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Summary

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Napoleone Ferrara has been awarded the 2009 Pezcoller Foundation-AACR International Award for Cancer Research.
He is Research Fellow at the Genentech Inc. of San Francisco, particularly engaged in extensive projects in the field of regulation of angiogenesis.

Dr Ferrara has been honored for his groundbreaking research in the mechanisms of tumor angiogenesis, going from pioneering basic science to creating novel therapies for diseases with unmet medical needs. His main research focus has been the study of vascular endothelial cell growth factor (VEGF) and the biology of the human VEGF protein. Studying angiogenesis, Dr Ferrara and his team identified and cloned the gene for human VEGF (in 1989) and then characterized this molecule as a major regulator of angiogenesis in a variety of circumstances, including cancer, embryonic development, reproductive functions and endochondral bone formation. He also has demonstrated that VEGF is a key mediator of tumor angiogenesis. The development of a humanized anti-VEGF antibody, bevacizumab, rhuMAb-VEGF, stems from these studies, and is the first molecule clinically effective in targeting angiogenesis.
Dr. Ferrara delivered an important Pezcoller lecture entitled "Tumor angiogenesis; VECF-dependent and independent mechanisms" in Denver at the 2009 AACR Annual Meeting on April 19th . He also gave the Korsmeyer lecture in Padova at VIMM, Venetian Institute of Molecular Medicine, to honor the memory of the late Stanley Korsmeyer who received the Pezcoller-AACR Award in 2004.
On May 8th 2009, Dr. Ferrara was given the Award during a solemn ceremony in the prestigious 16th century reception hall of the Buonconsiglio Castle in Trento.

This year's Selection Committee met in Philadelphia in December 2008 and was composed of: Dr. Joe W. Gray , Division Director at the Lawrence Berkeley National Laboratory - Chairman - Dr. Riccardo Dolcetti, Head of the Immunovirology and Biotherapy Department of the Cancer National Institute of Aviano - Prof. Giampaolo Tortora, Chair of Oncology, Department of Molecular and Clinical Oncology and Endocrinology, University Federico II of Napoli - Dr. Sotiriou Christos, Chief of Research Unit of genomics and transnational research of Jules Bordet Institute in Brussels (Belgium) - Prof. Joanna L. Groden, Ohio State University, Molecular Virology Department - Prof. Mary-Ann Bjornsti, St. Jude Chrildren's Research Hospital in Memphis (Tennessee) Molecular Pharmacology Department - Prof. Michael Karin, Pharmacology, University of California, San Diego.

In June 11-13, we will hold the 21st Pezcoller Symposium entitled, "Unconventional Therapeutic Targets in Cancer". The Symposium will be co-chaired by David Livingston (Dana Farber Cancer Institute, Boston, MA), Enrico Mihich (Roswell Park Cancer Institute, Buffalo, NY) and Pier-Paolo Pandolfi (Harvard Medical School di Boston, MA). The focus of the Symposium will be on identifying new targets for anticancer drugs action, and on utilizing new concepts and methods for drug design and development. The topics to be discussed will include protein/protein interactions, targeting transcription factors, targeting death pathways, new chemical screening of novel molecular targets, valid alternatives to known target proteins, and unexpected effects of disrupted angiogenesis. Each talk will be followed by an equal time for discussion thus providing ample opportunities for interactions and cross-fertilization among participants. The speakers are: Zaver Bhujwalla, Nika Danial, Vishva Dixit, Steven Elmore, Peter Finan, Todd Golub, William Hahn, Emilio Hirsch, William Kaelin, Robert Kerbel, Wilhelm Krek, David Livingston, Enrico Mihich, Sean Morrison, Pier Paolo Pandolfi, Thomas Roberts, David Sabatini, Charles Sawyers, Bruce Spiegelman, Giulio Superti-Furga, Gregory Verdine.
The abstracts of this symposium are in the following pages.
Gios Bernardi, MD
The Pezcoller Foundation President
and Editor of the Journal

Picture on front page:
AACR Meeting, Denver, April 19th 2009 – Pezcoller Foundation-AACR International Award for Cancer Research. From the left: Gios Bernardi, Napoleone Ferrara and Joe Gray.

21st Pezcoller Symposium

Unconvetional Therapeutic Targets in Cancer

Trento, Italy, June 11-13, 2009

ABSTRACTS OF ORAL PRESENTATIONS

Tumorigenic potential is a common attribute of melanoma cells rather than a property of a rare subpopulation of melanoma stem cells

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A fundamental question in cancer biology is whether cells with tumorigenic potential are common or rare within human cancers. Studies on diverse cancers, including melanoma, have indicated that only rare human cancer cells (0.1% to 0.0001%) have tumorigenic potential when transplanted into NOD/SCID mice. However, the extent to which NOD/SCID mice underestimate the frequency of tumorigenic human cancer cells has been uncertain. Here we show that modified xenotransplantation assay conditions, including the use of more highly immunocompromised NOD/SCID IL2Rγ^{null} mice, can increase the detection of tumorigenic melanoma cells by several orders-of-magnitude. In limiting dilution assays, approximately 25% of unselected melanoma cells from 12 different patients, including cells from primary and metastatic melanomas obtained directly from patients, formed tumors under these more permissive conditions. In single cell transplants, an average of 27% of unselected melanoma cells from four different patients formed tumors. Xenotransplantation assay modifications can therefore dramatically increase the detectable frequency of tumorigenic cells, demonstrating that they are common in some human cancers. Our preliminary data indicate that melanoma is not unusual in having very high frequencies of tumorigenic cells and that, depending on the cancer, high frequencies of tumorigenic cells can be detected in some cases even in NOD/SCID or in fully immunocompetent mice. Overall, our results suggest that some cancers follow a cancer stem cell model, marked by epigenetically distinct and hierarchically organized subpopulations of tumorigenic and non-tumorigenic cells, while many other cancers do not.

Cancer and the Control of Systemic Metabolism

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Cancer affects systemic metabolism and recent data indicates that the incidence of cancer is affected greatly by the metabolic status of the individual. Tumors are typically more dependent on glycolytic metabolism than their normal counterpart tissues. This is often called the Warburg effect. Humans

with obesity and insulin resistance also have a greater incidence of cancer, especially tumors of the breast and prostate. Our lab is interested in the mechanisms underlying the defects in oxidative metabolism and tumors as well as new approaches to energy balance and obesity in humans.

Obesity arises when there is an imbalance between energy intake and energy expenditure. Energy expenditure includes physical movement and other pathways outside of movement. These include basal metabolism and thermogenesis mediated by brown fat. Mice ablated in brown fat or UCP-1 have an increase in body weight and obesity. Recent data shows that humans have copious amounts of brown fat, and hence control of brown fat formation represents a new and promising way to control obesity and the incidence of cancer in humans. We have recently identified a "master" regulator of brown fat formation, the large zinc-finger protein PRDM16. This molecule is both necessary and sufficient to convert white fat cell precursors into brown fat cells. Moreover, very recent data shows that brown fat is actually a one gene conversion from muscle cells. This overturns the prevailing models that had suggested white and brown fat cells arise from a common precursor cell. We are exploring using PRDM16 to drive brown fat formation and energy expenditure in a variety of ways in vivo.

2-Oxoglutarate-dependent Dioxygenases as Therapeutic Targets in Cancer

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Most successful drugs are small organic molecules that inhibit the functions of particular proteins. Enzymes are particularly amenable to pharmacological attack. 2-oxoglutarate-dependent dioxygenases are a recently recognized superfamily of enzymes that have now been linked to a variety of biological processes. These enzymes can be inhibited with small organic molecules that compete with 2-oxoglutarate or which interfere with their utilization of reduced iron. In the presence of oxygen the alpha subunit of the HIF transcription factor is hydroxylated on one (or both) of two prolyl residues by the 2-oxoglutarate-dependent dioxygenase EglN1 (also called PHD2). This modification creates a binding site for the VHL tumor suppressor protein, which then targets HIFα for proteasomal degradation. When oxygen levels are low, or the VHL protein is crippled, HIFα accumulates, binds to HIFß, and transcriptionally activates genes that promote survival in a low oxygen environment. Drugs that inhibit EglN1 are currently being tested for the treatment of anemia, while drugs that activate EglN1 (thereby lowering HIF levels) are being explored as anticancer agents. We recently discovered that EglN2 (PHD1), a paralog of EglN1, indirectly regulates Cyclin D1 in a HIF-independent manner. Inhibition of EglN2 leads to loss of Cyclin D1, decreased proliferation, and impaired tumorigenesis in vivo. Regulation of Cyclin D1 by EglN2 requires EglN2 catalytic activity and impaired proliferation in cells lacking EglN2 can be rescued by exogenous Cyclin D1 or by loss of pRB. Therefore Cyclin D1 causes, and does not merely correlate with, the proliferation defect in cells lacking EglN2. RBP2 (JARID1A) is a pRB-binding protein linked to differentiation control by pRB. A number of JmjC-containing proteins, including RBP2, are 2-oxoglutarate-dependent dioxygenases that serve as histone demethylases. Our preliminary data suggest that inhibition of RBP2 leads to impaired proliferation, promotion of differentiation, and loss of tumorigenesis.

Deubiquitinases as therapeutic targets in cancer

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Proto-oncogenes ETV1, ETV4, and ETV5 belong to the PEA3 family of ETS transcription factors and have been identified recently in prostate cancer chromosomal rearrangements that result in their overexpression. Little is known, however, about their post-translational

regulation. Here we show that ETV1, ETV4, and ETV5 interact with the ubiquitin ligase COP1. COP1 together with its binding partner DET1 ubiquitinated ETV1, resulting in ETV1 proteasomal degradation. Importantly, we show that truncation of ETV1 by the TMPRSS2:ETV1 translocation found in prostate cancer removes its COP1 binding motif and thereby enhances its stability. Consistent with a critical role for COP1 in regulating PEA3 transcription factors, COP1 deficiency in primary mouse prostate cells produced prostate intraepithelial neoplasia in vivo. The relationship between COP1 and ETV1 was also evident in human prostate cancer samples where loss of COP1 expression correlated with elevated ETV1 protein.

Drugging "Undruggable" Targets Using Stapled Peptides

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One of the most vexing problems in life science is that of "undruggability," the difficulty of targeting certain biological macromolecules in vivo using existing drug or ligand discovery technologies. It has been estimated that as many as 80-90% of all potential targets, including many that have been extensively validated in humans and in animal models, are undruggable. The Verdine laboratory is developing powerful new chemistry-based platform technologies to address these undruggable targets. Specifically, the lab is developing "synthetic biologics," molecules that, like biologics, possess the ability to target large flat surfaces, but that, like small molecules, are fully synthetic and hence can be modified at will. Progress on the development of one class of synthetic biologics – hydrocarbon-stapled alpha-helical peptides – will be reviewed in this talk.

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Metabolism Meets Apoptosis: A Role for Pro-Apoptotic BAD in Glucose Sensing

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Cellular energy homeostasis requires careful orchestration of ATP production and utilization. Glycolysis and mitochondrial oxidative phosphorylation (OXPHOS) are two major cellular ATP regenerating pathways whose predominance is controlled by the availability of nutrients and oxygen. An improper shift in utilization of these pathways, including suppression of mitochondrial oxidative capacity, is associated with metabolic aberrations in both cancer and diabetes. Identification and characterization of molecules that govern the cell's choice of ATP regenerating systems will provide important insights into targeting energy metabolism in disease.
We have previously shown a novel role for the BCL-2 family protein BAD in glucose oxidation. Recent studies indicate that the BH3 domain of BAD, previously known as its minimal death domain, is *required* and *sufficient* for glucose oxidation. Importantly, phosphorylation of a defined residue within this domain constitutes a physiologic switch between BAD's metabolic and apoptotic functions.

