most often inhibits growth of cells, and also promotes apoptosis, matrix production and cell differentiation. TGF-β has a complicated role in cancer. Initially, it is a tumor suppressor through its ability to inhibit cell growth and to promote apoptosis. At later stages of tumor progression, however, TGF-β becomes a tumor promoter by induction of epithelial-mesenchymal transition (EMT), which makes cancer cells invasive, and by stimulating angiogenesis and inhibiting the immune system. We aim to elucidate the molecular mechanism of TGF-β, and to explore whether selective TGF-β antagonists can be developed. This would inhibit the tumor-promoting effects of TGF-β while leaving the tumor-suppressive effects unperturbed. Our ultimate goal is to investigate the clinical usefulness of such antagonists.

Another line of research at Ludwig Uppsala is to elucidate the role of the microenvironment in tumorigenesis. In particular, we study the matrix molecule hyaluronan and its receptor CD44, as well as their interactions with growth factor receptors. The groups in Uppsala are supported by expertise in proteomics and mass spectrometry.During the past year the group leader for the PDGF Translational Research Group, Carina Hellberg, left for a position as reader at the University of Birmingham, and the group leader of the Protein Structure Group, Ulf Hellman, retired after more than 25 years of work at Ludwig Uppsala. We thank Carina and Ulf for their excellent contributions to the Institute.

In January 2012 a new group leader, Ingvar Ferby, will start in Uppsala. We expect that his research program on modulatory mechanisms of signaling via epidermal growth factor receptor will fit in very well with our scientific program.

C.H. Heldin

PDGF translational research

Carina Hellberg, Ph.D.

In the PDGF Translational Research Group, we focus on PDGF receptors as cancer drug targets and on the modulation of PDGF β-receptor signal transduction.

Signaling via tyrosine kinase receptors regulates cell growth, adhesion, migration and differentiation as well as angiogenesis. Tyrosine phosphorylation is thus necessary for tumor growth and metastasis. These findings have led to development of antitumor drugs that specifically target tyrosine kinases.

Receptor tyrosine kinase inhibitors sensitize melanoma tumors to taxol

Targeting tumor vasculature by combining inhibition of PDGF and vascular endothelial growth factor receptors reduces the growth of B16 tumors. We have now found that inhibition of the tyrosine kinase receptor c-Kit selectively sensitizes melanoma cells to paclitaxel. When grown *in vivo*, B16 tumors do not respond to paclitaxel treatment, not even when it is combined with treatment with imatinib, which inhibits the kinases of PDGF receptors and c-Kit. Instead, a combination of imatinib and vatalanib, a vascular endothelial growth factor receptor kinase inhibitor, sensitized these tumors to low doses of paclitaxel.

Modulation of PDGF β-receptor signal transduction

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. Because PDGF-BB also increased the anchorage-independent growth of H-Ras-transformed fibroblasts, it is possible that the altered receptor trafficking augments cell transformation.

Involvement of protein tyrosine phosphatases in cell regulation

Screening for protein tyrosine phosphatases that regulate PDGF β-receptor phosphorylation and signal transduction has identified the receptor-like

protein tyrosine phosphatase LAR as a positive regulator of PDGF β-receptor phosphorylation. Inhibition of c-Abl kinase in LAR deficient 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, we identified novel substrates. The glycolytic enzyme pyruvate kinase M2 (PKM2) was found to interact with T cell phosphatase. Because PKM2 mediates the shift to aerobic glycolysis in tumor cells, known as the "Warburg effect," we investigated the possibility that T cell phosphatase regulates tumor cell metabolism. HeLa cells in which T cell phosphatase was knocked down showed increased PKM2 activity compared with control cells. Intracellular concentrations of several glycolytic intermediates were also affected, indicating that this phosphatase could participate in regulation of aerobic glycolysis.

PDGF signal transduction

Johan Lennartsson, Ph.D.

We aim to identify signaling pathways downstream of PDGF receptors and to elucidate their functional importance.

