Summary

- Editorial June 2010
- 22nd Pezcoller Symposium:
  Abstracts of oral presentations
  Abstracts of posters
- Call for: 2011 Pezcoller Foundation-AACR
  International Award for Cancer Research
June 2010

Dr. Joseph Schlessinger - William H. Prusoff, Professor and Chair of Pharmacology, Yale School of Medicine - is the recipient of the 2010 Pezcoller Foundation-AACR Award for Cancer Research.

This year the Selection Committee met in Trento on November 22, 2009 and was composed of: Dr. Giampaolo Tortora, Chairperson, Professor, Division of Medical Oncology, University of Naples Medical School, Federico II; Dr. Alberto Amadori, Department of Oncology and Surgical Sciences, University of Padova; Prof. David J. Kerr, Rhodes Professor of Therapeutics, Department of Clinical Pharmacology, University of Oxford; Dr. Sylvie Ménard, Department of Experimental Oncology, Molecular Targeting Unit, Istituto Nazionale Tumori, Milan; Dr. David Sidransky, Lab Director, Otolaryngology, Johns Hopkins Medical Institutions, Baltimore; Michael A. Calliguri, Professor of Cancer Research, The Ohio State University, Columbus; Lisa M. Coussens, Professor of Pathology, Cancer Research Institute, San Francisco; Danny R. Welch, Professor of Pathology, Cell Biology, and Pharmacology & Toxicology at the University of Alabama, Birmingham.

Schlessinger has an exceptional record of superb research accomplishments. With a creative and elegant experimental approach he achieved groundbreaking and field-defining fundamental discoveries in cancer research. His group discovered the main mechanisms of activation of membrane growth factor receptors, such as receptor dimerization and autophosphorylation, identified adaptor proteins that allow the flow of information from activated receptors to intracellular compartments, and defined the functioning of several tyrosine kinase receptors at atomic resolution, providing the conceptual foundation for identifying new tyrosine kinase inhibitors. Prof. Schlessinger was also highly successful in translating these findings into clinical practice designing drugs, including sunitinib and PLX-4032, which have already impacted patient treatments.

Joseph Schlessinger was introduced at the 2010 AACR Annual Meeting in Washington where he delivered the Pezcoller Lecture: “Cell Signaling by Receptor Tyrosine Kinase: From Basic Principles to Cancer Therapy” to a large audience.

On May 7, 2010 Schlessinger was given the Award during a solemn ceremony in the prestigious reception hall of the Buon Consiglio Castle in Trento, Italy. In the same period of time he also gave the Korsmeyer Lecture in Padova at VIMM to honor the memory of the late Stanley Korsmeyer who received the Pezcoller-AACR in 2004 and the Tecce Lecture in Rome at the “Regina Elena” Cancer Institute.

The 22nd Pezcoller Symposium entitled “RNA Biology and Cancer” will be held in Trento from June 10 to June 12, 2010.

The program of the Symposium has been developed by the Pezcoller Program Committee: Dr Enrico Mihich (Dana Farber Cancer Institute, Boston), Dr David Livingston (Dana Farber Cancer Institute, Boston) Dr René Bernards (National Cancer Institute, Amsterdam) and Dr Witold Filipowicz (Friedrich Miescher Institute for Biomedical Research, Basel. The focus of the Symposium will be on the functional importance of various non-coding RNA molecules in cancer.

The topics to be discussed will include the opportunities offered by small RNA as tools for cancer drug development, the role of non-coding RNAs, the biochemistry of small RNAs, the function of micro-RNA in cancer, and RNAs as diagnostics and therapeutics in cancer.

The speakers are: Reuven Agami, The Netherlands Cancer Institute, Amsterdam; Alan Ashworth, Breakthrough Research Centre, London; René Bernards, National Cancer Institute, Amsterdam; Irene Bozzoni, University La Sapienza, Rome; Dalia Cohen, Rosetta Genomics, Philadelphia; CarloCroce, Ohio State University, Columbus; Witold Filipowicz, Friedrich Miescher Institute for Biomedical Research, Basel; Manel Esteller, Spanish National Cancer Center, Madrid; Scott Hammond, University of North Carolina, Chapel Hill; Gunther Hartmann, University of Bonn; Eva Hernandez, New York University Langone Medical Center, New York; William Kaelin, Dana Farber Cancer Institute, Boston; Sakari Kaupinnen, Santaris Pharma a/s, Copenhagen; Wilhelm Krek, Institute of Cell Biology, Zurich; Anna Krichevsky, Brigham and Women’s Hospital, Boston; Joachim Lingner, Swiss Institute for Experimental Cancer Research (ISREC), Lausanne; David Livingston, Dana Farber Cancer Institute, Boston; Yi Liu, UT Southwestern Medical Centre, Dallas; Gunter Meister, Max-Planck Institute of Biochemistry, Martinsried; Enrico Mihich, Dana Farber Cancer Institute, Boston; Luigi Naldini, San Raffaele Telethon Institute for Gene Therapy (HSRTIGET), Milan; Pier Paolo Pandolfi, Harvard Medical School, Boston; Marco Pierattti, Istit Nazionale Tumori, Milan; Carola Ponzetto, University of Torino; Frank Slack, Yale University, New Haven; Nadia Zaffaroni, Istituto Nazionale Tumori, Milan; Lars Zender, Hannover Medical School, Hannover.

The abstracts of this symposium are in the following pages.

Gios Bernardi
Editor and Pezcoller Foundation President

Picture on the front page: 2010 Pezcoller Foundation AACR Award for Cancer Research, from the left J. Schlessinger, winner; E. Blackburne, Nobel Prize, G. Bernardi, Pezcoller Foundation President
RNAi screens to discover potential new therapeutic targets and biomarkers of response

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We have been using high- and medium-throughput RNAi screens to uncover new therapeutic targets for cancer as well as biomarkers of patients who might respond to specific treatments. In particular I will discuss the integration of genomic, gene expression and RNAi data to generate functional maps of cancer cell lines.

Understanding mechanism of drug resistance through RNAi based genetic screens

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Unresponsiveness to therapy remains a significant problem in the treatment of cancer, also with the new classes of cancer drugs. In my laboratory, we use functional genetic approaches to identify biomarkers that can predict responsiveness to targeted cancer therapeutics; drugs that specifically inhibit molecules or pathways that are often activated in cancer. These drugs are often highly selective for the cancer cells that harbor these activated pathways. Nevertheless, it remains poorly explained why a significant number of tumors do not respond to these therapies. We aim to elucidate the molecular pathways that contribute to unresponsiveness to targeted cancer therapeutics using a functional genetic approach. This will yield biomarkers that may be useful to predict how individual patients will respond to these drugs. Furthermore, this work may allow the development of drugs that act in synergy with the established drug to prevent or overcome drug resistance. To identify biomarkers that control tumor cell responsiveness to cancer therapeutics, we use multiple complementary approaches. First, we use genome wide loss-of-function genetic screens (with shRNA interference libraries) in cancer cells that are sensitive to the drug-of-interest to search for genes whose down-regulation confers resistance to the drug-of-interest (resistance screens). In addition, we use single well siRNA screens with a low dose of the drug to screen for genes whose inhibition enhances the toxicity of the cancer drug (sensitizer screens). As a third approach, we use gain of function genetic screens in which we search for genes whose over-expression modulates drug responsiveness. Once we have identified candidate drug response biomarkers in
relevant cell line models, we ask if the expression of these genes is correlated with clinical response to the drug-of-interest. For this, we use tumor samples of cancer patients treated with the drug in question and whose response to therapy is documented. Examples of genetic screens to identify mechanisms of resistance to different cancer drugs will be presented.