When phosphorylated, the BAD BH3 domain engages a metabolic program marked by glucose phosphorylation, mitochondrial respiration and ATP production. When dephosphorylated, the BAD BH3 domain binds and inactivates the anti-apoptotic BCL-2 and BCL-X_L, lowering the threshold for apoptosis. We further highlight the pharmacologic relevance of phosphorylated BAD BH3 domain by demonstrating the metabolic activity of hydrocarbon-stapled BAD BH3 helices. Our studies define an alternative target and function for the BAD BH3 domain and emphasize the therapeutic advantage of its mimetics. The significance of BAD's dual functionalities in different cell types, including pancreatic beta cells, will be discussed.

Allowing Sick Cells to Die: Targeting Bcl-2 Family Proteins to Treat Cancer

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Blocks in apoptotic signaling are a common requirement for oncogenesis, tumor maintenance and chemoresistance. This enables cancer cells to survive insults (genomic instability, oncogene activation, aberrant cell cycle progression, chemotherapy, etc.) that cause normal cells to undergo apoptosis. Death signals originating from a multitude of sources converge on the mitochondria where Bcl-2 family proteins act as critical regulators of apoptosis. Dynamic binding interactions between the pro-apoptotic (Bax, Bak, Bad, Bim, Noxa) and anti-apoptotic (Bcl-2, Bcl-x_L, Mcl-1) family members control commitment to cell death. Alterations in the balance between these opposing factions serve as one means by which cancer cells undermine normal apoptosis to gain a survival advantage.
Using structure based drug design we have discovered small molecule antagonists of anti-apoptotic Bcl-2 family proteins (1-4). ABT-263 is a potent (K_i of $\leq$ 1 nM), orally bioavailable small molecule inhibitor of Bcl-2/Bcl-x_L/Bcl-w that is currently in multiple Phase 1/2 clinical trials. Cellular studies reveal that ABT-263 disrupts Bcl-2/Bcl-x_L interactions with pro-apoptotic proteins (e.g., Bim) leading to the rapid initiation of Bax translocation, cytochrome *c* release and apoptotic cell death that is Bax/Bak dependent. In cell-based assays, ABT-263 is effective as a single agent against cell lines dependent on Bcl-2 and/or Bcl-x_L for survival (e.g. lymphoma, leukemia and small cell lung cancer (SCLC) but is less potent as a single agent in most solid tumors (5-8). However, by effectively lowering the apoptotic threshold, ABT-263 synergistically enhances the killing effect of chemotherapeutic agents in a wide variety of tumor types. The in vitro and in vivo single agent and combination activity of ABT-263 in hematologic and solid tumors along with the underlying mechanisms of action will be discussed.

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Microtubule-based VHL tumor suppressor mechanisms

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Inactivating germline mutations in the von Hippel Lindau (VHL) tumor suppressor gene are responsible for VHL disease, a multisystem cancer syndrome that manifests in the development of several benign and malignant tumors and cysts in different organ systems, including heamangioblastomas, pheochromocytomas, renal cysts and carcinomas. Biallelic *VHL* inactivation is also a common feature of sporadic renal cell carcinoma. How the *VHL* gene product, VHL, exerts its tumor suppression function remains incompletely understood. The most commonly accepted view attributes this function to a role of VHL in the control of transcription programs, extracellular matrix assembly and microtubule (MT) cytoskeleton (1).
We have previously reported that VHL associates with interphase MTs in vitro and in vivo and promotes MT stability (2). The MT stabilization function of VHL is compromised by certain naturally-occurring VHL mutations and has been linked to primary cilium maintenance (3) and suppression of renal cyst formation in mouse models (4), implying that regulation of MT stability by VHL is a critical aspect of its tumor suppressing activity. Based on these observations, we have gone on to investigate whether other cellular processes that depend on proper MT dynamics, in particular mitosis, are affected by loss of VHL function. Interestingly, we found that VHL localizes to the mitotic spindle and functions to suppress spindle mis-orientation and to promote chromosomal stability by positively regulating Mad2 mitotic checkpoint protein expression. These two newly identified functions of VHL are distinctly disrupted in naturally-occurring VHL mutants that are linked to MT-dependent and MT-independent activities of VHL, respectively. An association between *VHL* inactivation, reduced Mad2 levels and increased aneuploidy was also found in human renal cancer, implying that this newly identified functions of VHL in promoting proper spindle orientation and chromosomal stability likely contribute to tumour suppression (5).

More recently, we have investigated whether VHL affects the dynamics of MTs and how the dynamics may be specifically deregulated by disease-causing mutations of VHL. In this context, we have developed quantitative live cell imaging of MT growth by tracking the plus-end marker EB3-GFP in conjunction with spatiotemporal clustering of growth tracks to measure microtubule dynamic instability *in vivo*. Our experiments demonstrate that the sensitivity of computer-based live-cell readouts is becoming sufficient to pinpoint the subtle implications of individual protein domains on cell function *in vivo*. The practical simplicity of our assay brings high-content in situ live cell measurements of MT dynamics even into the realm of high-resolution screening applications, which will significantly expand our knowledge on the physiology of MTs and their roles in cancer as well as allow the identification of new anti-cancer agents that target distinct aspects of MT dynamic instability.

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Genetic modeling of PI3K inhibition

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Phosphoinositide 3-kinases (PI3K) are crucial elements needed for receptor-mediated signal transduction and modification of PI3K signaling is emerging as a key element in cancer development, because of the ability of PI3K to trigger a complex panoply of responses impinging on cell survival and proliferation (Hirsch et al., 2008). PI3K consist of heterodimers of a 110 kD catalytic (p110) as well as a regulatory/adapter subunit and are required for the production of a membrane bound phosphorylated lipid (PIP3) that acts as a critical secondary messenger molecule. Class I p110s (p110α, β, γ and δ) share significant homology but studies using genetically engineered mice show that they all play non-redundant roles. While these reports recently provided support for PI3K catalytic activity as a promising drug target they also unexpectedly revealed that these proteins not only work as kinases but also as scaffolds for protein-protein interactions (Hirsch et al., 2009). For example, we showed that p110γ plays a crucial role in the mounting of inflammatory reactions but is also part of a complex that, independently of its kinase function, controls cardiac contractility (Patrucco et al., 2004). Similarly, the ubiquitously expressed p110β appears to act not only as a kinase but also as a key element for protein-protein interactions (Ciraolo et al., 2008; Jia et al., 2008). Although genetic deletion of the p110β gene causes embryonic lethality, the expression of a catalytically inactive p110β is compatible with life. Interestingly, while the absence of p110β blocks proliferation of fibroblasts, inhibition of its kinase activity does not affect growth of these cells, thus indicating that a kinase-independent function is involved in this process. Indeed, p110β has been implicated in the endocytic process for long and we recently confirmed that the p110β protein is required for endocytosis of the EGF receptor independently of its kinase activity. The precise mechanism by which p110β controls EGF receptor endocytosis is not yet fully understood but clathrin-coated vesicle production is severely impaired in the absence of p110β and, on the contrary, is normal when p110β catalytic function is ablated. Despite this potential function as a scaffold protein, p110β catalytic activity is clearly involved in signal transduction events triggered by both tyrosine kinase and G protein-coupled receptors. For example, in fibroblasts, Akt phosphorylation mediated by the GPCR agonists LPA and S1P relies on p110β catalytic activity. Furthermore, mice expressing a catalytically inactive p110β develop peripheral insulin resistance, suggesting an involvement of this enzyme in insulin signaling. Similarly, males of this genetically modified strain show infertility due to abnormal activation of the c-Kit-dependent signaling pathway. Finally, p110β appears to be required for oncogenic Erbb2-mediated mammary gland cancer development as mice expressing inactive p110β show dramatically delayed mammary gland cancer development when crossed with transgenic mice expressing active Erbb2 in the mammary gland. In these compound mutants, not only latency of tumor appearance is reduced but also cancer growth as well as the number of affected glands per animal. Of note, reduction of primary tumor cell proliferation was confirmed by treatment with selective p110β inhibitors and similar effects were obtained even when cancer cells lost the oncosuppressor gene PTEN, thus suggesting a link between p110β and PTEN function. These results show that, although p110β shows kinase independent functions, its kinase activity can still play a critical role in cancer. This suggests that pharmacologic targeting of p110β might be beneficial in Erbb2-positive breast cancer and that such treatment will potentially cause tolerable and manageable side effects. Efforts to produce and test such drugs are under way and clinical trials are foreseen for the next future.

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The therapeutic potential of the mTOR patway

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mTOR is the target of the immunosuppressive drug rapamycin and the central component of a nutrient- and hormone-sensitive signaling pathway that regulates cell growth and proliferation. We now appreciate that this pathway becomes deregulated in many human cancers. We have identified two distinct mTOR-containing proteins complexes, one of which regulates growth through S6K and another that regulates cell survival through Akt. These complexes, mTORC1 and mTORC2, define both rapamycin-sensitive and insensitive branches of the mTOR pathway.
I will discuss new results from our lab on the regulation of the mTORC1 and mTORC2 pathways.
I will provide an overview of mTOR signaling as well as discuss the regulation of mTORC1 by insulin and nutrients and the role of mTORC2 in cancer. We have recently identified new upstream components of the mTORC1 pathway that are involved in amino acid sensing and are starting to understand some of the molecular mechanisms involved in this process. Current evidence suggests that amino acids regulate the mTORC1 pathway by controlling the intracellular localization of mTORC1. Specifically, amino acids promote the movement of mTORC1 to a part of the endomembrane system that also contains its activator Rheb. Amino acids signal through the conserved Rag family of small GTPases that directly interact with the raptor component of mTORC1 in fashion that depends on the GTP-loaded status of the Rags. In addition, I will present evidence that inhibition of mTORC1 by the novel mTOR-interacting protein Deptor is a mechanism for hyperactivating PI3K signaling in multiple myeloma.

Targeting PI3Kinase Isoforms in Cancer

Jean J Zhao and Thomas M Roberts
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The phosphatidylinositol 3 kinase (PI3K) signaling axis is frequently activated in adult solid tumors, including those of the breast, ovary, prostate, colon and brain. PI3 kinases constitute a small family of lipid kinases that is commonly subdivided into three classes by substrate specificity and subunit composition. Of these, the only class associated with cancer is the class 1A, consisting 3 enzymes termed p110α,p110ß and p110δ that are capable of signaling downstream from receptor tyrosine kinases (RTKs), G protein coupled receptors (GPCRs) and oncoproteins. Pathway activation in tumors is most commonly achieved through activating mutations in one of the PI3K catalytic subunit isoforms, p110α,▫or▫via inactivating mutations in the PTEN tumor suppressor, a lipid phosphatase that reverses the action of the PI3 kinases. Recent studies have delineated distinct but overlapping functions in cell signaling and tumorigenesis for p110α and p110ß, the two major catalytic subunits of PI3K expressed in the tissues of origin for the common tumor types. In most cell types studied, p110α carries the majority of the PI3K signal in classic RTK signal transduction, while p110ß responds to GPCRs. Both p110α and p110ß function in cellular transformation induced by alterations in components of PI3K pathway. Specifically, p110α is essential for the signaling and growth of tumors driven by PIK3CA mutations and/or oncogenic RTKs/Ras, whereas p110ß is the major isoform in

mediating PTEN-deficient tumorigenesis. While pan-PI3K inhibitors are currently being tested in the clinic, p110 isoform-specific inhibition holds forth promise as a therapeutic strategy. Our labs have been using conditional knockouts of p110α and p110ß to study the isoform requirements for tumor formation in a variety of mouse models. Results from recent studies will be discussed.