Activation and regulation of Erk5 MAP kinase downstream of PDGF receptor

It is well established that Mek5 lies upstream of Erk5 mitogen-activated protein kinase (MAPK), but the details of how Mek5 is activated are unclear. To clarify this, we have initiated a chemical biological screen using a panel of kinase inhibitors to assay which pathways contribute to Erk5 activation. In parallel, we also use an array of PDGF receptor mutants to identify mutants that are defective in Erk5 activation, and a short interfering RNA screen targeting potential kinases upstream of Mek5 that have been identified from the literature. The goal is to delineate the pathway from the receptor to Mek5. By understanding this pathway, we hope to clarify the mechanism by which Erk5 mediates its functions and cross-talks with other pathways, including Erk1/2 and Akt.

Dephosphorylation of MAPKs is an important mechanism of control that restricts the extent of their signaling. MAPK phosphatase 3 (MKP3) is a dual-specificity phosphatase that is selective for MAPK Erk1/2. We found that silencing of MKP3 in fibroblasts resulted in an elevated basal level of Erk1/2 phosphorylation, but we did not observe any difference compared with control cells after PDGF stimulation. Furthermore, we observed that PDGF-induced MKP3 expression depended on both Erk1/2 and PI3-kinase. Reducing the level of MKP3 using short interfering RNA or by inhibiting Erk1/2 with a low-molecular-weight inhibitor resulted in increased Erk5 and Akt phosphorylation. This suggests that there is negative cross-talk between Erk1/2 and Erk5 that involves MKP3. Erk1/2 is important for expression of MKP3, which then can negatively regulate Erk5. Activation of PI3-kinase was also necessary for MKP3 expression, and we found that MKP3 negatively regulated Akt phosphorylation. Functionally, we observed that silencing of MKP3 enhanced the chemotactic response toward PDGF, but did not influence the mitogenic response.

Fer is necessary for PDGF-induced Stat3 phosphorylation and colony formation in soft agar

Fer is a cytoplasmic tyrosine kinase that is activated by several cytokines and growth factors including PDGF. We have confirmed that Fer is activated by PDGF receptor and expanded this finding to show that Fer is activated in a sustained manner and independently of Jak and Src kinase activities. Silencing of Fer selectively abolished the ability of PDGF to promote Stat3

phosphorylation. Using cells expressing a kinase-deficient version of Fer, we concluded that the role of Fer in PDGF induced Stat3 phosphorylation was independent of Fer kinase activity and only dependent on expression of Fer protein. Inhibition of Src kinase activity also reduced Stat3 phosphorylation, and we found that Src activation was dependent on Fer. This suggests that Fer may serve a scaffolding function important for Src activation and subsequent Stat3 phosphorylation. Functionally, we showed that silencing of Fer in cells with an autocrine loop involving PDGF abolished the ability of these cells to form colonies in soft agar. Thus our data suggest that Fer has a critical role in PDGF-induced Stat3 signaling and cell transformation.

TGF-β signaling

Aristidis Moustakas, Ph.D.

Our research covers signal transduction and cancer biology. We study the developmental process of EMT and its links to tumor metastasis and cancer stem cell biology. We also study the TGF-β and BMP pathways.

TGF-β signaling and cancer

TGF-β regulates cell growth and differentiation via Smad signaling proteins, intracellular kinases and GTPases. Smads signal from TGF-β receptors, enter the nucleus and regulate transcription. TGF-β signaling suppresses early-stage tumors but promotes late-stage ones. We investigate the function and regulation of TGF-β responsive genes to explain tumor suppression or progression by TGF-β and other pathways, such as Notch.

Regulation of TGF-β/BMP receptor signaling by protein kinases

TGF-β induces salt-inducible kinase (SIK), which regulates TGF-β receptor turnover. We now show that Smurf ubiquitin ligases and the inhibitory Smad7 are SIK cofactors during TGF-β receptor downregulation. SIK operates under the control of the kinase LKB1, and we have uncovered how cross-talk among TGF-β, BMP and LKB1 signaling occurs the receptor level. We also study the mitogen and stress-activated kinase family and its cooperation with p53 during epithelial cell death and survival. For these projects, we collaborate with Patrick Micke, Christer Busch and Fredrik Pontén (Uppsala University), Peter ten Dijke (Leiden University) and George Pyrowolakis (Freiburg University).