References:


Epigenetic control of miRNAs involved in molecular circuitries with a relevant function in Duchenne Muscular Dystrophy pathogenesis

Cacchiarelli D., Martone J., Girardi E., Cesana M., Incitti T., Morlando M. and Bozzoni I. Department of Genetics and Molecular Biology, “Sapienza” University, Rome.

miRNAs are recognized as important regulators of gene expression in the differentiation commitment of several cell types and have been shown to occupy very high hierarchical positions in the cascade of regulatory events controlling cell specification. Proper muscle tissue development and function was also described to depend on the controlled expression of specific families of miRNAs; in several cases it has been also shown that their ectopic expression can direct cells towards specific differentiation programmes. Moreover, altered levels of miRNAs were found in several muscular disorders such as myocardial infarction, Duchenne Muscular Dystrophy and other myopathies.

Duchenne Muscular Dystrophy is a severe genetic disorder caused by mutations in the dystrophin gene. The disruption of the Dystrophin-Associated Protein Complex at the muscle membrane, due to dystrophin deficiency, represents the primary event that leads to the disease pathogenesis: muscle fiber breakage and degeneration is then accompanied by a complex series of events including activation of satellite cells, inflammatory infiltration and intense fibrosis. Even though a cure is not yet available, several different therapeutic strategies are nowadays entering human experimentation. In particular, exon skipping has been proven to be very powerful in restoring dystrophin expression and conferring benefit in animal models. Surprisingly, the beneficial effects observed on muscle function and morphology could be obtained also with low levels of protein rescue, suggesting that partial protein re-localization to the membrane could be only part of the story.

Taking advantage of a controlled rescue of dystrophin synthesis through exon skipping in mdx mice, we discovered that molecular circuitries, important for muscle differentiation and tissue integrity, are directly controlled by dystrophin through epigenetic modulation of a specific class of miRNAs.

MicroRNAs in Diagnostics and Therapeutics

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In cancer, different microRNA (miRs) expression patterns have been shown to be associated with different tumor types. Due to their high tissue specificity and their involvement in different biological processes, miRs can serve as a basis for the development of diagnostic tools and for a novel class
of therapeutic targets. Hepatocellular carcinomoma (HCC) is the fifth most common type of cancer in the world and new therapeutic interventions are of highly unmet medical need. Expression profiling of miRs was performed on RNA extracted from liver tissue samples of HCC patients and compared to matched adjacent liver samples to identify differentially expressed miRs. Hsa-miR-191 was found to be a potential drug target for HCC. Inhibition of this miR caused decrease cell proliferation and induction of apoptosis in vitro and a significant reduction of tumor mass in vivo in an orthotopic liver xenograft model. This miR was also found to be up-regulated by dioxin, a known liver carcinogen and to be a key regulator of cancer related pathways. In addition to the potential of miRs as novel drug targets, they can also serve as diagnostic and prognostic biomarkers.

Microrna Dysregulation in Cancer

Causes and Consequences of Microrna Dysregulation in Cancer

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During the past several years it has become clear that alterations in the expression of microRNA genes contribute to the pathogenesis of most, perhaps all, human malignancies. These alterations can be caused by a variety of mechanisms, including deletions, amplifications or mutations involving microRNA loci, by epigenetic silencing or by dysregulation of transcription factors targeting specific microRNAs. Since malignant cells show dependence on the dysregulated expression of microRNA genes, which in turn control or are controlled by dysregulation of multiple protein coding oncogenes or tumor suppressor genes, these small RNAs provide important opportunities for development of future microRNA based therapies.

Epigenetic and genetic disruption of ncRNAs in cancer

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MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression by targeting messenger RNA (mRNA) transcripts. miRNAs play important roles in several cellular processes, such as proliferation, differentiation, apoptosis, and development, by simultaneously controlling the expression levels of hundreds of genes. In human cancer, miRNA expression profiles differ between normal tissues and derived tumors and between tumor types and it has been shown that miRNAs can act as oncogenes or tumor suppressors. Importantly, an miRNA expression profile of human tumors has emerged that is characterized by a defect in miRNA production and global miRNA downregulation. Recent studies have shed some light on possible mechanisms that could explain this miRNA deregulation in cancer: failure of miRNA posttranscriptional regulation, CpG island promoter hypermethylation-associated transcriptional silencing (Lujambio et al., 2007), transcriptional repression by oncogenic factors and mutational impairment of the TARBP2 miRNA processing gene (Melo et al., 2009). This last finding is particularly relevant because if enzymes and cofactors involved in miRNA processing pathways are themselves targets of genetic disruption, they might represent a new class of tumor suppressor genes. Recent studies have also linked different sets of miRNAs to metastasis through either the promotion or suppression of this malignant process. Interestingly, epigenetic silencing of miRNAs with tumor suppressor features by CpG island hypermethylation is also emerging as a common hallmark of human tumors. Thus,
we wondered whether there was a miRNA hypermethylation profile characteristic of human metastasis (Lujambio et al., 2008). We used a pharmacological and genomic approach to reveal this aberrant epigenetic silencing program by treating lymph node metastatic cancer cells with a DNA demethylating agent followed by hybridization to an expression microarray. Among the miRNAs that were reactivated upon drug treatment, miR-148a, miR-34b/c, and miR-9 were found to undergo specific hypermethylation-associated silencing in cancer cells compared with normal tissues. The reintroduction of miR-148a and miR-34b/c in cancer cells with epigenetic inactivation inhibited their motility, reduced tumor growth, and inhibited metastasis formation in xenograft models, with an associated down-regulation of the miRNA oncogenic target genes, such as C-MYC, E2F3, CDK6, and TGF2. Most important, the involvement of miR-148a, miR-34b/c, and miR-9 hypermethylation in metastasis formation was also suggested in human primary malignancies because it was significantly associated with the appearance of lymph node metastasis. Our findings indicate that DNA methylation-associated silencing of tumor suppressor miRNAs contributes to the development of human cancer metastasis (Lujambio and Esteller, 2009).

References

Mechanisms and Regulation of miRNA Repression and miRNA metabolism in Mammalian Cells

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miRNAs regulate gene expression post-transcriptionally by causing translational repression or mRNA degradation. Mature miRNAs are incorporated into RNP complexes, miRNPs, which are responsible for silencing of mRNA targets, but mechanistic details of how miRNPs repress protein synthesis are still poorly understood. Proteins of the GW182 family emerged recently as particularly important factors involved in miRNA repression in metazoans. They directly interact with Ago proteins, key components of miRNPs, and also form part of P-bodies, structures implicated in translational repression and mRNA degradation. Deletion analysis of the human GW182 protein TNRC6c and Drosophila dGW182 revealed that their C-terminal fragments encompassing DUF and RRM domains act as potent mediators of both translational repression and mRNA decay. We will discuss the progress on characterization of the features of these C-terminal regions responsible for repression of protein synthesis and factors mediating their function. More recently, we also initiated projects aimed towards characterization of miRNAs regulated during light and dark adaptation in the mouse retina (projects carried out in collaboration with Dr. Botond Roska of the Friedrich Miescher Institute). We have identified miRNAs regulated by different light levels in the retina, independent of circadian time. We also characterized mRNA targets which are controlled by the light-regulated miRNAs in vivo. We will discuss possible mechanisms which underly the light-mediated regulation of miRNA levels in retinal neurons. We will also discuss our recent data on the metabolism of miRNAs in hippocampal and cortical neurons.