Molecular networks and chemical proteomics: charting drug targets

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Physiology relies on the concerted action of a number of molecular interactions of gene products and metabolites operationally organized in so-called pathways and in yet larger molecular networks. However, current appreciation of the "wiring diagram" of these pathways is scanty and drug discovery does not yet make use of the new postgenomic appreciation of physiology and patho-physiology. Through integrated approaches using proteomics as central "glue" it is possible to obtain physical, functional and "knowledge" maps of human disease pathways. We use affinity proteomics using TAP-mass spectrometry to chart protein complexes and molecular networks [1] and use drugs to establish target profiles [2]. The combination of these approaches allows not only to map drug/ligand- target relationships but also to position these elements onto molecular pathways. The promiscuity of clinical drugs entails the danger of side effects, but also the opportunity of additional medical uses. We have investigated five clinical drugs/drug candidates that are in use for chronic myeloid leukemia (CML), which is caused by the deregulated tyrosine kinase activity of the fusion oncoprotein BCR-ABL [3]. The targeted BCR-ABL inhibitor imatinib showed significant therapeutic success. However, both primary and secondary resistance necessitated the development of second- (nilotinib and dasatinib) and third-generation (bosutinib and INNO-406) inhibitors, which display varying degrees of specificity and potency. We identified the disease-relevant protein target profiles of these small molecule drugs by chemical proteomics, which combines drug affinity-chromatography with mass spectrometry, probing primary patient cell material. We identified several novel kinase and/or non-kinase targets for each one of the drugs [3-5]. One of these non-enzymatic targets, NQO2, is inhibited by imatinib at nanomolar concentrations [3, 6] and is seen to bind the drug in the crystal structure in a conformation that is different to how the drug binds the Abl kinase [7]. The interaction profiles of the five drugs display strong differences, overlapping only at the ABL kinases, with dasatinib and bosutinib being particularly promiscuous. As BCR-ABL forms the central cognate target of these five drugs, we additionally characterized its molecular machine by a systematic immunoprecipitation/proteomics approach. We purified endogenous Bcr-Abl complexes, analyzed their composition by LC-MSMS and identified a set of 7 stoichiometric core interactors [8]. The significant occurrence of these core proteins was confirmed by statistical analysis of triplicate large-scale IP/LC-MSMS data. In addition, we generated a comprehensive dataset based on tandem affinity purification using these 7 interactors as baits and generation of network models. iTRAQ experiments show that only some interactions of these 7 components with Bcr-Abl are abolished following treatment drug treatment, yielding a "post-drug" complex that may still be competent for signaling [8]. There are several general consequences of these studies: 1. Even "modern" targeted drugs are quite promiscuous, 2. Drug targets may generally be likely to be part of larger protein complexes, 3. Binding partners may influence drug action on the complex and in turn be affected (providing an additional rationale for the number of genes with personal "pharmacogenomic" effects on drug action), 4. In light of cellular "proteo-stasis", the complex components that may

become available after drug treatment may redistribute and affect other signaling pathways, 5. Drugs are likely to be understood as systems perturbators and not "erasers" of protein activity (with some gain-of-function effects matching loss-of-function). A better understanding of these perturbations should form the basis for an informed combination-type of therapy. Performed at a larger scale, these type of studies can contribute to an appreciation of the cellular interactome(s) relevant for disease, annotated with chemically tractable nodes as well as with the dynamic consequence on the network of chemical perturbation. We believe that approaches as these can pave the way for a real systems-level post-genomic molecular pharmacology.

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Approaches to dissecting signaling pathways by chemical genetics

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One of the greatest challenges facing the pharmaceutical industry is the identification of new therapeutic targets within disease linked pathways. With some noticeable exceptions, most marketed chemically derived pharmaceuticals perturb a small subset of target clasess such as ion channels, GPCRs, kinases, proteases and other enzymes [1]. This represents only a fraction of genes contained in the human genome and whilst many of these genes will remain refractory to therapeutic modulation there is a need within the medical research community to expand the number of targets which can be modulated with low molecular weight modulators. One approach to this is to systematically interrogate key signaling pathways through the creation of cell based assays which are amenable to high throughput screening and to couple this screening strategy to technology platforms that allow the identification of target-compound pairs and elucidation of the mechanism of action of modulators. We have used this approach to identify low molecular weight inhibitors of the Wnt pathway. Deregulated Wnt pathway activity has been implicated in many cancers, making this pathway an attractive target for anti-cancer therapies. However, the development of targeted Wnt pathway inhibitors has been hampered by the limited number of pathway components that are amenable to small molecule inhibition. In this study, we used a chemical genetic screen to identify modulators which selectively inhibits β-catenin-mediated transcription. Using a quantitative chemical proteomic approach, we discovered that these chemical modulators inhibit a member of an enzyme family not been previously associated with the Wnt pathway. This target hypothesis was confirmed through RNAi studies and assessing the effects of the Wnt modulators in model organisms. Thus, our studies provide novel mechanistic insights into the regulation of the Wnt

pathway and highlight an approach that can be taken to identify new therapeutic targets for drug discovery programs.
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Systematic functional approaches to target *KRAS*

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Recent advances in genomics now make it possible to consider enumerating all of the genetic lesions in specific cancers. While these approaches will yield critical information regarding the identify, number, and types of alterations found in human tumors, a complementary approach to decipher the molecular basis of malignant transformation depends upon the application of genome scale tools to annotate the function of genes involved in cancer initiation and progression. Over the past several years, we have developed genome scale RNAi libraries and open reading frame expression libraries that permit a systematic evaluation of genes involved in cancer initiation and maintenance (Moffat et al., 2006). Using these libraries, we have now performed screens in a panel of human cancer cell lines to systematically identify cancer vulnerabilities. By combining these functional approaches with information derived from mapping the structural abnormalities present in cancer genomes, we have identified several new oncogenes that contribute to cancer development (Boehm et al., 2007; Firestein et al., 2008; Luo et al., 2008).
However, many commonly occurring and well-validated oncogenes and tumor suppressor genes remain refractory to molecularly targeted therapies. For example, the proto-oncogene *KRAS* is mutated in a wide array of human cancers, most of which are aggressive and respond poorly to standard therapies. An alternative strategy for targeting *KRAS* is to identify gene products that, when suppressed or inhibited, result in cell death only in the presence of an oncogenic allele. Through the use of systematic RNAi screens, we have identified two kinases, *TBK1* and *STK33* that act in a synthetic lethal manner to selectively kill cancer cell lines that depend on mutant *KRAS* (Barbie et al.; Scholl et al., 2009). Taken together, these studies suggest that combining forward and reverse genetic approaches with information derived from the cancer genome anatomy mapping projects will yield a comprehensive list of cancer vulnerabilities and establish a general approach for the rational identification of oncogenic and co-dependent pathways in cancer.

Signatures for Small Molecule Discovery

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The application of genomic approaches to the study of cancer holds tremendous promise for improved diagnostic and prognostic tests, and for the elucidation of new therapeutic targets by building a molecular taxonomy of the disease. More recently, we have addressed the challenge of using gene expression data in the drug discovery setting. That is, having defined a gene expression signature of a biological state of interest (e.g. tumor subtype or state of pathway activation), could a small molecule library be screened to identify compounds capable of modulating the signature of interest – and by inference, modulate the biological state under study. We piloted this idea, termed Gene Expression-based High Throughput Screening (GE-HTS), and applied it to the discovery of compounds capable of inducing the myeloid differentiation of acute myeloid leukemia cells. Importantly, the discovery of these compounds did not require a specialized phenotypic assay, nor did it require prior knowledge of the mechanism by which differentiation occurs. We have subsequently applied this GE-HTS concept to the discovery of compounds that inhibit the activity of the Ewing Sarcoma oncogene EWS/FLI and that abrogate androgen receptor signaling in prostate cancer. These experiments establish the feasibility of using a gene expression signature as the read-out of a primary small-molecule screen.

Extending on this concept of signature-based chemical screening, we have recently established the feasibility of using a database of gene expression profiles to systematically connect signatures of diseases to signatures of gene product function or signatures of drug action. We refer to this project as the Connectivity Map project. By querying a centrally generated database of gene expression profiles, users can find 'connectivity' between a query signature of interest and one or more treatments (perturbagens) in the database. The data and tools are available at www.broad.harvard.edu/cmap, and we have used the method to discover relevant connections in dexamethasone-resistant childhood leukemia, androgen response in prostate cancer, and connections to HDAC inhibition in various cell types. These experiments demonstrate the feasibility of the Connectivity Map approach, and suggest the value of creating a larger, more extensive, publicly accessible Connectivity Map database.

Targeting the cancer initiating cell for therapy

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Failure to eradicate the cancer-initiating cell (CIC) and, specifically, the quiescent CIC population is at the core of disease relapse upon conventional and targeted therapies. We will discuss here how the elucidation of a novel PTEN/PI3K signaling regulatory network for the control of stem cell-ness has allowed us to propose and experimentally test novel concepts for CIC eradication of immediate therapeutic applicability.

Novel Approaches to Prostate Cancer Therapy

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Resistance to anti-androgen therapy is associated with increased expression of androgen receptor (AR) mRNA, AR gene amplification or AR mutation. Increased AR levels are necessary and sufficient to promote hormone-refractory growth in models and, paradoxically, alter the cellular response to classic AR antagonists such that they function as weak agonists. Therefore, second generation antiandrogens must overcome these resistance mechanisms. We searched for novel AR antagonists that might retain function in the context of increased AR expression through a cell-based screen. Using the high affinity AR agonist RU59063 as a starting point, we synthesized and screened over 200 compounds to construct a structure/activity profile that defines features of the scaffold essential for receptor binding and for maximal antagonism. We focused our further efforts on a novel compound RD162, which retains potent antiandrogen activity in cells expressing increased levels of AR, blocks AR function in mice and impairs the growth of LNCaP and LAPC-4 xenografts engineered to express high levels of AR, whereas bicalutamide had minimal activity. RD162 inhibits AR with 10-fold greater affinity than bicalutamide and functions through a novel mechanism of action that impairs nuclear translocation and DNA binding. An RD162 derivative MDV3100 has shown clinical activity in a Phase I-II clinical trial of ~140 men with castrate-resistant prostate cancer. About 50 percent of patients have had reductions of PSA by greater than 50% sustained for more than 12 weeks, including some men with radiographic responses and a much larger proportion with stable disease.
One important AR target gene is the TMPRSS2-ERG fusion gene found in ~50 percent of invasive prostate cancers. Interestingly, TMPRSS2-ERG is less commonly detected in the histologic precursor lesion prostatic intra-epithelial neoplasia (PIN), raising the question of whether TMPRSS2-ERG is sufficient to initiate disease or whether it plays a role in early disease progression. We generated transgenic mice expressing the TMPRSS2-ERG fusion most commonly found in patients and find that mice expressing this fusion develop PIN, but only in the context of PI3-kinase pathway activation mediated by either

Pten loss or Akt activation. We also find that TMPRSS2-ERG positive tumors from patients are highly enriched for PTEN loss, suggesting that these two events cooperate in human prostate tumorigenesis. Of note, MDV3100 (but not bicalutamide) impairs growth of the TMPRSS2-ERG positive cell line VCAP in culture and induces cell death by apoptosis but PI3K pathway activation may blunt the efficacy of antiandrogen therapy.

Disclosures: Dr. Sawyers is a co-inventor of MDV3100 and owns stock in Medivation.