Regulation of nuclear TGF-β/BMP signaling

We study enzymatic regulators of nuclear Smad function such as LKB1, which phosphorylates Smad4, and the poly-ADP-ribose polymerases, which regulate chromatin and transcription, the latter in collaboration with Michael Hottiger (University of Zurich). We analyze Smad ubiquitination by HECT-domain ligases and the prolyl-isomerase Pin1 in collaboration with Takeshi Imamura (Ehime University). We want to understand how Smad post-translational modifications change between normal and cancer cells. We apply *in situ* proximity ligation widely to enhance sensitivity in detecting endogenous protein interactions and post-translational modifications, in collaboration with Ola Söderberg (Uppsala University).

Molecular mechanisms of epithelial-mesenchymal transition

In exploring the relationship between EMT and cancer progression, we found that TGF-β promotes mammary EMT, enhances tumor growth and

enriches tumors in differentiated mammary cells, suggesting the presence of mammary stem cells. Molecular analysis of EMT showed that nuclear factor HMGA2 is induced by TGF-β, binds to Smads and induces other EMT factors, for example Snail and Twist. We now study whether HMGA2 and Smads cause chromatin remodeling and DNA methylation during EMT, and regulation of EMT by p53, LKB1 and SIK. Finally, we have demonstrated how the oncogenic DNA replication factor Cdc6 causes EMT.

Mechanisms of self-renewal of tumor-initiating cells

We analyze the role of TGF-β and HMGA2 in the self-renewal of breast- and brain-tumor-initiating cells by studying the interplay between HMGA2 and microRNA synthesis in regulation of tumor-initiating cell self-renewal. We also develop mouse xenograft models of human glioblastoma multiforme after overexpression of EMT regulators, in collaboration with Bengt Westermark and Karin Forsberg-Nilsson (Uppsala University). By studying BMP-regulated genes we also want to explain how BMP suppresses glioblastoma.

TGF-β pathway modeling

Using mathematical modeling of the TGF-β pathway with Zhike Zi (Freiburg University), Edda Klipp (Humboldt University) and Xuedong Liu (University of Colorado), we uncovered changes between graded and on-off switch–like signaling behavior.

Apoptotic signaling

Maréne Landström, M.D., Ph.D.

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

Type I TGF-β receptor recruits ubiquitin ligase TRAF6 to activate a cancer-specific invasive pathway

We have previously reported that type I TGF-β receptor (T-βRI) interacts with TGF-β-associated kinase 1 (TAK1), and that activation of TAK1 requires Lys63-dependent polyubiquitination by the ubiquitin ligase TRAF6. This post-translational modification of TAK1 determines its biological responses and provides a molecular mechanism for a non-Smad signaling pathway initiated by TGF-β receptors. Smad7 is a negative modulator of TGF-β signaling. However, we have identified Smad7 as an adaptor to assemble a TGF-β-induced signaling complex causing activation of TAK1, MAPK kinase 3 and p38 MAPK pathway in prostate cancer cells. Interestingly, TGF-β-induced activation of the p38 MAPK pathway is not dependent on the kinase-activity of T-βRI. We have found a consensus binding site for TRAF6 in T-βRI, explaining how TRAF6 associates with T-βRI. The canonical Smad versus the Smad independent pathways can thus be distinguished from each other. Our studies provide data to help us understand how ligand-induced oligomerization of the receptor complex causes autoubiquitination and activation of TRAF6, followed by Lys63 dependent polyubiquitination and activation of the TAK1-p38 MAPK pathway.