Post-transcriptional regulation of microRNAs

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MicroRNAs (miRNAs) are short, non-coding RNAs that post-transcriptionally regulate gene expression. Over 900 miRNA genes have been identified in the human genome. We have undertaken the study of miRNA function in mammals. Using a custom microarray platform, we investigated miRNA expression patterns in mammalian development and in cancer
(Thomson et al. 2004). In early development, several families of miRNA are highly expressed. This includes the oncogenic cluster miR-17-92 (He et al. 2005). Regulation of this cluster occurs largely at the level of transcription, with E2F and Myc playing a critical role (Woods et al. 2007). A second group of miRNA are not expressed in early development, but are induced strongly during mid-gestation. This includes tissue restricted miRNAs, and also widely expressed miRNAs in the Let-7 and miR-125 families. These same miRNAs are often reduced in expression in cancer. We have investigated the regulation of these miRNAs in development and in cancer (Thomson et al. 2006). Let-7 biogenesis is regulated post-transcriptionally at distinct steps. A major component of this regulation is via the RNA binding protein Lin28 (Newman et al. 2008). We have characterized the binding site for Lin28 on the Let-7 stem loop. We have defined the regulatory mechanism of Lin28 action on Let-7 biogenesis. This work establishes a post-transcriptional regulatory network for tumor suppressor miRNAs.


RIG-I drives immunogenic tumor cell apoptosis

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Effective tumor therapy requires selective elimination of tumor cells while keeping normal cells intact. One situation in which selective killing of self cells in mandatory is viral infection. The recent literature shows a dramatic increase of knowledge how viral infection is detected in the cytosol, and how this detection is translated into specific killing of virally-infected cells. Detection of virus calls outside and inside processes that enable our body to eliminate virus-infected cells: from outside, it guides antigen-specific as well as innate effector cells to the target virus-infected cells, and from inside, proapoptotic signaling decreases the threshold of virus-infected cells to undergo apoptosis. A picture now evolves in which this recent insight in the antiviral defense mechanisms can be exploited to develop strategies that allow to specifically eliminate tumor cells in the same way as they would be virally-infected cells. Thus, micking viral infection becomes an attractive approach to significantly advance tumor therapy.

The innate immune system detects viruses based on the recognition of viral nucleic acids. Immunorecognition of viral nucleic acids leads to the initiation of early antiviral immune responses that limit viral replicaton and are essential for eliciting acquired immune responses to virus specific antigens. Receptors involved in immunorecognition of viral nucleic acids include four members of the family of toll-like receptors (TLR3, TLR7, TLR8 and TLR9) which are localized in the outer cell membrane (TLR3) or the endosomal membrane (TLR7, TLR8, TLR9). While those TLRs are preferentially expressed in immune cell subsets (except TLR3), all cells are capable of recognizing the presence of viral nucleic acids in the cytosol. Detection of viral nucleic acids in the cytosol is based on the family of the so-called RIG-I-like receptors (RLRs). Of all RLRs most is known about the ligand of RIG-I. We found that RNA with a triphosphate group at the 5´end (3pRNA) is the ligand for RIG-I, and that the helicase domain is required for binding of 3pRNA (1). In the literature, the exact structure of RNA activating RIG-I is controversial. We established a chemical approach for 5´triphosphate

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oligoribonucleotide synthesis and found that short double strand conformation with base pairing of the nucleoside carrying the 5’ triphosphate was required for recognition by RIG-I. RIG-I ligand binding and activation was impaired by a 3’ overhang at the 5’ triphosphate end. These results explain how RIG-I detects negative strand RNA viruses which lack long double-stranded RNA but do contain panhandle blunt short double-stranded 5’ triphosphate RNA in their single-stranded genome (2). Recently we were able to resolve the molecular structure of the RIG-I-RNA interaction by studying crystals of RIG-I and RNA (unpublished).

With regard to function we demonstrate that RIG-I-RNA ligand interaction not only activates type I IFN, but also induces inflammasome activation (3) and pro-apoptotic signaling (4). Furthermore we found that RIG-I activation in primary ovarian cancer cells induces immunogenic tumor cell death (unpublished). Based on these activities, RIG-I ligands are promising candidates for the therapy of viral infection and cancer. We developed short interfering RNA (siRNA) containing triphosphate groups at the 5′ ends (3p-siRNA) for the therapy of melanoma. The 3p-siRNA used comprises two distinct and independent functional activities in one molecule: silencing of anti-apoptotic bcl-2 and activation of the cytosolic helicase RIG-I. Systemic treatment with bcl-2-specific 3p-siRNA elicited strong anti-tumor activity in a metastatic melanoma model. Like TLR agonists, RIG-I ligation by 3p-siRNA activated innate immune cells such as dendritic cells; unlike TLR agonists, activation of RIG-I directly induced a type I IFN response and apoptosis in murine and human tumor cells; RIG-I-induced apoptosis of tumor cells synergized with apoptosis induced by siRNA-mediated silencing of bcl-2 in tumor cells. In vivo, these mechanisms acted in concert to provoke massive apoptosis of tumor cells in lung metastases. The overall therapeutic activity of 3p-siRNA in vivo required NK cells, type I IFN and silencing of bcl-2 as evidenced by downregulation of bcl-2 protein in tumor cells in vivo (5). Thus, 3p-siRNA represents a novel single molecule-based combinatorial approach for tumor therapy that makes use of immunological and gene silencing properties of RNA oligonucleotides.

Without the knowledge of how to mimic viral infection, the natural and most powerful way to eliminate unwanted self cells, tumor therapy must have failed in the past, and there is great hope that with this recent insight, a new era of effective tumor therapies is just about to take off.

1. Hornung V, ... and Hartmann G. 5′-Triphosphate RNA Is the Ligand for RIG-I. Science 2006; Nov 10; 314:994-7.
Melanoma incidence and associated mortality continue to increase worldwide. Outcomes for metastatic melanoma patients remain very poor, and the current standard-of-care treatment options consistently fail to demonstrate efficacy in terms of overall survival. The lack of progress in drug development for stage IV melanoma is due, at least in part, to an incomplete characterization of the molecular mechanisms that initiate and promote metastasis. Recent evidence indicates that microRNAs (miRNAs) play a role in melanoma progression. Using miRNA arrays, we identified miRNAs 30b and 30d, which form a cluster in chromosome 8q24, as overexpressed in metastatic melanoma, with higher levels correlating with increased stage and recurrence rate in primary tumors. Functional assays demonstrated that ectopic expression of miR-30b or miR-30d in vitro enhances the invasive capacity of melanoma cells without affecting proliferation. In vivo studies revealed that transient downregulation of miR-30d represses the metastatic capacity of B16/F10 cells, whereas miR-30d ectopic expression produces the converse effect. Transcriptional profile of miR-30d-transduced melanoma cells evidenced alterations in predicted targets involved in cell-to-cell adhesion and extracellular matrix remodeling. CESLR3, TWF1 and GALNT7 were found direct miR-30d targets by reporter assays, and silencing of the glycosyl transferase GALNT7 mimicked miR-30d pro-invasive effects. Moreover, miR-30d upregulation in melanoma cells enhanced STAT3 phosphorylation correlating with increased IL10 synthesis and secretion. In accordance with the immunosuppressive effects of IL10, B16F10/miR30d cells recruited significantly less activated DCs and more regulatory T cells and myeloid-derived suppressor cells (MDSCs) to the metastatic site in vivo. Our results unravel a complex miRNA-mediated mechanism of invasion and immune modulation underlying melanoma metastasis that might have important therapeutic implications.