Conventional imaging to guide therapy of unconventional targets

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Current advances in multi-modality imaging, contrast development, and molecular biology, are revolutionizing the applications of imaging in cancer therapy. In our program the identification of specific targets in cancer is driving advances in novel image-guided platforms such as liposomes, microencapsulation devices, and nanoplexes to deliver siRNA to down regulate specific targets and pathways. Another exciting development is in the development, synthesis and application of a novel prototype agent for image-guided prodrug therapy. This prodrug enzyme platform can be combined with siRNA targeting. Damage to normal tissue is a major limiting factor in chemotherapy as well as radiation therapy and numerous strategies to protect normal tissue while maximizing damage to cancer cells have been actively pursued. Prodrug enzyme activation systems, where enzymes delivered to the tumor convert a nontoxic prodrug to a cytotoxic drug are one of the most attractive of these strategies but to date have resulted in limited success. Imaging the delivery of the enzyme so that the prodrug is administered when enzyme levels are highest in the tumor and lowest in systemic circulation and normal tissues would be of significant importance for optimizing such a therapeutic strategy. We have synthesized a prototype agent consisting of a cancer therapeutic prodrug enzyme labeled with multimodal magnetic resonance (MR) and optical imaging reporters. The prodrug enzyme, cytosine deaminase, converts a non toxic prodrug 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU). The prodrug 5-FC and its conversion to 5-FU can be detected noninvasively by 19F magnetic resonance spectroscopy. This prototype agent has been used to demonstrate the feasibility of image-guided prodrug enzyme therapy using magnetic resonance imaging (MRI); imaging can be used to time the administration of the prodrug when the enzyme has cleared from normal tissue but is still present in the tumor. Such an approach minimizes cytotoxic side-effects. We are expanding this platform to combine prodrug enzyme delivery, and the delivery of siRNA. The optical reporter is especially useful to track the conjugate in cells and tissue using microscopy, while the MR reporter provides potential clinical translatability of this approach.
One common metabolic feature identified by MRS in most cancers is the elevation of total choline. This elevation of choline compounds provides a unique target to exploit for therapy; such targeting can be imaged noninvasively with MRS. We are developing molecular and molecular imaging based approaches to target choline metabolism, specifically choline kinase activity, which is the first step in choline phospholipid biosynthesis. The approaches include targeting choline kinase using siRNA delivered using lentiviral vectors, and performing image-guided targeting of choline kinase using siRNA in combination with the prodrug enzyme cytosine deaminase using a multi-modal imaging platform. These advances can be used to target specific pathways, microenvironments, and cell types within tumors under image-guidance.

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Some Unexpected Outcomes of Therapeutically Interfering with VEGF Pathway Function

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One of the most significant developments in medical oncology over the last 5 years has been the approval of a number of antiangiogenic drugs for treatment of a variety of cancers including colorectal cancer, non small cell lung cancer, breast cancer, renal cell carcinoma and hepatocellular carcinoma(1). These drugs include bevacizumab, the monoclonal anti-VEGF antibody, and sunitinib and sorafenib, both oral small molecule multitargeting receptor tyrosine kinase inhibitors which target VEGF and PDGF receptors, among a number of others(1). The progression free or overall survival clinical benefits induced by these drugs are modest and as such this fact has stimulated considerable interest in the mechanisms by which resistance to them develops(2). However, another way in which their potential clinical benefits can be reduced (or in some cases eliminated?) is by drug-induced increases in tumor growth, invasion, and metastasis, subsequent to causing an initial anti-tumor benefit, e.g. slowing down tumor growth. One way in which this change in tumor biology can occur is by the drugs causing increased tumor hypoxia – which is what they are essentially designed to achieve(3-5). This can result in the adaptive upregulation of a number of genes which induce angiogenesis, cell invasion and metastasis, through increases in the hypoxia-induced transcription factor, HIF-1(6, 7), something which may help explain the high rate of highly invasive glioblastomas at recurrence in patients treated with bevacizumab, as first shown in preclinical models(5).

In addition to this tumor-associated hypoxia-dependent mechanism, we have been studying a fundamentally different mechanism which, in theory, can also contribute to both drug resistance and increases in malignant aggressiveness(8). It involves the systemic induction in host tissues of multiple circulating growth factors, cytokines and chemokines as a result of administration of antiangiogenic drugs such as sunitinib(9). These factors, in mice, include not only VEGF and PlGF – similar to what is seen in cancer patients(10, 11) but also 'off target' or collateral changes such as SDF-1, G-CSF and osteopontin(9); such changes are reversible and dose-dependent and parallel drug potency, as defined by anti-tumor activity in previous therapy experiments. Since many, if not all, of these aforementioned factors can promote tumor growth and angiogenesis we reasoned that there may be circumstances where antiangiogenic drug activity may not only be diminished or lost altogether but they might also eventually increase tumor aggressiveness. This stimulated us to assess the effects of short-term sunitinib treatment in preclinical models of neoadjuvant or aduvant-like therapy of micrometastatic breast cancer where we recently reported evidence of accelerated metastatic growth – in contrast to established (primary) tumors, the growth of which were suppressed(8).

From our results we concluded that adjuvant treatment of early stage cancers with antiangiogenic drugs, after surgery, may be less or even non effective compared with established metastatic disease(8). The disappointing results recently announced of the first adjuvant trial of antiangiogenic therapy(12, 13) suggest this hypothesis may have merit. However, they also suggest a number of strategies to improve the impact of antiangiogenic drugs for both early and late stage disease; one approach we have been studying is the combination of antiangiogenic drugs with metronomic chemotherapy(14, 15).

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ABSTRACTS OF POSTERS

Novel "vascular markers" highly expressed by human tumor endothelial cells

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Molecules expressed by the tumor microenvironment provide opportunities for the development of innovative therapeutic strategies. This is exemplified by the number of approved agents affecting the tumor vasculature, whose administration improved the therapeutic efficacy of both standard chemotherapy and radiotherapy and is becoming standard of care for many patients. Yet, different tumor types display variable degree of responsiveness and may become resistant. Thus, novel targets ought to be identified to develop new agents and overcome the unexpected negative features associated with the currently used drugs.

Our effort has been aimed at the molecular characterization of tumor associated endothelial cells to identify novel markers, suitable for targeting the tumor vasculature. Endothelial cells (EC) were isolated from tumor and normal tissue specimens and gene expression profiles analyzed (Affymetrix GeneChip®). The robust expression of many transcripts defined "typical endothelial markers" endorsed the endothelial origin of the cells.
Microarray results indicated that one hundred fifty-eight genes (involved in a variety of biological functions including basement membrane/extracellular matrix degradation and remodeling, proliferation and differentiation, cell-cell and cell-matrix interaction) were expressed to a much greater extent in EC isolated from cancer specimens with respect to normal tissue derived EC. Along with molecules known to be expressed by tumor endothelium (e.g. urokinase-plasminogen activator, CD44, endothelin-1 and 2, endothelin receptor-A, tissue factor, VEGF), we have identified some transcripts that might represent novel markers of tumor vasculature.

Our investigation focused onto four genes: ADAM23 [a disintegrin and metalloproteinase domain 23], FAP [fibroblast activation protein], GPNMB [glycoprotein nmb] and PRSS3 [serine protease 3].
RT-RealTime-PCR analyses of newly purified ECs confirmed their higher expression by tumor derived EC, both in the absence and in the presence of an *in vitro* reconstituted "tumor/angiogenic" environment (i.e. enriched in growth factors and extracellular matrix proteins).
Further recent investigation established that PRSS3 expression is regulated by pro-angiogenic factors FGF2, VEGF and EGF which determine a 4-fold induction.
In addition to tumor EC, two transcripts could be expressed at higher levels by other stromal cells such as fibroblasts (FAP and GPNMB) and smooth muscle cells (GPNMB). These differences are likely to have *in vivo* relevance. By in situ hybridization, transcripts such as ADAM23, GPNMB, and PRSS3 were found expressed and associated to the blood vessels of human cancer specimens but not by vessels of non neoplastic tissues.
This work evinces some of the distinctive features of tumor-derived endothelium, and identifies potential vascular/stroma markers that may provide the groundwork for novel therapeutic strategies.

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High-throughput technology for the identification of cancer-related microRNAs

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MicroRNAs (miRNAs) are a class of small noncoding RNA molecules that regulate gene expression at the post-transcriptional level by partially complementary base pairing to sequences located in the 3'UTR regions of mRNAs. MiRNAs have been implicated in a wide variety of physiological processes including development, proliferation and tissue differentiation. Growing evidence suggests that miRNA disregulation plays an important role in cancer, where these molecules can act both as oncogenes and oncosuppressors. We chose two widely different types of tumors, rhabdomyosarcoma (pediatric, soft tissue origin) and gastric cancer (adult, epithelial origin), to assess the potential of the new generation sequencing technologies for the identification of both known and new tumor-related miRNAs. To this aim we generated small RNA libraries from primary tumor samples, cell lines and normal tissues and deep-sequenced them using 454 and Solexa platforms. Here we report the results of the analysis of close to one hundred million independent small RNA-derived reads from 46 different libraries. All sequences were then analyzed by MiRDeep, which allowed us to identify a number of previously uncharacterized miRNA candidates. Our data, besides showing that the miRNA expression signature clearly distinguishes malignant from normal tissues, reveal putative oncogenic and oncosuppressor miRNAs (currently under validation) that are differentially expressed in tumors with respect to controls and highlight the extraordinary potential of high-throughput approaches for the identification of miRNAs that might play a key role in developing new therapeutic strategies against cancer.

Urokinase targeting in human hepatocellular carcinoma by shRNAs/ miR23-b and proteomic identification of lasp1 as u-PA effector.

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After the identification of urokinase (u-PA: urokinase type plasminogen activator) as unfavourable prognostic marker of human hepatocellular carcinoma (HCC) and possible therapeutic target (Cancer Res. 1998, Int. J. Cancer 2000), we used shRNA technology for u-PA with the objective to develop new therapeutics for HCC. We designed and characterized 2 different short-hairpin RNAs (shRNAs) for u-PA mRNA targeting at its 5'terminus. We developed stable expression of these u-PA shRNAs by plasmid expression vectors with pPolIII or pPol II promoter (pS and pH constructs; pPolII constructs); and we studied the effects of u-PA silencing in cultured HCC cells, also in HCC xenografts induced in nude mice (Mol. Cancer Ther. 2004, Tumor Biol. 2007). The results obtained showed that: 1) both u-PA shRNAs were

similarly effective in silencing u-PA, mainly at protein level (by 90% protein inhibition); 2) u-PA plays an essential role in the migration and proliferation abilities of cultured HCC cells; 3) u-PA silencing in HCC xenografts in nude mice delayed tumour development and affected tumour growth with a sequence-specific and long-term effect; after 11 weeks of tumour development, each nodule contained the construct and no toxic side effect was recorded in mice developing tumours; 4) u-PA silencing was associated with fibronectin fibrils induction in cultured cells and in HCC xenografts; 5) u-PA silencing was associated with induction of BAX pro-apoptotic protein expression along with a decrease of BCL-X_L antiapoptotic protein and the induction of BCL-X_S. Further, studying the protein effectors of u-PA, we recently reported the proteomic identification of LASP1 downregulation after RNAi uPA silencing in HCC cells. LASP1 is a scaffold protein known to be involved in cytoskeleton dynamics. The ectopic expression experiments revealed that both u-PA and LASP1 overexpression increased the motility of the cells; and that u-PA upregulation enhanced LASP1 expression, but not vice versa thus suggesting LASP-1 as uPA effector (Neoplasia 2009).