We have further explored the role of TRAF6 in TGF-β signaling and have found that T-βRI is cleaved by the metalloprotease tumor necrosis factor–converting enzyme just outside of the cell membrane, in a TRAF6 and protein kinase C-ζ-dependent manner. This event generates an intracellular domain that acts as a part of a transcriptional complex in nuclei of cancer cells to activate proinvasive genes, such as Snail1 and matrix metalloproteinase-2, promoting tumor cell invasion. Thus, TGF-β induces several different signaling pathways. We are investigating the molecular mechanisms whereby TRAF6 determines the specificity of cellular responses induced by TGF-β.

Inflammation in relation to tumor biology

Many studies have shown that chronic inflammation increases the risk of developing several forms of cancer, including cancer of the prostate, stomach and colon. Thus, tumor-promoting inflammation is one of the hallmarks of cancer. We study the underlying molecular mechanisms of

how inflammatory cytokines promote tumor progression. We have found that ubiquitination of Lys34 in TAK1, via Lys63-linked polyubiquitin chains, is important for its activation by TGF-β, tumor necrosis factor-α and interleukin-1β (IL-1β). Interestingly, prostate cancer cells and monocytes use similar pathways to initiate NF-κB activation, as signals downstream of activated Toll-like receptor 4 by bacterial lipopolysaccharides also initiate the same cascade of events, where active NF-κB causes secretion of IL-6 and IL-8. This could favor tumor progression *in vivo.*

Development of novel treatment strategies for patients with advanced prostate cancer

Improved therapeutic strategies are urgently needed for patients with advanced and metastatic prostate cancer as no efficient therapy currently is available. We currently investigate whether inhibition of specific kinases, such as TAK1 and protein kinase C, could be useful for inhibition of invasive prostate cancer growth.

Molecular pathology

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

We are interested in the cell-type specificity and context dependency of TGF-β family members. We focus on Smad proteins, which are central mediators of intracellular signal transduction of TGF-β family members. The DNA-binding affinities of Smad proteins are relatively low, and these proteins interact with other DNA-binding cofactors to cooperatively regulate a specific set of target genes. We now analyze Smad-binding sites on a genome-wide level by using ChIP followed by sequencing (ChIP-seq) and try to characterize molecular mechanisms of how Smad proteins regulate transcription of their target genes.

Analysis of Smad-binding sites by ChIP-chip/ChIP sequencing

To reveal cell-type-specific function of TGF-β, we compared Smad2/3-binding sites in HaCaT cells with those in hepatocytes. We found that hepatocyte nuclear factor HNF4-α, one of the master regulators of hepatocyte differentiation and liver function, contributed to a hepatocyte-specific binding pattern of Smad2/3. *MIXL1* was identified as a new combinatorial target of HNF4-α and Smad2/3, and both HNF4-α protein and its binding motif were required for induction of *MIXL1* by TGF-β in HepG2 cells. Interestingly, 32.5% of the Smad2/3-binding regions overlapped with those of HNF4-α. This goes against the simple model in which cell-type-specific master regulators recruit Smad proteins to their binding sites and determine their function.

We are also interested in cross-talk between TGF-β and other signaling pathways. For example, TGF-β is a well-characterized inducer of EMT, and we and other groups have revealed that the Ras/Erk MAPK pathway plays important roles in TGF-β-induced EMT. To explore the functional relationship among TGF-β, Ras/Erk MAPK and other signaling pathways, we have obtained genome-wide binding maps of Smad2/3, with and without activation by Ras, another important inducer of EMT. Based on these data, we are currently investigating the molecular mechanism of TGF-β-induced EMT.

Regulatory mechanisms of angiogenesis by TGF-β family signaling

Dysregulated BMP signaling in endothelial cells (ECs) and pulmonary arterial smooth muscle cells (PASMCs) is implicated in human genetic disorders. We reported Smad1/5-binding genomic regions in ECs and PASMCs obtained by ChIP-seq and their characteristics. Cell-type-selective Smad1/5-binding patterns seemed determined mostly by cell-type-specific differences

in baseline chromatin accessibility patterns. We also found that several Notch signaling pathway–related genes were induced by BMP specifically in ECs. Among them, a Notch ligand, Jagged1, was regulated directly by Smad1/5, transactivating Notch signaling in the neighboring cells. These findings suggest that the cross-talk between BMP and Notch signaling plays important roles in the pathogenesis of vascular lesions of certain genetic disorders.