Terra-Telomeric Repeat Containing Rna

Alix Christen, Sascha Feuerhahn, Nahid Iglesias, Brian Luke, Andrea Panza, Antonio Porro, Sophie Redon, Patrick Reichenbach, Joachim Lingner

The ends of eukaryotic chromosomes, known as telomeres, function as guardians of genome stability, cellular clocks and tumor suppressors. Telomeres consist of simple repetitive DNA repeats and specialized proteins that are crucial for telomere function. In addition, as we discovered recently in eutherian mammals and subsequently in the yeast Saccharomyces cerevisiae, telomeres contain a large noncoding RNA termed TERRA (for telomeric repeat containing RNA). TERRA transcription occurs by RNA polymerase II at several are all chromosome ends from the subtelomeric region towards the ends of chromosomes. It has been proposed that TERRA may regulate telomere chromatin structure. In addition, TERRA may regulate telomerase. Furthermore, TERRA may interfere with replication of telomeric DNA when upregulated. We have investigated the roles of TERRA in telomerase control. We demonstrate that endogenous TERRA is bound to human telomerase in cell extracts. Using in vitro reconstituted telomerase and synthetic TERRA molecules we demonstrate that TERRA binds to the RNA template sequence of the telomerase RNA moiety (hTR). Binding to the template involves the 5′-UUAGGG-3′ repeats which are present near TERRA 3′ ends. In addition we demonstrate that TERRA binds to the telomerase reverse transcriptase (TERT) protein subunit independently of hTR. In vitro studies demonstrate that TERRA acts as a potent competitive inhibitor for telomeric DNA in addition to exerting an uncompetitive mode of inhibition. Our data identify TERRA as a telomerase ligand and natural direct inhibitor of human telomerase. Telomerase regulation by the telomere substrate may be mediated via its transcription.

Diverse small RNA biogenesis pathways in fungi

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RNA interference pathways use small RNAs
to mediate gene silencing in eukaryotes. In addition to small interfering RNAs (siRNA) and microRNAs, several types of endogenously produced small RNAs play important roles in gene regulation. In the filamentous fungus *Neurospora*, we discovered qiRNA, a type of small RNA that is induced after DNA damage. Production of qiRNAs requires the RdRP QDE-1, the Werner/Bloom RecQ DNA helicase homolog QDE-3 and dicers. qiRNA biogenesis also requires DNA damage-induced aRNAs as precursor. Surprisingly, our results suggest that the RdRP QDE-1 is the DNA-dependent RNA polymerase that produces aRNAs. By comprehensively analyzing small RNA associated with the Argonaute protein QDE-2, we also show that diverse pathways generate miRNA-like small RNAs (milRNAs) and Dicer-independent small interfering RNAs (disiRNAs) in *Neurospora*. milRNAs are processed by at least four different mechanisms that use a distinct combination of factors, including Dicers, QDE-2, the exonuclease QIP and a novel RNase III domain-containing protein MRPL3. In contrast, disiRNAs originate from loci producing overlapping sense and antisense transcripts, and do not require any of the known RNAi pathway components for their production. Taken together, these results uncover several novel pathways for small RNA production in filamentous fungi, shedding light on the diversity and evolutionary origins of eukaryotic small RNAs.

MicroRNAs In Cancer

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Summary: We are focused on the role of let-7 and other microRNA (miRNAs) in regulating proto-oncogene expression during development and cancer, and on using miRNAs to diagnose and suppress tumorigenesis.

Background: miRNAs are a large family of small regulatory RNAs found in multicellular eukaryotes, including humans that regulate gene expression to control important aspects of development and metabolism such as cell differentiation, apoptosis and lifespan. miRNAs, while poorly understood, have been shown to be important regulators of development and even implicated in human cancers [1]. miRNAs control gene expression through complementary elements in the 3' UTRs of target miRNAs [2]. let-7, a founding member of the miRNAs is required for timing of the developmental switch from larval to adult cell fates in C. elegans [3]. The let-7 miRNA is phylogenetically conserved and temporally expressed in many animals [4] and human let-7 has been implicated in cancer. We showed that the let-7 family of miRNAs controls expression of RAS, through sequences in its 3'untranslated region. The 3' UTRs of the human RAS genes contain multiple LCSs, allowing let-7 to regulate RAS expression. let-7 expression is lower in lung tumors than in normal lung tissue while RAS protein is significantly higher in lung tumors, providing a possible mechanism for let-7 in cancer [5]. The RAS family makes up major oncogenes in various cancers, including lung cancer. We showed that human let-7 is poorly expressed or deleted in lung cancer, but let-7 is highly expressed in lung tissue. This work suggests that the level of expression of the let-7 miRNA might be an important factor in limiting or contributing to oncogenesis. Inhibiting let-7 function leads to increased cell division in A549 lung cancer cells and in vivo in a mouse model of lung cancer [6, 7], providing evidence that let-7 functions as a tumor suppressor in lung cells. Over-expression of let-7 in cancer cell lines alters cell cycle progression and reduces cell division [6]. While let-7 regulates the expression of the RAS lung cancer oncogenes, we also showed that multiple genes involved in cell cycle and cell division functions are also directly or indirectly repressed by let-7 [6]. This work reveals the let-7 miRNA to be a master regulator of cell proliferation pathways. This was also the first step in a possible cancer therapy, targeting RAS with a miRNA.

Lung cancers do not respond to current chemotherapy and radiotherapy and novel therapeutic options are desperately needed. We showed that delivery of a miRNA to lung cancers in vivo can slow their growth [8] and reverse the tumor phenotype [7], the first time any microRNA has been shown to have therapeutic efficacy in vivo in cancer. This opens up the possibility of miRNA replacement therapy for cancer patients. Most lung cancer patients beyond stage 1 have a poor outcome, but very few lung
cancers are diagnosed at stage I and screening mechanisms are expensive, demonstrating the desperate need for markers of individuals with a greater risk of developing lung cancer. We identified a single nucleotide polymorphism (SNP) in the KRAS 3'UTR that disrupts let-7 miRNA binding, and is associated with a 2-fold increased risk of developing lung cancer [9]. This is the first solid genetic marker for inherited lung cancer risk, and also suggested that 3'UTRs are an unmined area for variation associated with cancer risk.