Since uPA upregulation may be coordinated with c-met overexpression in HCC (Int J Cancer 2000), we investigated whether the expression of these genes might be coregulated by specific microRNAs. miRNAs are small non-protein-coding RNAs implicated in negatively gene expression regulation. Each miRNA can bind to several mRNA targets and a given mRNA can be recognized by different microRNAs. By bioinformatics approach, using the algorithm PicTar, we predicted that hsa-miRNA-23b could recognize 2 sites in the 3'UTR of uPA and 4 sites in the c-met 3'-UTR. The pre-miRNA-23b expression analysis in human tumour and normal cells revealed an inverse trend with uPA and c-met expression; therefore indicating that uPA and c-met negative regulation might depend on miR-23b expression. Transfection of miR-23b molecules in HCC cells (SKHep1C3) led to the protein expression inhibition of the target genes and caused a decrease of cell migration and proliferation capabilities. Furthermore, anti-miR-23b transfection in human AB2 dermal fibroblasts up-regulated the expression of endogenous uPA and c-met. Co-transfection experiments of the miR-23b with the pGL4.71 Renilla luciferase reporter gene constructs, containing the putative uPA and c-met 3'UTR target sites, showed a decrease of the relative luciferase activity. This would indicate that miR-23b can recognize target sites in the 3'UTR of uPA and of c-met mRNAs and translationally repress the expression of uPA and c-met in HCC cells. miR-23b exerted a negative regulation of uPA and c-met also in AB2 normal cells. Our findings suggest a regulatory mechanism involving molecules, miR-23b, uPA and c-met, until now not known to interact (FEBS J. June 2009). Further in order to determine the potential role of miR-23b in human HCC we assessed the miR-23b expression level in HCC biopsy specimens and corresponding peritumoral tissues (PT). Data obtained showed dysregulated expression of miR-23b in HCC specimens. Among the samples derived from 17 HCC patients there are two subsets: one (14/17, 82%) with miR-23b downregulation in HCC specimens as compared with PT tissues with the average expression level of miR-23b in HCC specimens about two-fold lower than in PT tissues ($P<0.01$); and another (3/13,18%) showing miR-23b upregulation in HCC specimens. Taken together these results are a novel contribution to gene expression regulation of uPA and c-met and on their negative coregulation in human cells. As pharmacological regulation of uPA and c-met has proved to be a challenge in cancer, this regulatory mechanism may potentially be a new tool with which to alter the expression of uPA and c-met as well as other molecules (e.g. shRNA, uPA inhibitors, and tyrosine protein kinase inhibitors).

Polycystin-1 Controls mTOR and its downstream effectors p70S6K and 4EBP1 in an ERKs-dependent, Akt-independent manner

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We have previously shown that over-

expression of Polycystin-1 (PC-1) in renal epithelial cells (MDCK) induces reduced proliferation rates, resistance to apoptosis and spontaneous tubulogenesis. More recently, we have shown that expression of PC-1 in MDCK cells reduces cell size in a cell cycle independent manner. Furthermore, we found that expression of PC-1 results in downregulation of phosphorylaton of p70S6K and 4EBP1, downstream effectors of mTOR, one of the most studied pathways able to control cell size (Distefano et al, *Mol Cell Biol*, 2009 **29:**2359-71). Despite the capability of PC-1 to upregulate Akt kinase activity Akt phospho-specific sites in Tsc2 are not altered in our system. However, we have shown that PC-1 is able to strongly inhibit the MEK-ERKs signaling pathway and that constitutively active forms of MEK are able to rescue ERKs phosphorylation, p70S6K and 4EBP1 phophorylation as well as cell size. Furthermore, we isolated Mouse Embryonic Fibroblasts from *Pkd1*+/+ and -/- mice and showed that knock-out cells present increased cell cycle progression, cell-cycle independent increase in cell size, enhanced activity of the MEK-ERKs pathway and increased phosphorylation of p70S6K and 4EBP1. Finally, we have shown that PC-1 is able to control cell size in a *Tsc2*-dependent manner, as expression of PC1 in *Tsc2*+/+, but not in *Tsc2*-/- MEFs, results in decreased cell size. Finally, we have demonstrated that Tuberin phosphorylation in Ser664 (ERKs-dependent site, kindly provided by Dr. PP Pandolfi) is decreased in PC-1 expressing MDCK cells as compared to controls and consistently it is increased in Pkd1-/- as compared to *Pkd1*+/+ MEFs. Next, we tested the physiological relevance of our findings by generating a mouse model of ADPKD. We engineered a mouse line carrying a floxable *Pkd1* allele and crossed it with a kidney-specific Cre line (Ksp-Cre). *Pkd1*$^{flox/flox}$:Ksp-Cre mice present with massive renal cystogenesis after birth, ultimately leading to death by the second week of life. Biochemical analysis of these tissues showed that both ERKs and the mTOR pathway are upregulated in polycystic kidneys, while Akt is not, further supporting our conclusions.
From all these data we conclude that PC-1 regulates the mTOR pathway through an unusual mechanism mediated only by the ERKs component and not by Akt.

Identification of natural compounds as potent inhibitors of 26S proteasome assembly in vitro.

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The Ubiquitin-Proteasome System (UPS) is the main non-lysosomial proteolytic machinery in the eukaryotic cells. Prior to their degradation proteins are adressed to the catalytic 26S proteasome complex composed of a 20S "core" that contains proteolytic activities, assembled with one or two 19S regulatory particles that bind proteins to be degraded. Due to its central role of in regulating cell cycle progression and signal transduction the UPS has been proposed as a suitable target for antineoplastic strategies . To date only catalytic inhibitors of proteasome, targeting proteolytic activities of the 20S core particle, have been characterized and/or have emerged as potent anticancer drugs *(1)*. However, the capacity of the UPS to degrade proteins not only depends of the catalytic activities of the 20S core, but also of the 26S proteasome assembly, a dynamic and tightly controlled cellular process. Thus, compounds impairing 26S proteasome assembly could represent an original class of proteasome inhibitors.
With the aim of identifying such molecules we first set up a robust biochemical assay allowing to follow 26S proteasome assembly *in vitro*. The 26S proteasome was purified from Hela cells using affinity chromatography followed by gel filtration. Then, experimental conditions were determined for *in vitro* 26S disassembly into its constitutive sub-complexes (19S+20S) as well as its re-assembly. Disassembly/assembly of 26S was analysed *in vitro* using as the readout the differential CTL-activity between 26S *vs.* 20S proteasomes *(2)*, and was further confirmed using in-gel overlay experiments and western-blotting.
Using this biochemical assay:
a) we showed that catalytic inhibitors of the proteasome such as epoxomicin, that induce complete break down of CTL-activities linked to both 20S and 26S entities, do not affect 26S proteasome assembly.

b) we identified structurally-related natural compounds, epidithiodioxopiperazine and diketodithiopiperazine derivatives, as potent inhibitors of the 26S proteasome assembly without significantly affecting 26S and 20S catalytic activity *in vitro*.
Our research efforts now aim at understanding: -i) the molecular mechanisms by which these molecules inhibit proteasome assembly in cells, and –ii) how this inhibition can be correlated with their antiproliferative effects.

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Hormone-dependent nuclear export of estradiol receptor and DNA synthesis in breast cancer cells

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The most important features that differentiate eukaryotic cells from prokaryotic cells is the presence of distinct intracellular compartments, organelles and nucleus.
The *shuttling* nucleocytoplasmatic of proteins plays a critical role in the regulation of important biological functions. Steroid receptors continuously shuttle between cytoplasm and nucleus. Although the import mechanism of steroid receptors has been extensively dissected, their export mechanism is still unclear.
In breast cancer cells, estradiol induces in rapid succession nuclear translocation and nuclear exit of the estradiol receptor (ERα). We have analyzed in MCF-7 cells the nucleo-cytoplasmic shuttling of the estradiol receptor focusing on its export from the nucleus.
We identified, in MCF-7 cells, a nuclear export sequence (NES) in ER alpha and showed that its export is dependent on both estradiol-mediated phosphatidylinositol-3-kinase (PI3K)/AKT activation and chromosome region maintenance 1 (CRM1). CMR1 is the best-characterized export receptor and has been implicated for some steroid receptors. A Tat peptide containing the ER-alpha NES prevents *in vitro* estradiol-alpha dependent ER/CRM1 interaction and inhibits nuclear export of ER-alpha and estradiol-induced DNA synthesis in MCF-7 cells. Furthermore, experiments in NIH3T3 fibroblasts show that an NESERα mutant fails to induce S-phase entry of cells, whereas it is able to activate gene transcription.
Confocal microscopy analysis shows that ERα colocalizes with forkhead in nuclei of estradiol-alpha treated MCF-7 cells. A forkhead mutant, unphosphorylatable by Akt, is trapped together with ER wt in nuclei and blocks estradiol induced S phase entry in MCF-7 cells. In turn, the Erα NES mutant fails to exit nuclei and prevents forkhead wt nuclear export.
In conclusion, ERα nuclear export is associated with forkhead exit and regulates G1-S transition in breast cancer cells. Estradiol stimulation of the PI3K/Akt pathway facilitates the export of the two transcription factors in breast cancer cells.
Our results highlight the studies on regulation of ERα intracellular trafficking and point to a key role of estradiol in modulating this process.
Therefore, these observations offer a powerful tool to modify the intracellular distribution of
ER and reveal much about the biological functions of this receptor. On this basis, the strategy of trapping ER in nuclear compartment might offer a potential approach to the therapy of human breast cancers.

Insulin-like growth factor 2 targeted ligand traps based on soluble versions of domain 11 of the IGF2 receptor.

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Ligands transported by the mannose 6-phosphate/insulin-like growth factor 2 receptor (IGF2R) include IGF2 and mannose 6-phosphate-modified proteins. Increased extra-cellular supply of IGF2, either secondary to loss of the clearance function of IGF2R, loss of IGF binding protein function through protease cleavage, or increased IGF2 gene expression, can all result in embryonic overgrowth and cancer promotion. Human tumours over-express *IGF2* often as a result of loss of imprinting e.g. sarcoma, Wilms, colorectal, breast and prostate cancers. IGF2R loss of function occurs frequently in hepatocellular, breast and lung cancers, and an exon specific polyG tract can disrupted in mismatch repair defective colorectal cancer. Reduced supply of IGF2 was detrimental to tumor growth promoted by T-antigen (RIP-Tag), $Apc^{Min/+}$ [1] and $Ptch^{+/-}$ in the mouse, suggesting that gain of function of unbound IGF2 is a molecular target for human cancer therapy. IGF2R domain 11 has a conserved ß-barrel structure that binds IGF2 with high specificity and affinity via an evolutionary aquired binding pocket formed by structural loops (AB, CD, HI and FG). Structural and mutagenesis studies have shown that substitution of glutamic acid for lysine at residue 1554 of the AB loop results in a 6-fold higher affinity for IGF-II (20.5 nmol/L) than native domain 11 (119 nmol/L) [2]. Here, we have generated novel high-affinity IGF2 ligand trap by fusion of mutated human 11(E1554K) to a COOH-terminal human IgG1 Fc domain (11(E1554K)-Fc)[3]. The resulting homo-dimer has a significantly increased affinity for IGF2 (1.79 nmol/L) when measured by surface plasmon resonance. IGF2 signaling via the IGF-I receptor and the proliferative effect of IGF2 were specifically inhibited by 11(E1554K)-Fc in both HaCaT and $Igf2^{-/-}$ mouse embryonic fibroblast cells[3]. In order to optimize affinity through 'off rate' selection, structural approaches[4,5] and a novel Pichia Pastoris yeast display system have generated higher affinity versions of domain 11 that in an Fc fusion generated affinity in the picomolar range. These data confirm that a novel engineered and soluble IGF2R-11(E1554K)-Fc protein functions as an IGF2 specific and high-affinity ligand trap in vitro and that an optimized version of this protein has potential application as an IGF2 specific antagonist for cancer therapy.

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Aberrant Choline Metabolism in Ovarian Cancer: Identification of New Therapy Targets.