Through analysis of the ChIP-seq data, we also identified several novel target genes of Smad1/5 in either ECs or PASMCs. We are currently collaborating with the zebrafish facility of the SciLifeLab, Uppsala University, to determine the *in vivo* relevance of our findings using the zebrafish angiogenesis model.

We work in close cooperation with the Genome Science Division of the Research Center for Advanced Science and Technology and the Department of Molecular Pathology in the Graduate School of Medicine at the University of Tokyo, as well as the TGF-β Signaling, Protein Structure and Cancer Signaling Groups at Ludwig Uppsala.

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 the past year, we have focused on complexes formed by components of the TGF-β GFt-Smad and MAPK-AP-1 signaling pathways, both in cell lines and biopsies of human cancer patients. Secondly, we have in collaboration with the Molecular Pathology Group used ChIP-seq to try to identify Smad complexes and target genes that could mediate EMT and invasion.

BMP-7 inhibits TGF-β-induced invasion through inhibition of integrin β_3 expression

TGF-β acts as a tumor suppressor in the initial phase of tumorigenesis, but stimulates invasion and metastasis at later stages. BMP members of the TGF-β superfamily can also have dual effects on cancer, and BMP-7 inhibits bone metastasis of breast cancer cells. We previously developed a TGF-β-dependent spheroid invasion assay using (pre)malignant MCF10A1 breast epithelial cell derivatives (M2 and M4) embedded in collagen. We found that (i) BMP-7 inhibits TGF-β-induced invasion of metastatic M4 cells but not of premalignant M2 cells, and (ii) BMP-7 inhibits TGF-β-induced expression of integrin α_v and integrin β_3 in collagen-embedded M4 spheroids. Targeting of this integrin by a small nonprotein inhibitor or lentiviral knockdown of integrin β_3 negatively affected TGF-β-induced invasion. Overexpression of integrin β_3 counteracted the inhibitory effect of BMP-7 on TGF-β-induced collagen invasion. Thus, BMP-7 may exert antimigratory actions by inhibiting TGF-β-induced expression of integrin β_3.

Analysis of role of MAP kinase and AP-1 in TGF-β-induced invasion

The MCF10A model system also allowed us to functionally study the role of TGF-β/BMP pathway components, components of the MAPK signaling cascade and components of the AP-1 transcription factor family. Various MAPK and AP1 components were found to be regulated by TGF-β and/or BMP signaling, suggesting that some of them also may be involved in invasion via regulation of TGF-β induced invasion genes.

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

Interactions between the Jun, Fos and ATF components of transcription factor AP-1 exhibit specific and critical functions in regulation of cell

proliferation, differentiation and survival. Previously, we validated the proximity ligation assay for its ability to specifically visualize and quantify changes in endogenous c-Jun/c-Fos, c-Jun/ATF2, and c-Jun/Fra1 complexes. In collaboration with the group of Ulf Landegren, University of Uppsala, we have used the proximity ligation assay technique to study the involvement of different type of Jun, Fos and ATF complexes in TGFb-induced invasion. In collaboration with the Surgery Department of the Leiden University Medical Centre in the Netherlands, we have tried to detect AP-1 complexes in biopsies of human breast cancer patients.

Matrix biology

Paraskevi Heldin, Ph.D.

Our project aims to explore the mechanisms whereby the host microenvironment supports tumor progression. In particular, we investigate how hyaluronan synthase 2 (HAS2)-induced hyaluronan synthesis and CD44 support cancer cell metastasis.