In vivo RNAi to dissect signaling pathways in liver cancer and liver regeneration

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Target cells for transformation in human hepatocellular carcinoma are either bipotential liver progenitor cells (oval cells in rodents) or differentiated hepatocytes, whereas 60-80% of all human HCCs are thought to be derived from hepatocytes. Current work revealed striking differences between progenitor cell- and hepatocyte derived hepatocellular carcinomas in mice: Whereas oncogenic Nras (NrasG12V) efficiently triggers hepatocellular carcinomas derived from p53-/- liver progenitor cells, almost no tumor growth is observed, when NrasG12V is delivered into p53-/- hepatocytes. However, aggressive hepatocellular carcinomas develop after short latency, when oncogenic Nras is delivered into p19Arf-/- hepatocytes, thus suggesting that p19Arf mediates p53-independent tumor suppressive functions in differentiated hepatocytes. To identify mediators of such p53-independent tumor suppressive functions of p19Arf in the mouse liver we set up an in vivo RNAi screen. As a proof of principle we could show that co-delivery of NrasG12V and p19Arf-shRNAs into p53-/- hepatocytes was able to phenocopy NrasG12V-driven hepatocarcinogenesis in p19-/- livers. To perform the screen, a focused shRNAmir library was compiled based on differentially expressed genes in NrasG12V; p53-/- mouse livers compared to NrasG12V; p19Arf-/- livers. From NrasG12V; p53-/- mouse livers compared to NrasG12V; p19Arf-/- livers. This focused shRNA library was divided into several low complexity pools (n=48) and subjected to an in vivo positive selection screen in a p53--/ background (co delivery with NrasG12V). From NrasG12V; p53--/ tumors we identified several shRNAs knocking down new candidate genes mediating p53-independent tumor suppressive functions of p19Arf in the mouse liver. Functional validation experiments using single hairpins have already been completed for some candidates, among these are genes involved in the spindle assembly checkpoint. In a second part of my talk I will present a new mouse model, which allows to perform in vivo RNAi screens for molecular modulators of liver regeneration. Combining the well characterized FAH-/- mouse model with third generation microRNA based shRNA (shRNAmir) technology, we show that mouse livers can be stably repopulated with complex shRNAmir libraries and quantification of shRNAmir
representation in shRNAmir populations and chimeric mouse livers can be accomplished at any given time point by Illumina/Solexa deep sequencing. A first screen was performed using a thematically focused shRNAmir library consisting of 631 constructs targeting 362 genes. Mouse livers were stably repopulated with the shRNAmir library and after repopulation, mice were subjected to chronic CCl4 treatment to induce chronic liver damage. ShRNAmir representation in the starting library pool, in the liver directly after repopulation and after four weeks of CCl4 treatment was deconvoluted using deep sequencing. While the majority of shRNAmir did not change representation during liver repopulation or CCl4 mediated liver damage, we identified several shRNAmir which showed strong enrichment or depletion during regeneration and therefore pinpoint new regulators of liver regeneration. Identified candidate genes were functionally validated in vivo regarding their role in modulating the regenerative capacity of murine hepatocytes. Interestingly, a couple of strongly enriched candidate shRNAmir target kinases and for some of them pharmacological inhibitors are readily available.

Targeting of microRNAs for therapeutics

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MicroRNAs act as important post-transcriptional regulators of gene expression by mediating mRNA degradation or translational repression. There is now ample evidence that perturbations in the levels of individual or entire families of miRNAs are prevalent in and strongly associated with the development of a variety of human diseases. Apart from cancer, miRNAs have also been implicated in viral infections, cardiovascular disease and neurological disorders. Hence, disease-associated miRNAs could represent a potential new class of targets for oligonucleotide-based therapeutics, which may yield patient benefits unobtainable by other therapeutic approaches.

LNAs comprise a class of bicyclic high-affinity RNA analogues, in which the ribose ring in the sugar-phosphate backbone is locked in an RNA-like, C3'-endo conformation by the introduction of a 2'-O,4'-C methylene bridge. This results in high binding affinity of single-stranded LNA-modified oligonucleotides to their complementary miRNA targets. Moreover, LNAs combined with a phosphorothioate backbone show high biostability and improved pharmacokinetic properties in vivo. Here, we describe an approach that enables miRNA knock-down using 7 to 8 nucleotide fully LNA-modified phosphorothioate oligonucleotides, termed tiny LNAs, complementary to the miRNA seed region. Transfection of tiny LNAs into cells results in successful inhibition of miRNA seed families with concomitant up-regulation of direct targets. In addition, systemically delivered, unconjugated tiny LNAs show uptake in many tissues and in breast tumors in mice, coinciding with long-term miRNA silencing, while transcriptomics and proteomics data from off-target analyses suggest that tiny LNAs do not significantly affect the output from mRNAs with perfect match binding sites. Our data validates the use of tiny LNA-based knockdown in exploring miRNA function with important implications for the development of therapeutic strategies aiming at pharmacological inhibition of disease-associated miRNAs. Finally, we will present an update on the therapeutic development of antimiR-122 for treatment of hepatitis C virus infection.

Selective control of miRNA accessibility by RNA binding proteins

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microRNAs (miRNAs) interact with 3'-Untranslated regions (3'UTRs) of messenger RNAs (mRNAs) to control the expression of a large proportion of the protein coding genome during normal development and cancer. RNA-binding proteins (RBPs) potentially control the biogenesis, stability, and activity of miRNAs. In the past we have demonstrated that miRNA accessibility to target mRNAs can be controlled by RBPs, a process required for proper differentiation and survival of primordial
germ cells. We now have performed a large-scale screen to identify RBPs whose expression controls miRNA accessibility to target mRNAs. This has led us to the identification of RBPs required for proliferation of cancer cells as well as for optimal p53 function.

The role of microRNAs in glioblastoma pathogenesis

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MicroRNAs (miRNAs) are fundamental regulators of gene expression that direct processes as diverse as cell metabolism, lineage specification or cell differentiation. miRNAs are frequently mis-expressed in cancer. In several cancer types including breast cancer and glioblastoma, a cell population with stem cell-like properties has been identified and termed cancer stem cells. Such cancer stem cells are believed to be the cause of relapse after therapy and contribute to treatment resistance. Here we report the miRNA expression profile of CD133+ glioblastoma stem cells. We find that both miR-9 as well as miR-9* are highly abundant in glioblastoma stem cells. We further find that inhibition of miR-9/9* promotes neuronal differentiation suggesting that miR-9/9* inhibit differentiation of glioblastoma stem cells and maintain their stemness. We identify the calmodulin-binding transcription activator 1 (CAMTA1) as miR-9/9* target. CAMTA1 over-expression substantially reduces colony formation demonstrating that CAMTA1 is a novel tumor suppressor in glioblastomas. Finally, we analyzed CAMTA1 expression in a large number of glioblastoma samples and find that CAMTA1 expression correlates with patient survival. Since tumor stem cells often survive therapy, our findings could provide a basis for novel therapeutic strategies.