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Introduction - In spite of substantial progress in clinical oncology, epithelial ovary cancer (EOC), the gynaecological malignancy at highest death rate in industrialized countries, is often discovered only at advanced stages, with frequent occurrence of relapse, onset of drug resistance and a 5-year survival still remaining at about 44%. The development of more effective molecular imaging approaches could facilitate our understanding of molecular mechanisms underlying onset and development of this disease, improve clinical diagnosis, and allow identification of new prognostic factors and novel targets to therapy. Detection and characterization by magnetic resonance spectroscopy (MRS) of aberrant phosphatidylcholine (PC) metabolism in tumors fostered in the last decade novel areas of investigation in cancer cell biology and allowed identification of new indicators of in vivo tumor progression by application of choline-based MRS and PET diagnostic approaches. In particular, increase in the ^{1}H

MRS resonance centered at 3.2 ppm, mainly due to headgroups of choline-containing metabolites (tCho), has been reported as a common feature of several cancer types, including breast, prostate, colon and ovary carcinomas, bone and soft tissue sarcomas and neuroepithelial tumors[1-4]. By comprising signals from different PC metabolites, such as phosphocholine (PCho), glycerophosphocholine (GPC) and free choline (Cho), the tCho spectral profile and its changes reflect the overall basal or perturbed contents and fluxes of Cho derivatives in the PC-cycle pathways.
Aims of this study: a) to investigate the molecular mechanisms underlying the aberrant choline metabolism in ovary cancer; b) to evaluate the role of PC-cycle enzymes, as a basis for the possible design of new therapies.
Materials and Methods – *Cells*: Human EOC cell lines (OVCAR3, IGROV1, CABAI, SKOV3); normal ovary surface epithelium (OSE) cells and non tumoral immortalized cell variants (IOSE, hTERT) collectively named as EONT.
Results – Quantification of PC metabolites in cell extracts showed higher levels of phosphocholine (PCho, 3- to 8 x, P< 0.0001) and tCho (2.0 to 4.4 x, P< 0.0001) in EOC as compared to EONT cells[5]. The increase in PCho content in cancer cells was associated with activation of enzymes involved in both biosynthetic and catabolic pathways. In particular, we observed a strong increase (up to 25 fold) in the activity of choline kinase (chok) with respect to the non tumoral hTERT cells. The two catabolic pathways mediated by phospholipase D (pld) and glycerophosphocholine-phosphodiesterase (GPC-pd) contributing to the direct formation of free choline, increased only 2-4 times in some (but not all) EOC, as compared to EONT cells. We showed for the first time a striking increase (up to 17-fold) in the activity of phospholipase C (PC-plc) in EOC cells. The order of increase in PC-plc activity was comparable to that measured for chok. Exposure of OVCAR3 cells to tricyclodecan-9-yl-potassium xanthate (D609; 200μM 24h,) led to a 90% decrease in PC-plc activity, associated with a 37 % drop in PCho content (P = 0.028) and reduced cell proliferation (P=0.001), in the absence of apoptosis. Furthermore, evidence in our laboratory also showed that: a) activation of PC-plc was associated in EOC cells with accumulation of this enzyme on the external plasma membrane; b) decrease in PC-plc activity was associated with decrease in the S-phase fraction in serum-deprived EOC cells, both parameters reverting to their respective basal levels upon cell restimulation and c) D609 induced long-lasting block in the recovery of both PC-PLC activity and S-phase fraction in receptor re-stimulated cancer cells [6]. These findings warrant further investigation on the role of PC-plc in constitutive EOC cell stimulation and proliferation.
Conclusions - Major contributions to PCho accumulation in EOC cells can be attributed to both upregulation/activation of chok and increase in PC-plc activity. Emerging knowledge on selective genomic/biochemical regulation of PC-cycle enzymes may open novel ways to targeted anticancer therapies in EOC. Activation of both biosynthetic and catabolic enzymes occurs in the PC-cycle during ovary cancer progression. Evidence of abnormal PC metabolism has implications in cancer biology and may provide an avenue to the development of non-invasive *in vivo* clinical methods (MRS and choline-based PET) for diagnosis and treatment follow-up. PC-plc may represent a novel target for the design of new therapeutic strategies in ovary cancer.

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Compensatory induction of menin-oncosuppressor : a post-transcriptional mechanism that opens the possibility of a RNA-based therapy in MEN1 gene mutation carriers.

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Multiple endocrine neoplasia type 1 (MEN1) syndrome is characterized by tumours of the

parathyroids, of the neuroendocrine cells of the gastro entero pancreatic tract, and of the anterior pituitary. MEN1 gene, a tumour suppressor gene, encodes menin protein. Loss of heterozygosity at 11q13 is typical of MEN1 tumours. We analysed the MEN1 mRNA and menin expression in fibroblasts from normal skin biopsies and from MEN1 patients [two with a frame-shift 738del4 (exon 3) mutation, introducing a premature stop codon, and an individual with a R460X (exon 10) nonsense mutation]. The expression of full length menin protein did not differ between MEN1 and normal fibroblasts with both wild type and mutated alleles mRNAs being expressed in MEN1 patients, suggesting a mechanism of compensation for mRNA loss by up regulating the expression of menin oncosuppressor at a post transcriptional level. In vitro menin recognized its mRNA and a specific RNA proteins complex bound to MEN1 mRNA, thus indicating that induction of menin oncosuppressor compensation could have been regulated through RNA protein driven post transcriptional mechanisms. The binding of miR- 24 to the 3'-UTR of MEN1 mRNA opens the possibility of involvement of this microRNA in the mechanism of compensation. This model offers the possibility of using in vitro catalytic RNAs, siRNAs and RNA antagomirs for the MEN1 gene also opening new avenues for future developments of RNA-based strategies in the in vivo control of tumorigenesis in MEN1 carriers

Activation of PAF/PAF-like receptor dependent pathways within melanoma microenvironments contribute to melanoma growth and interferes with tumor response to chemotherapy

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Phagocytosis of apoptotic bodies or apoptotic self-cells by macrophages induces TNF-α and IL1β pro-inflammatory cytokines downregulation. Previous data from our group suggested that macrophages become suppressed after phagocytosis via PAF-R-mediated pathways. This study aimed to show the effect of apoptotic cell inoculation or apoptosis induction by the cytotoxic agents dacarbazine (DTIC) and cisplatin (CDDP) in the treatment of BF16F10 melanoma experimental model combined or not to the PAF-Receptor antagonist WEB2170. *In vivo,* WEB2170 alone or in association with DTIC significantly reduced tumor volume. In addition, the survival of tumor bearing mice was significantly improved by the combination of DTIC and WEB2170. *In vitro,* co-cultures of murine peritoneal macrophages with B16F10 exposed to cisplatin and WEB2170 presented more hypodiploid cells than co-cultures exposed to cisplatin alone. Immunohistochemistry showed that COX-2 and galectin-3 positive cells and microvascular density within the tumor mass were significantly reduced by treatment with WEB2170 or DTIC alone or the combination of both agents. These results suggest that PAF-R dependent pathways are activated within the tumor mass and were negatively modulated by treatment with WEB or DTIC alone or in combination. We hypothesize that PAF-R dependent pathways are activated during experimental tumor growth, modifying tumor macrophages phenotype and the microenvironment in such a way to favor tumor growth. Combination therapy with PAF-R antagonist and a chemotherapeutic drug may represent a new strategy in cancer treatment and deserves further investigation.

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Sensitivity of tumor cells to hypoxia and outcome of anti-vegf therapy

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Anti-angiogenic therapy has recently been added to the panel of cancer therapeutics. In pre-clinical models and patients as well, benefits from angiogenesis inhibitors, mainly targeted to the VEGF pathway, have generally been short-term and mechanisms of intrinsic as well as acquired resistance have been proposed. In animals, the prediction that anti-angiogenic therapy would cause tumor starvation by hitting the vasculature followed by increased hypoxia and impairment of nutrients supply has been substantially fulfilled. In patients, however, mechanisms might differ, especially considering that VEGF neutralization has therapeutic efficacy mainly if combined with conventional chemotherapy. These considerations highlight the need of more in depth information about how tumors react and adapt to angiogenesis blockade, in order to administer anti-angiogenic drugs in a smart fashion and improve therapeutic outcome.
Hypothetically, the response of tumor cells to severe hypoxia could affect the outcome of anti-angiogenic therapy, although this possibility has not been carefully investigated so far. In a previous study, we identified ovarian cancer cells endowed with different metabolic phenotypes and correlated this feature with resistance or sensitivity to severe hypoxia in vitro. These cells, which are prototypes of highly and poorly glycolytic cancer cells, have been exploited to investigate the outcome of VEGF neutralization on tumors with different glycolytic phenotypes. Results disclosed depletion of glucose levels and exhaustion of ATP in highly glycolytic tumors following short-term anti-VEGF therapy. This associated with detection of larger necrotic areas compared to controls and delayed tumor growth. In contrast, poorly glycolytic tumors were resistant to hypoxia in vitro and, in spite of comparable vascular changes, did not regress following anti-VEGF therapy in vivo. Altogether, these results describe a novel mechanism of intrinsic resistance to anti-VEGF therapy, represented by a distinct metabolic phenotype of the cancer cells.

Innovative phase I-II study of concomitant and consecutive treatment with Dasatinib and MK-0457 (Aurora kinase-inhibitor) in refractory Ph+ CML and ALL patients

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Background. MK-0457 is a pan-aurora kinase inhibitor with demonstrated activity against wild-type and mutated BCR-ABL, including the T315I form, as well as FLT3 and JAK-2. It is a promising molecule for the management of Ph+ leukemias, in which the emergence of mutations in the ABL kinase domain still represents the main mechanism of resistance to TK inhibitors. In CML and ALL patients treated with a 5-day continuous infusion of MK-0457 at doses of 24-40 mg/m2/hr every 14 days, an increased incidence of related adverse events was shown.
Aim In our Institution, an innovative Phase I clinical study of sequential and concomitant treatment with Dasatinib, previously administered for three months, and MK-0457 has been conducted. This combined activity suggests that MK-0457, in association with Dasatinib, would suppress the emergence of T315I and other resistant clone, improving upon the response rate for Dasatinib and the durability of response. The trial investigated two schedules of therapy: patients who achieved and maintained a major hematologic response after three months of therapy with Dasatinib (70 mg twice daily) received a 6-hour biweekly infusion of MK-0457 at 64 mg/m2/hr, whereas patients who failed to achieve a major hematologic response received a 5-days continuous infusion of MK-0457 at 10 mg/m2/hr, every 4 weeks. Biologically, the first schedule was demonstrated to suppress the emergence of

Dasatinib-resistant clones, through a stronger inhibition of BCR-ABL, whereas the second one was showed to inhibit more potently Aurora Kinase activity.
Results Two patients with Ph+ ALL and one patient with CML in myeloid blast crisis, previously unsuccessfully treated with imatinib, were enrolled in the protocol. The first two patients, both in hematologic response after three months of treatment with Dasatinib, subsequently received the 6-hour biweekly schedule, maintaining the haematological response. No haematological toxicity was described. The third patient, enrolled in progression disease, received the 5 days MK-0457 schedule of treatment. His peripheral blood count was consistent with a severe pancytopenia, which required frequent platelets and red blood cells transfusions. His bad clinical performance status was compromised by a severe hemorrhagic pleural effusion, responsible for moderate dyspnoea and severe asthenia. After one cycle of MK-0457, a complete recovery of the pulmonary disease and a complete hematologic response were obtained.
Conclusions The sequential and concomitant innovative administration of Dasatinib and MK-0457 represents a promising therapeutic strategy for refractory Ph+ CML and ALL, showing a relevant haematological activity in a limited number of patients. Assessment of the benefit risk profile for this combination remains to be determined.
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Gene profiling, expression and mutations of Epidermal Growth Factor Receptor (EGFR) in androgen-dependent prostate cancer.