HAS2 expression is correlated with breast cancer progression

The extensive accumulation of hyaluronan that occurs in various malignant and inflammatory conditions is correlated with the severity of the pathological condition. Recent studies have demonstrated that *HAS2* gene rearrangements are correlated with breast cancer malignancy as well as with a skin phenotype and periodic fever syndrome of shar pei dogs. For novel treatment strategies, we need to understand the signaling mechanisms involved in the control of *HAS* gene expression and the events that regulate HAS activities. 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 the HAS2 protein. Our studies on the mode of regulation of HAS2 revealed that HAS2 activity is regulated through monoubiquitination at Lys190 and oligomerization. The adhesion of cancer cells to microvascular and lymphatic endothelium is a prerequisite for their intravasation into the vasculature and subsequent extravasation in secondary organs. Bone metastasis is prevalent in advanced breast cancer. A critical step in the metastatic process of breast cancer cells, and any other carcinoma, is remodeling of the basement membrane. We aim to explore the role of hyaluronan in molecular mechanisms that underlie the degradation of basement membrane and translocation of tumor cells.

To validate the role of HAS2 expression in the invasive potential of a clone of breast cancer cell line MDA-MB-231 that forms bone metastases, we used an *in vivo*–like basement membrane model. We found that knockdown of HAS2 completely suppressed the invasive capability of these cells by induction of tissue metalloproteinase inhibitor-1 and dephosphorylation of focal adhesion kinase. This study provides new insights into a possible mechanism whereby HAS2 can enhance breast cancer invasion.

Elucidation of molecular mechanism of signaling via CD44

CD44 is an adhesion receptor with an extracellular hyaluronan-binding domain, a stalk region of variable length because of differential splicing, and an intracellular part with FERM-, ankyrin- and PDZ-binding motifs.

CD44 is 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 cross-talk between CD44 and growth factor receptors, including the receptors for PDGF-BB, TGF-β, hepatocyte growth factor and epidermal growth factor.

To elucidate the molecular mechanisms underlying the multiple functions of CD44, we used a peptide-based pull-down assay to identify proteins that interact with the its intracellular domain. 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. Hyaluronan-mediated migration of fibroblasts was found to be IQGAP1 dependent. Rac1 was found to play a key role in this process.

Protein structure

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The Protein Structure Group has solid experience in peptide synthesis, general chromatography work and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

Synthetic peptides and connected affinity chromatography

Synthetic peptides are used to generate antipeptide antibodies, as substrates or inhibitors, or as ligands in affinity chromatography experiments. An important extension has developed over the last years: we now also carry out affinity purification of antipeptide antibodies in collaboration with other groups of our branch. We also, in collaboration with other groups in Uppsala, perform affinity-based searches for interacting partners using immobilized ligands (for example, a modified synthetic peptide carrying a given motif). The combination of the biotin-streptavidin system and magnet beads has proven powerful.

MALDI mass spectrometry

We use a top-of-the-line MALDI-TOF MS. Our present instrument (Bruker Ultraflex III TOF/TOF), which was installed in March 2007, offers a dramatic increase in sensitivity and accuracy for both MS and MS/MS, user friendliness and peptide sequencing possibilities compared to the previous instrument.

Sample preparation for mass spectrometry

Most of the samples for analysis by MALDI-TOF/TOF-MS are delivered as bands or spots from one- or two-dimensional SDS-PAGE gels. The gel-separated proteins are prepared for MS analysis by in-gel tryptic digestion. The work-up of samples via C18 μZipTip allows us to get significant identities of minute amounts of sample. We typically identify proteins represented by single weak silver-stained spots from one two-dimensional gel.

Peptide mass fingerprinting and analysis of post-translational modification

After generation of a proteolytic digest and determination of peptide masses by MALDI-TOF MS, we search for a matching protein in sequence databases. When a significant mass spectrum is obtained, we can almost always identify the protein with high confidence. However, we often confirm the identity by subjecting a few peptides to fragment analysis followed by MS/MS search. Should we fail to identify the target protein, the identity may be established by obtaining the amino acid sequence of one or more tryptic peptides. A sequence homology search by BLAST is, in contrast to peptide

mass fingerprinting, tolerant of amino acid substitutions. We also study post-translational modifications, an important branch of proteomics. The nature of the modification determines the difficulty involved: we have been successful with, for example, methylation, acetylation, ubiquitylation and phosphorylation.

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