Exploiting and antagonizing microRNA for therapeutic applications

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microRNA are post-transcriptional regulators of gene expression which bind in a sequence-specific manner to messenger RNAs and mediate down-regulation of their expression. Despite the relatively low extent of down-regulation of individual target mRNA, each miRNA may regulate hundreds of targets in a cell and profoundly impact important pathophysiological processes such as cell growth, transformation and metastasis. We have shown that by incorporating target sites for a specific microRNA into a gene transfer vector, its expression becomes susceptible to regulation in cells where that microRNA is expressed. This strategy allows de-targeting the expression of exogenously delivered genes from unwanted cell types and can improve tremendously the specificity and efficacy of gene and stem cell therapy. Vectors designed to undergo microRNA regulation can also serve as sensitive reporters for the activity of microRNA at the single-cell level, in rare cells among heterogeneous cell populations, and in in vivo models of disease. By this approach we have identified and profiled the activity of several microRNAs specifically expressed in normal hematopoietic stem cells and their leukemic counterparts. We determined that miR-130a and miR-126 were specifically expressed in mouse and human HSC and early progenitors, defined according to surface markers and their biological activity. miR-126 expression was maintained in a subset of human cord blood progenitors during in vitro culture and allowed prospective isolation of cells capable of long-term engraftment in immunodeficient mice. We then exploited the miR-126 expression pattern to regulate HSC gene transfer. By making the vector responsive to miR-126 regulation, transgene expression was suppressed in HSC while sustained at high levels in mature cells. This vector design allowed for the first time successful gene therapy in a mouse model of globoid cell leukodystrophy, which previously failed due to transgene toxicity in HSC and early progenitors. We have then probed the function of these microRNAs by
knocking-in and knocking-out their activity in vivo and investigating the resulting phenotype. Knock-in was accomplished using vectors that forced microRNA expression in constitutively or exogenously regulated manner. Knock-out was performed by exploiting a vector design that over-express microRNA target sites to inhibit the function of an endogenous microRNA. In this case, the target-containing transcripts are made to accumulate in a cell and act as a decoy or sponge for the cognate microRNA, thus interfering with the ability of the microRNA to regulate its natural targets. This technology provides a versatile way to investigate microRNA biology and study the role of microRNAs in the hematopoietic stem cell niche.

MicroRNAs as Regulators of Gliomagenesis and Targets for Glioma Therapy

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MicroRNA (miRNA) is a class of small RNA molecules involved in the regulation of expression of at least 30% of human genes and, therefore, it controls all principal cellular processes, including cell division, metabolism and cell death. MicroRNA expression profiling studies revealed a number of dysregulated miRNAs in malignant brain tumor, glioblastoma (GBM); molecular mechanisms underlying functions of these miRNAs are mainly unknown.

In my talk, I will describe our approach to identify key miRNA regulators of gliomagenesis. We have previously identified miR-21 as a miRNA most commonly upregulated in GBM tumors (Chan et al., 2005). Loss- and gain-of-function experiments in glioma cells established that miR-21 regulates multiple genes associated with cell cycle, apoptosis, migration, and invasiveness, including RECK and TIMP3, tumor suppressors and inhibitors of matrix metalloproteinases (MMPs) (Gabriely at el 2008). Specific inhibition of miR-21 with antisense oligonucleotides reduces MMP activities in vitro and in a model of human glioma in nude mice. These data validate an entirely novel layer of gene regulation by miRNA involved in the progression and maintenance of these malignancies. Furthermore, they present proof-of-principles that inhibition of an oncogenic miRNA like miR-21 could provide a novel therapeutic approach for “physiological” modulation of multiple proteins whose expression and activity is deregulated in cancer (Krichevsky and Gabriely, 2009). Nevertheless, inhibition of miR-21 alone is insufficient for complete eradication of glioma tumor growth. Our large-scale analysis of miRNome in multiple glioblastoma samples identified additional dysregulated miRNAs, or a “miRNA signature” of glioma. Among them we investigate miRNAs that regulate glioma cell viability, cell cycle progression and apoptosis, and affect tumor growth in mouse models of human GBM. miRNAs are involved in controlling various aspects of glioma progression. We have also performed a screen for endothelial miRNAs that control glioma-associated angiogenesis. Identified in this screen miR-296 is upregulated in primary tumor endothelial cells isolated from human brain tumors compared to normal brain endothelial cells, and in endothelial cell co-cultured with glioma cells in vitro. By targeting the hepatocyte growth factor regulated tyrosine kinase substrate (HGS) mRNA, miR-296 controls sorting and accumulation of growth factor receptors on endothelial cells, and thus tumor-associated angiogenesis (Wurdinger et al 2008). These examples suggest a therapeutic potential for this novel class of molecules.

References:
A coding-independent function of gene and pseudogene mRNAs regulates tumour biology

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The canonical role of messenger RNA (mRNA) is to deliver protein-coding information to sites of protein synthesis. However, given that microRNAs bind to RNAs, we hypothesized that RNAs could possess a regulatory role that relies on their ability to compete for microRNA binding, independently of their protein-coding function. As a model for the protein-coding-independent role of RNAs, we describe the functional relationship between the mRNAs produced by the PTEN tumour suppressor gene and its pseudogene PTENP1 and the critical consequences of this interaction. We find that PTENP1 is biologically active as it can regulate cellular levels of PTEN and exert a growth-suppressive role. We also show that the PTENP1 locus is selectively lost in human cancer. We extended our analysis to other cancer-related genes that possess pseudogenes, such as oncogenic KRAS. We also demonstrate that the transcripts of protein-coding genes such as PTEN are biologically active. These findings attribute a novel biological role to expressed pseudogenes, as they can regulate coding gene expression, and reveal a non-coding function for mRNAs.
ABSTRACTS OF POSTERS

MicroRNAs involvement in fludarabine refractory chronic lymphocytic leukemia

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Abstract
Background. Fludarabine, is one of the most active single agents in the treatment of chronic lymphocytic leukemia (CLL). Over time, however, virtually all CLL patients become fludarabine-refractory. To elucidate whether microRNAs are involved in the development of fludarabine resistance, we analyzed microRNA expression of 29 CLL patients either before and after 5-days of fludarabine mono-therapy. Based on NCI criteria, we enrolled a cohort of patients which responded to fludarabine treatment and a cohort of refractory patients.

Results. By comparing the expression profiles of these two groups of patients, we identified a microRNA signature able to distinguish refractory from sensitive CLLs. The expression of some microRNAs was also able to predict fludarabine resistance. Based on NCI criteria, we enrolled a cohort of patients which responded to fludarabine treatment and a cohort of refractory patients.

Conclusions. This is the first report that reveals the existence of a microRNA profile that differentiate refractory and sensitive CLLs, either before and after fludarabine mono-therapy. A p53 dysfunctional pathway emerged in refractory CLLs and could contribute in explaining the observed miRNA profile. Moreover, this work suggests that specific microRNAs can be used to predict fludarabine resistance, therefore establishing an important starting point for future studies.

Analysis of microRNA expression in human T cells at different stages of maturation.

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At present, little is known about the role of miRs during the physiologic development of human T cells, nor their possible significance in T cell-derived malignancies. In order to identify miRs involved in the differentiation of T lymphocytes, we analyzed...
the miR expression profiles of human T cell progenitors at different stages of maturation: Double Positive (DP), Single Positive CD4+ (SP CD4) and Single Positive CD8+ (SP CD8). This array-based analysis showed that each thymic population displays a distinct miR expression profile, which reflects the developmental relationships among thymocytes. In addition, small-RNA libraries were generated from unsorted and DP thymocytes, as well as CD4+ and CD8+ mature peripheral blood T lymphocytes. Computational analysis of the 29,744 small-RNA sequences obtained by 454 massive parallel sequencing-by-synthesis of the four libraries led to the identification of 139 sequences corresponding to known miRs and 98 sequences of new candidate miRs. By comparing the array and sequencing data, we identified a group of known miRs consistently regulated during normal T cell maturation. The modulation of their expression during T cell differentiation was further validated by qRT-PCR. Eventually, we studied the functional effects of the over-expression of miR-150, one of the top up-regulated miR during T maturation, in T-cell acute lymphoblastic leukemia (T-ALL) cell lines. MiR-150 is known to be regulated during both B and T lymphocyte maturation and activation (1) and has been reported to be frequently down-regulated in ALL (2). Indeed, we found MiR-150 expressed at very low levels in all the T-ALL cell lines tested. Forced expression of miR-150 in Jurkat cells caused a significant reduction of the proliferation rate associated with the accumulation of cells in the G2 phase of cell cycle. This work identifies a group of miRs which are modulated during human T cell maturation and whose role in this process remains to be investigated. Moreover, we identify a potential anti-proliferative function in T-ALL of miR-150, one of the top-up-regulated miRs during T cell differentiation.