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We analyzed mutations in Epidermal Growth Factor Receptor (EGFR) Tyrosine kinase (TK) domain, EGFR expression and gene profiling in prostate carcinoma (PC) in order to find out molecular prognostic markers and supply a rational for EGFR targeted therapies.
100 glyofix-fixed, paraffin-embedded PC specimens were recovered after radical prostatectomy from locally advanced PC patients. Exons from 18 to 21 of EGFR TK domain were amplified and sequenced. For the whole cohort, EGFR protein evaluation by immunohistochemistry was performed. Gene expression profile was analyzed on 51 out of 100 samples by whole genome microarray. Statistical tests were performed in order to detect any significant association between EGFR iperexpression and prognosis. None of 100 specimens presented mutations in exon 18; 2 point mutations were identified in exon 19, 5 in exon 20 and 6 in exon 21. In addiction, 58 out of 100 patients had the same silent mutation, at codon 787 in exon 20. EGFR iper-expression was found in 36% of specimens. Gene profiling analysis on mutated samples selected 29 modulated genes which separated significantly mutated EGFR+ from mutated EGFR- samples; 4 down-regulated genes, EAF2, ABCC4, KLK3 and ANXA3 and one up-regulated gene, FOXC1, were involved in prostate cancer progression. Our findings suggest that a subgroup of PC patients could potentially benefit of EGFR targeted therapies. The EGFR protein evaluation could contribute to identify PC relapsers. The identification of 29 genes signature may give more informations about outcome and development of new target therapies in prostate cancer.

Mast Cells and Myeloid-derived Suppressor Cells as alternative unconventional targets to treat prostate cancer

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A common feature of tumors is the ability to evade the immune response through components of the innate and adaptive immune system, which also provide trophic factors fostering tumor progression. The identification and targeting of such immune cells might provide a new therapeutic opportunity against cancer.
From TRAMP (Transgenic Adenocarcinoma of the Mouse Prostate) mice, we derived two novel cell lines that phenocopy prostate adenocarcinoma and neuroendocrine variants proper of the human counterpart, named T1525 and T23, respectively. Phenotypic and gene expression analyses indicate that T1525 has an epithelial profile, while T23 displays the molecular signature typical of epithelial-to-mesenchymal transition, a process involved in tumor invasion and metastasis. In vivo sub-cutaneus injection of the two lines shows that they enact different mechanisms to dampen the immune response: T1525 is infiltrated with mast cells (MC), while T23 induces the expansion of myeloid-derived suppressor cells (MDSC).
MC are granulocytic cells mostly known as the primary responders in allergic reactions, but that have also shown beneficial or detrimental activity on tumor growth. The in vivo progression of T1525 largely depends on MC, since this tumor fail to develop in MC-deficient Wsh mice. MC infiltration of T1525 may depend on c-Kit ligand (SCF), produced abundantly by T1525 cells as both membrane-bound and soluble molecule. To target MC in T1525 tumors, mice were treated with sodium cromoglycate, which inhibits MC degranulation. We found that sodium cromoglycate, administered daily from tumor onset, effectively delays tumor outgrowth.
Differently, T23 tumor-bearing mice are characterized by an expansion of MDSC. These cells are a heterogeneous population of immature myeloid cells, which expand, from the bone marrow, during infections and cancer and that are able to actively suppress the immune response. One of the most relevant molecules involved in MDSC expansion is VEGF, which is abundantly expressed by the T23 cell line. We are currently trying to target VEGF to hamper MDSC expansion in T23-bearing mice, by using amino-bisphosphonates. These drugs inhibit matrix-methalloprotease 9 (MMP-9), which makes tumor-produced VEGF free from extracellular matrix entrapment, and has been shown effective in reducing both MDSC and tumor progression in a transgenic model of mammary adenocarcinoma.

Targeting p53 to combat cancer: from lab bench to patient

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Rescue of the p53 tumor suppressor function by small molecules might open new therapeutic avenues against cancer. Using cell-based screens of chemical libraries we have identified several compounds which can reactivate p53 in cancer cells *in vitro* and *in vivo*. We focused our studies on the most potent and selective compounds PRIMA-1 and RITA, which have different mechanisms of action. PRIMA-1 restores the function of mutant p53 proteins by preventing their unfolding (1), whereas RITA blocks MDM-2 mediated degradation of wild type p53 (2). We have taken PRIMA-1 (commercial name APR-246) from pre-clinical level through animal toxicity testing into a clinical Phase I trial, which is going to start in Sweden in May 2009 in patients with hematological malignancies and prostate cancer. Mechanistic and pre-clinical studies on RITA are currently ongoing. An unbiased examination of the effects of RITA in cells using DNA microarrays revealed high specificity of RITA in targeting p53 (2). We detected major changes in gene expression in wild-type p53 expressing cells including the differential regulation of known p53 target genes. In contrast, no significant changes in gene expression in p53-negative HCT116 *TP53-/-* cells were detected. We uncovered a previously unrecognized role of MDM2 by showing that MDM2 released from p53 by RITA promotes degradation of p21 and the p53 cofactor hnRNPK, required for p21

transcription. Functional studies identified MDM2-dependent inhibition of p21 as a key switch regulating apoptosis induction upon p53 reactivation by RITA (2). Further, we found a potent inhibition of crucial oncogenes by RITA-activated p53. p53 unleashes transcriptional repression of anti-apoptotic proteins Mcl-1 Bcl-2, MAP4, and survivin, blocks Akt pathway on several levels and downregulates c-Myc, cyclin E and β-catenin (3). Notably, we found that MDM2 is present on p53-repressed promoters and controls p53-mediated transrepression even tighter that transactivation. In addition to targeting p53/MDM2 interaction, RITA is able to prevent p53/HDMX interaction. Thus, RITA could be useful for treatment of tumors with high expression of HDMX. Our mechanistic studies revealed that RITA binds to the N-terminal domain of p53 outside of MDM2-binding site, inducing a conformational change which prevents the binding of p53 inhibitors MDM2 and HDMX. Unexpected mechanism of p53 activation by RITA might open a way to design novel compounds with the similar mechanism. Our results emphasize the utility of targeting p53 protein itself as a promising approach for anticancer therapy.

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MicroRNA-based, p53 dependent post-transcriptional circuits: mechanisms, targets and inter-individual variation.

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The tumor suppressor p53 is a sequence specific master regulatory transcription factor that responds to a variety of environmental stresses or to DNA damage. p53 differentially regulates the expression of vast number of target genes associated with cell cycle control, apoptosis, DNA repair, angiogenesis, and other cellular processes. Approximately 50% of all cancers bear a mutation in p53. Since p53 mutant status is linked to adverse prognosis in different cancers, various intervention strategies are centered on it.
A number of recent studies identified direct p53 regulation of miRNAs and few related regulatory circuits. MiRNAs are small non-coding RNAs typically of 21-25 nucleotides in length, able to regulate gene expression either by inhibiting translation or repressing stability of target messenger RNAs. Differences in miRNA expression profiles are associated with various cancers and could contribute to tumorigenesis.
Using bioinformatics approaches, we have identified an additional group of candidate miRNAs for direct p53 transcriptional control. Furthermore, some of those miRNAs can be predicted to target mRNAs in genes relevant to p53-mediated responses. Notably, we found examples of miRNA seed binding sequences at target 3'UTRs that contain single nucleotide polymorphisms (SNPs) predicted to modulate miRNA binding. Our work aims at the validation of p53-mediated control of the newly predicted miRNA genes and related circuitries that would provide additional negative and/or positive feedback loops for p53 regulation. The validation involves assays for establishing the functionality of the predicted p53 response elements (REs) identified in miRNA promoters, the outcome of p53-dependent regulation of the miRNAs on cellular responses and the analysis of the impact of the identified SNPs. We will present results of functional assays evaluating the potential for p53, p63 and p73 transcriptional control of 15 miRNA genes, not previously described to be under control of this family of transcription factors. In particular, we employed the *delitto perfetto* approach for *in vivo* mutagenesis in the baker's yeast *S. cerevisiae*, a method that utilizes oligonucleotides and exploits homologous recombination to rapidly generate isogenic derivative strains, harboring the predicted p53 REs derived from miRNAs promoters upstream of the firefly luciferase reporter gene. Human wild type p53 or the related transcription

factors p63 e p73 are then expressed from a centromeric, selectable vector, using the inducible *GAL1,10* promoter and the transactivation potential towards the various REs is assessed by quantifying the luciferase activity.
Overall, our work aims at establishing transcriptional and post-transcriptional regulatory circuits engaged in cellular responses to stress conditions and explore the functional impact of inter-individual variation in regulatory elements within these circuits. The knowledge gain achieved would contribute to the identification and validation of new target genes and miRNAs and the rational selection of intervention strategies pivoting around the p53 transcription factor.

Differentiation therapy of rhabdomyosarcoma by myomiR-1/206

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The recent discovery that the genomic landscape of the most common cancers is highly heterogeneous has cast doubts that drugs targeting a single gene will be active against the majority of solid tumors. In this work we show that primary rhabdomyosarcomas (RMS) do not express miR-1 and that this muscle-specific microRNA, and its virtually identical paralog miR-206, fail to be induced in RMS cell lines upon serum deprivation. Moreover, re-expression of microRNA-1/206 can fully rescue differentiation of RMS cells and can block tumor growth by inducing a major switch of the global expression profile toward that of mature muscle. Finally, we show that at the onset of normal myogenesis miR-1/206 downregulates the Met receptor, which is overexpressed in RMS and has been implicated in RMS pathogenesis. We propose that tissue specific microRNAs, giver their ability to modulate hundreds of transcripts, may override genome heterogeneity and ultimately hold greater therapeutic potential for some solid tumors than single-gene directed drugs by acting as non-toxic differentiative agents.

New circuits between miRNAs and transcription factors Hoxa9, Hoxa10, Hoxa11 and Pax8: possible identification of therapeutic targets for Epithelial Ovarian Cancer treatment

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Epithelial Ovarian Cancer (EOC), the most lethal of the gynaecological neoplasms, is a morphologically and biologically heterogeneous disease. Unfortunately, the specific molecular mechanisms involved in its development are still unclear. Unlike most cancers, epithelial ovarian tumours are more differentiated than cells of the likely precursor, the ovarian surface epithelium (OSE). Moreover, the major subtypes of EOCs (undifferentiated, serous, mucinous, endometrioid, clear cell) show morphologic features that resemble those of the müllerian duct-derived epithelia of the reproductive tract.
Recent studies have shown that some transcription factors (*Hoxa9, Hoxa10, Hoxa11* and *Pax8*) which normally regulate müllerian duct differentiation, are not expressed in normal OSE, but are expressed in different EOC subtypes according to the pattern of müllerian-like differentiation of these cancers. Ectopic expression of *Hoxa9* in tumorigenic OSE cells gave rise to papillary tumors resembling serous EOCs. In contrast,

Hoxa10 and *Hoxa11* induced morphogenesis of endometrioid-like and mucinous-like EOCs, respectively.
Therefore, inappropriate activation of a molecular program that controls patterning of the female reproductive tract could explain the morphologic heterogeneity of EOCs and their assumption of müllerian-like features. Increasing numbers of homeobox genes have been found to be aberrantly expressed in a variety of haematologic malignancies and solid tumours.
Thereby, our work is aimed at testing the hypothesis that *Hoxa9-11* and *Pax8* genes are silenced in OSE cells, while differently expressed in the various subtypes of Epithelial Ovarian Cancers.
With the aim of establishing cell models representative of the different EOC subtypes, we are currently analysing the levels of *Hoxa9*, *Hoxa10*, *Hoxa11* and *Pax8* mRNAs and proteins in a panel of 12 available EOC cell lines, and comparing these levels with those in cells representative of normal ovarian surface epithelium (IOSE cells).
Presently, the processes driving aberrant expression of *Hoxa9, Hoxa10, Hoxa11* and *Pax8* genes in EOCs remain completely unknown.
Recent publications hint at a role for microRNAs (miRNAs) in regulating *Hox* expression. MiRNAs are ~21 nt regulatory RNAs that control development and differentiation acting as post-transcriptional negative regulators of the expression of key target genes. It has been shown that there is altered expression of miRNAs in several human malignancies and that miRNAs may act as oncogenes or tumour suppressors. Moreover, several miRNA genes are located in *Hox* clusters.
We are trying thereby to define the role of miRNAs in ovarian cancer biology and to generate functional data with respect to these miRNAs, taking advantage of a miRNA microarray profiling. We are testing the hypothesis that miRNAs normally silence *Hoxa9-11* and *Pax8* genes in OSE cells, and are differently de-regulated in different subtypes of Epithelial Ovarian Cancers, focusing our attention on the study of 19 miRNAs that we predicted *in silico* to downregulate the expression of genes *Hoxa9*, *Hoxa10*, *Hoxa11* and *Pax8*.
Based on the outcome of this analysis, we will further investigate the possibility of restoring miRNAs transcriptional balance using two different strategies: in case of miRNAs overexpression, we plan to decrease the miRNAs levels by specific antagomirs; in case of miRNAs downregulation we will clone the miRNA and transfect it by gene delivery vectors.
The possibility that the transcription factors under study take part in regulatory networks involving some of the miRNAs will also be explored.