(1) Xiao C, Calado DP et al. MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. Cell. 2007; 131(1):146-59;

Ribonomics approach to study an complex inherited tumor predisposing disorder: the multiple endocrine neoplasia type as a model.

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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 the MEN1 mRNA, thus indicating that induction of menin oncosuppressor compensation could have been regulated through RNA protein driven post transcriptional mechanisms. A ribonucleoprotein structure in which multiple mRNAs were coordinately regulated by RNA binding proteins and small non-coding RNA (RNA regulons) could be hypothesized in the menin-mRNA-microRNA(s) complex. Moreover, in silico analysis with Target Scan, Miranda and Pictar-Vert softwares for the prediction of miRNA targets indicated miR-24 as capable to bind to the 3'UTR of MEN1 mRNA. Analysis of miR-24 expression profiles performed in parathyroid and pancreatic endocrine tissues from MEN1 mutation carriers, in their sporadic non-MEN1
counters and in normal tissues, showed that the expression profiles of miR-24 mRNA and menin protein were inversely correlated, suggesting a negative post-transcriptional control of miR-24 on MEN1 expression. Interestingly, the interplay between menin and miR-24 suggested an autoregulatory feedback with functional significance in MEN1 tumorigenesis thus also opening new avenues for future developments of RNA-based strategies in the \textit{in vivo} control of tumorigenesis in MEN1 carriers.

\textbf{miRNAs expression profiles during ErbB2 driven mammary carcinogenesis}

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MicroRNAs (miRNAs) are noncoding RNAs that regulate global gene expression. They often act synergistically to repress target genes, and their deregulation can contribute to the initiation and progression of a variety of cancers. In view of the roles played by miRNAs in cancer progression, we performed a miRNAs microarray analysis aimed at identifying the modulation of miRNAs expression profiles during the progression of authochtonous mammary carcinomas arising in mice transgenic for the activated transforming rat ErbB2 oncogene (BALB-neuT mice). BALB-neuT mice constitute a suitable cancer-prone model, since inexorably the females develop an invasive and metastatic mammary cancer in all of their ten mammary glands with a step wide pattern and a systemic metastatic spread similar to that observed in human mammary cancer. Using Applied Biosystems Megaplex low density miRNA expression arrays miRNAs expression signature of diffused atypical hyperplasia (6 week-old BALB-neuT versus 6 week-old BALB-c females mammary glands) and invasive carcinoma (19 week-old BALB-neuT versus 19 week-old BALB-c females mammary glands) were identified. PCA shows a wide difference in miRNAs expression between diffused atypical hyperplasia and invasive carcinoma. Moreover, rank product analysis allowed the detection of 6 and 4 miRNAs whose expression is correlated to invasive carcinoma and to diffused atypical hyperplasia respectively. Among those that were found to be upregulated in invasive carcinoma miR-135a and miR-135b were found to be expressed also in several lines derived from BALB-neuT mammary tumors, suggesting their role in mammary cancer progression. By contrast, the expression of miR-741 resulted undetectable in all the mouse cell lines derived from BALB-neuT mammary tumors, even if, its expression was upregulated in mammary BALB-neuT tumors, suggesting its role in the tumor microenvironment phenotype. Modulation (over-expression or down-modulation) of miR-135b in breast cancer cells is under investigation in order to evaluate its role in \textit{in vivo} tumor growth, matrigel invasion \textit{in vitro} as well as lung metastasis formation \textit{in vivo}.

\textbf{Integrated analysis of microRNA and mRNA expression profiles in physiological myelopoiesis: role of hsa-mir-299-5p in CD34+ progenitor cells commitment}

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Hematopoiesis entails a series of hierarchically organized events that proceed throughout cell specification and terminates with cell differentiation. Commitment needs the transcription factors effort that, in concert with microRNAs, drives cell fate and responds to promiscuous patterns of
gene expression by turning-on lineage-specific genes and repressing alternate lineage transcripts. We obtained microRNAs profiles from human CD34+ hematopoietic progenitor cells and in-vitro differentiated erythroblasts, megakaryoblasts, monoblasts and myeloblasts precursors, that we analyzed together with their gene expression profiles. The integrated analysis of microRNA-mRNA expression levels highlighted an inverse correlation between microRNAs specifically up-regulated in one single cell progeny and their putative target genes, which resulted down-regulated. Among the up-regulated lineage-enriched microRNAs, hsa-miR-299-5p emerged as having a role in controlling CD34+ progenitors fate, grown in multilineage culture conditions. Gain- and loss-of-function experiments revealed that hsa-miR-299-5p participates the regulation of hematopoietic progenitors fate, modulating megakaryocytic-granulocytic versus erythroid-monocytic differentiation.

Identification of Mirnas Involved in Tumor Invasiveness

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MicroRNAs (miRNAs) are small non-coding RNAs involved in negative regulation of gene expression. They act at a post-transcriptional level by repressing translation of protein-coding mRNAs that display a partially complementary sequence in their 3'UTR. In this way, miRNAs regulate genes that drive cell proliferation, migration and apoptosis and behave as classical oncogenes or tumor suppressor genes. This implicates that, when aberrantly expressed, they can play a fundamental role in cell transformation and tumor progression. Indeed, miRNAs were recently found to be involved in the pathophysiology of many types of human cancers and their profiling has allowed the identification of signatures associated with diagnosis, prognosis and response to treatment.

It is, therefore, of outstanding interest to identify miRNAs involved in the molecular mechanisms underlying tumor progression that might represent both new cancer biomarkers and therapeutic targets. The present work is aimed at discovering miRNAs implicated in the metastatic process. We performed a miRNA expression profiling of tumour xenografts that displayed different metastatic ability. We isolated two miRNAs belonging to the same family that are more expressed in tumours with increased metastatic properties. Preliminary in vitro experiments showed that the ectopic expression of these miRNAs enhanced the ability of cells to scatter and to invade a collagen matrix. Accordingly, the over-expression of these small RNAs induced the down-regulation of E-cadherin, a molecule known to be implicated in cell-cell adhesion. A luciferase assay performed with a construct containing E-cadherin 3'UTR cloned downstream of the luciferase gene confirmed that E-cadherin is a target of these miRNAs. Moreover, by screening a panel of cancer cells of different histological origin we found a good anti-correlation between miRNAs and E-cadherin expression. Now, we are particularly interested in evaluating whether the expression of the identified miRNAs negatively correlates with E-cadherin also in human tumors and can be associated with the clinical outcome. To this aim, expression analysis in different types of human tumor specimens is in progress.