Tumor interaction with the nervous system

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The neuro-endocrine system is the superordinate regulatory organ of the body. The regulatory effects range from systemic, long-term effects, which are basically caused by hormones, to localized, tissue- or organ-specific short-term effects, which are mediated by neurotransmitters. The function of hormones in tumor growth is already well characterized and still a centre issue of cancer research, whereas in contrast the role of neurotransmitters in tumor growth and progression has been much less investigated so far. We have shown *in vitro* by our collagen-based three-dimensional migration assay and *in vivo* by experiments with athymic BALB/c nude mice, that various neurotransmitters enhance the migratory activity of carcinoma cells from breast, prostate and colon tissue origin [1], and promote the development of lymph node metastases [2], with the stress-related neurotransmitter norepinephrine being the most effective one. Being aware of these effects the resulting question is, how neurotransmitters are delivered to tumor tissue. Almost 40 years ago, the observation has been made that tumors release substances that initialize their vascularisation (neoangiogenesis) [3]. Only recently, an analogous mechanism has been demonstrated for the development of lymph vessels in tumors (lymphangiogenesis), and

both of these processes are supposed not only to facilitate tumor growth (because of sufficient nutrition), but also to relieve metastasis formation [4]. The innervation of growing tissues is regulated by the same and additionally related signal substances as those regulating neoangiogenesis and lymphangiogenesis. Our investigations show that tumor cells release a bunch of nerve growth factors, which have effects on neuronal-like cells towards innervation, *i.e.* the development of long neurites and chemotactic orientation towards the source of release. In summary, a blockade of interactions of tumor cells with the nervous system can be achieved in two ways: on the one hand, neurotransmitters receptors can be blocked on tumor cells, and on the other hand, the innervation of tumors can be inhibited by the blockade of the responsible growth factor receptors. The inhibition of the interaction of tumor cells with the nervous system or its signal substances might display an unconventional therapeutic target for the delay or inhibition of metastasis formation.

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Circulating tumor cells (CTCs) in metastatic renal cancer (mRCC): a Pilot Study in Sunitinib treated patients

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Background: Haematogenous spreading of tumor cells is a key step toward metastasis; the automated analysis of CTC by CellSearch platform represents an exciting improvement to serially investigate and accurately quantify these rare cells. At present, CTC count is indicated in the follow-up of metastatic breast cancer, colon rectal cancer and prostate cancer, so that cut-off values are now defined, predicting high risk of recurrence in metastatic disease. Moreover, variation in CTC can indicate a significant change in prognosis as early as the first treatment cycle and throughout the continuum of care. Preliminary reports indicate that CTC are present in patients with various metastatic carcinomas of epithelial origin with a wide range of incidences and frequencies but lack at presents extensively analysis of renal cell carcinoma (RCC) patients.
Until recently, patients with advanced, unresectable or metastatic RCC had very few therapeutic options. Cytokine therapy, consisting mainly of interferon-α and interleukin-2, was considered the mainstay of therapy. A better understanding of the biology of RCC has led to the development of novel therapeutic agents that target angiogenesis. Sunitinib, a new multitargeted tyrosine-kinase inhibitor (TKI), has shown high activity in RCC and is now widely used for patients

with metastatic disease. Sunitinib inhibits the vascular endothelial growth factor (VEGF) receptor and other tyrosine kinases, including the platelet-derived growth factor (PDGF) and c-kit receptor at nanomolar concentrations. First-line Sunitinib improves survival in patients with metastatic renal cancer patients, but no validated predictive markers of response are currently available.
Methods: Since the antiangiogenic properties of Sunitinib would suggest interference with extravasation and circulation of CTCs, we designed a pilot study to evaluate CTC count fluctuations and their correlation with radiological response in previously untreated patients with metastatic RCC. Blood draw will be collected at day 1 and 28 of the first two cycles, and sequentially at day 1 of the III, V and VII cycles of treatment or at progression of disease. The first clinical objective of the study is to correlate CTC count with major prognostic factors determined at diagnosis. To provide an integrated view of the angiogenic state in Sunitinib patients, data obtained with the CTC assay will be combined with measurement of circulating endothelial cells (CECs) by CellSearch. To gain further information on the biologic significance of CTC in patients with RCC we will also characterize the phenotypic profile of these cells, firstly regarding their metastatic potential.
Results and conclusions: The study started at may-2008. Data obtained in small group advanced/metastatic RCC patients indicate that:

- over 80% of RCC samples present CTC; no CTC were detected in healthy donors
- in the first eight evaluable pts CTC count correlated with radiological response;
- anti-M30 immunostaining seems to be feasible tool for apoptotic CTC monitoring during chemotherapy
- Although consensus on the phenotypic definition of CECs as well as on the optimal enumeration technique is still lacking, automated isolation and staining of these cells by CellSearch platform, which identify DAPI+CD105+CD146+CD45- cells seems to be useful tool to serially monitoring mature endothelial cells throughout the continuum of care.

Targeted Treatment of Malignant Tumors with the TGF-beta 2 Inhibitor Trabedersen (AP 12009)

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The current immunotherapeutic approaches against tumors concentrate on stimulating the immune system. Vaccination based on cancer-associated antigens is frequently used and alternative efforts rely on the improvement of antigen presentation, e.g. via dendritic cells. To overcome tumor-induced immunosuppression we focus on the blockade of transforming growth factor beta 2 (TGF-ß2) formation via the antisense oligonucleotide trabedersen (AP 12009). In highly aggressive tumors such as malignant brain tumors or pancreatic carcinoma TGF-ß2 promotes malignant tumor progression and metastasis via multiple mechanisms. Most importantly, TGF-ß2 induces escape from immunosurveillance. Therefore, blocking TGF-ß2 formation enables reactivation of antitumor responses of the immune system. The clinical development of trabedersen started in patients with high-grade gliomas (recurrent/refractory glioblastoma multiforme, GBM WHO grade IV and recurrent/ refractory anaplastic astrocytoma, AA WHO grade III). Trabedersen is infused directly into brain tumors via convection enhanced delivery (CED).
Three Phase I/II dose-escalation studies proved the safety and tolerability of trabedersen treatment. Furthermore, long-lasting tumor remissions were observed. In a multinational, randomized and active-controlled Phase IIb study trabedersen was superior in patients with AA compared to standard chemotherapy, with a significantly better progression rate at 14 months, a median overall survival benefit of 17.4 months and a two-fold higher 2-year survival rate (83.3 vs. 41.7%).
In GBM patients, trabedersen was as efficacious as standard chemotherapy and in a subgroup with prespecified prognostic factors (age ≤55 years, KPS >80%), trabedersen patients had a markedly higher 2-year survival rate compared to standard chemotherapy (44.4 vs. 13.3%).
A pivotal, randomized and active-controlled Phase III study named SAPPHIRE in patients with recurrent or refractory AA has started

recently and patient enrollment is ongoing.
A Phase III clinical study in patients with recurrent or refractory GBM is currently being prepared.
Intravenous application of trabedersen as 2nd to 4th line treatment in patients with advanced pancreatic cancer, malignant melanoma, and colorectal cancer is investigated in an ongoing Phase I/II dose-escalation study. Interim results underline that trabedersen is safe and well tolerated and very encouraging first efficacy results were observed. Further 24 patients with pancreatic carcinoma or malignant melanoma are currently being enrolled for the treatment with a defined dose.
In conclusion, trabedersen is a very promising new drug for the targeted and multi-modal treatment of patients with malignant tumors overexpressing TGF-ß2.

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2010 Pezcoller Foundation-AACR International Award for Cancer Research

The prestigious Pezcoller Foundation-AACR International Award for Cancer Research was established in 1997 to annually recognize a scientist:

- who has made a major scientific discovery in basic cancer research OR who has made significant contributions to translational cancer research;
- who continues to be active in cancer research and has a record of recent, noteworthy publications; and
- whose ongoing work holds promise for continued substantive contributions to progress in the field of cancer.

The Award is intended to honor an individual scientist. However, more than one scientist may be co-nominated and selected to share the Award when their investigations are closely related in subject matter and have resulted in work that is worthy of the Award. In the rare event that there are dual winners of the Award, the cash award will be shared equally between them, and the AACR Executive Committee will determine which of the two co-recipients will present the Pezcoller-AACR Award Lecture at the AACR Annual Meeting.

Candidates for the Award will be considered by a prestigious international Selection Committee of renowned cancer leaders appointed by the President of the AACR and the Council of the Pezcoller Foundation. The Committee will consider all nominations as they have been submitted; the Committee may not combine submitted nominations, add a new candidate to a submitted nomination, or otherwise make alterations to the submitted nominations. After careful deliberations by the Committee, its recommendations will be forwarded to the Executive Committee of the AACR and the Council of the Pezcoller Foundation for final consideration and determination.

Selection of the Award winner will be made on the basis of the candidate's scientific accomplishments. No regard will be given to race, gender, nationality, or religious or political view.

The Pezcoller Foundation was established in 1980 by Professor Alessio Pezcoller, a dedicated Italian surgeon who made important contributions to medicine during his career and who, through his foresight vision and generous gift in support of the formation of the Foundation, stimulated others to make significant advances in cancer research. Previously the Pezcoller Foundation, gave a major biennial award for outstanding contributions to cancer and cancer-related biomedical science, in collaboration with the ESO-European School of Oncology.

The American Association for Cancer Research (AACR) was founded in 1907 by eleven physicians and scientists dedicated to the conquest of cancer and now has over 28,000 laboratory, translational, clinical and epidemiological scientists engaged in all areas of cancer research in the United States and in more than 80 other countries around the world.

The AACR is dedicated to its mission of preventing and curing cancer through the communication of important scientific results in a variety of forums including publications, meetings and training and educational programs. Because of the commitment of the Pezcoller Foundation and the AACR to scientific excellence in cancer research, these organizations are now collaborating annually on the presentation of the Award. This will strengthen international collaborations and will be a catalyst for advancements in cancer research internationally.

The winner of the Pezcoller Foundation-AACR International Award for Cancer Research will

give an Award lecture during the AACR Annual Meeting 2010 in Washington, DC (April 17-21, 2010), and the memorial Korsmeyer Lecture at the VIMM in Padua and will receive the award in a ceremony at the Foundation's headquarters in Trento, Italy (May, 2010). The award consists of a prize of € 75.000 and a commemorative plaque.

Nomination Deadline: September 15, 2009

Questions about the nomination process: Monique P. Eversley, Staff Associate - American Association for Cancer Research, 17th Floor, 615 Chestnut Street, Philadelphia, PA 19106-4404 - Tel. +1 (267) 646-0576; E.mail: eversley@aacr.org - www.aacr.org

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