MicroRNAs impair met-mediated invasive growth

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MicroRNAs (miRNAs) are a recently identified class of noncoding, endogenous, small RNAs that regulate gene expression, mainly at the translational level. These molecules
play critical roles in several biological processes, such as cell proliferation and differentiation, development, and aging. It is also known that miRNAs play a role in human cancers where they can act either as oncogenes, down-regulating tumor suppressor genes, or as onco-suppressors, targeting molecules critically involved in promotion of tumor growth. One of such molecules is the tyrosine kinase receptor for hepatocyte growth factor, encoded by the MET oncogene. The MET receptor promotes a complex biological program named “invasive growth” that results from stimulation of cell motility, invasion, and protection from apoptosis. This oncogene is deregulated in many human tumors, where its most frequent alteration is overexpression. In this work, we have identified three miRNAs (miR-34b, miR-34c, and miR-199a*) that negatively regulate MET expression. Inhibition of these endogenous miRNAs, by use of antagomiRs, resulted in increased expression of MET protein, whereas their exogenous expression in cancer cells blocked MET-induced signal transduction and the execution of the invasive growth program, both in cells expressing normal levels of MET and in cancer cells overexpressing a constitutively active MET. Moreover, we show that these same miRNAs play a role in regulating the MET-induced migratory ability of melanoma derived primary cells. We are now trying to verify the possible involvement of these miRNAs in tumor progression: we hypothesize that their inhibition, due either to epigenetic modifications or to genetic alterations, could confer a more aggressive phenotype to cancer cells up-regulating, among the others, the expression of the MET gene. To this end, we are screening primary lung and colon tumors at different clinical stages for the expression of MET and of these miRNAs in order to assess a possible inverse correlation between them. In conclusion, we have identified miRNAs that behave as oncosupressors by negatively targeting MET and might thus provide an additional option to inhibit this oncogene in tumors displaying its deregulation.

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c-Myc-dependent regulation of miR-26a and histone methyltransferase EZH2 during acute myeloid leukemia differentiation

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MicroRNAs (miRNAs) are emerging as important player during the execution of the hematopoietic differentiation programs and their alteration has been associated to the establishment of leukemogenesis. In this study we show that mir-26a is downregulated in different subtypes of acute myeloid leukemia (AML). We found that mir-26a expression increases during myeloid differentiation of AML cell lines and that, as previously shown in Myc-driven lymphoma, c-Myc oncogene is responsible for mir-26a transcriptional repression. Mir-26a has a role as tumor suppressor in AML cell lines: enforced expression is able to inhibit proliferation and potentiate myeloid differentiation. However, conversely to what it was observed in lymphomas, mir-26a does not post-transcriptionally repress the Polycomb Repressive Complex core component EZH2. Decrease expression of EZH2 and c-Myc is required for AML cells to arrest cell cycle and undergo terminal differentiation. We observed a strong correlation between EZH2 and c-Myc protein levels during myeloid differentiation of AML cells and we demonstrated that c-Myc directly activate EZH2 at transcriptional level. Our results support the role of miR-26a as a tumor suppressor in hematological malignancies and identify a new direct link between c-Myc and EZH2 expression that may be relevant to AML development.

The microRNA profile of prostate carcinoma obtained by deep sequencing reveals potential miRNA targets

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Background: Prostate cancer is a leading cause of tumor mortality. In order to identify and characterize the underlying molecular mechanisms, we performed microRNA (miRNA) profiling of primary prostate cancers and non-cancer prostate tissue. Furthermore, using database analysis, we sought to identify regulatory targets of aberrantly expressed miRNAs.

Material and Methods: We performed comparative miRNA expression profiling in ten prostate cancer specimens and ten non-cancer prostate tissue samples using deep sequencing of cDNA libraries. The deregulation of selected miRNAs was validated in established prostate cancer cell lines by Northern blotting as well as in 26 paired samples of prostate cancer tissue and adjacent normal tissue by quantitative reverse transcription polymerase chain reaction (qRT-PCR). The regulative capabilities of selected miRNAs on potential target genes were analyzed in vitro using reporter gene constructs and transfection of miRNA expression vectors.

Results: Using deep sequencing method we found that 16 miRNAs were up-regulated more than 1.5-fold and 17 miRNAs were down regulated more than 1.5-fold. The most pronounced deregulation was noted for miRNAs miR-375 and miR-200c which showed an up-regulation of more than 4.5-fold and miR-143 and miR-145 which were down regulated 4-fold each. The differential expression of these miRNAs could be confirmed by Northern blot analysis of established prostate cancer cell lines by Northern blotting as well as in 26 paired samples of prostate cancer tissue and adjacent normal tissue by quantitative reverse transcription polymerase chain reaction (qRT-PCR). The regulative capabilities of selected miRNAs on potential target genes were analyzed in vitro using reporter gene constructs and transfection of miRNA expression vectors.

Conclusions: Our findings indicate that specific miRNAs are reproducibly found to be deregulated in different cohorts of prostate cancer specimens using independent experimental methods. This is the first report to show that miRNAs miR-143 and miR-145 are capable of negatively regulating the protein expression of Myosin VI.

Study of miRNAs and transcription factors Hoxa9, Hoxa10, Hoxa11 and Pax8: looking for new targets of therapeutic intervention in Epithelial Ovarian Cancer.

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Epithelial Ovarian Cancer (EOC) is a morphologically and biologically heterogeneous disease, which represents the most lethal of the gynaecological neoplasms. 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. Unlike most cancers, epithelial ovarian tumours are more differentiated than cells of the likely precursor, the ovarian surface epithelium (OSE). The specific molecular mechanisms involved in the development of EOC have remained elusive.

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. However, the processes driving...
aberrant expression of Hoxa9, Hoxa10, Hoxa11 and Pax8 genes in EOCs remain completely unknown.

Micro RNAs (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. Recent publications hint at a role for miRNAs in regulating Hox expression. Moreover, several miRNA genes are located in Hox clusters. Our work is aimed at 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.

With the aim of establishing cell models representative of the different EOC subtypes, we are currently analysing the levels Hoxa9, Hoxa10, Hoxa11 and Pax8 mRNAs and proteins in a panel of 16 available EOC cell lines, and comparing these levels with those in cells representative of normal ovarian surface epithelium (IOSE cells). In parallel, we are analysing the levels of several miRNAs, generating miRNA microarray profiling data of the cell lines.

In addition, by bioinformatic means we have predicted 18 miRNAs, which could down-regulate the expression of Hoxa9, Hoxa10, Hoxa11 and Pax8 genes in OSE and whose loss could be the cause of different EOC subtypes. We are currently confirming the levels of those miRNAs in the 16 EOC cell lines, by the means of real-time RT-PCR and validating the interaction of the miRNAs with Hoxa9-11 and Pax8 3’UTRs.

The identification of miRNA::HOX transcription factors circuitries with a role in EOC, might provide us with new targets of therapeutic intervention in EOC.

MicroRNA expression profiles of tumors, normal lung tissues and plasma samples from spiral-computed tomography (CT) trial for early lung cancer detection

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The benefits of lung cancer screening by spiral CT are still controversial and ongoing randomized trials will demonstrate the real impact of early detection on mortality of high risk individuals. Since the nature and life-threatening potential of CT-detected nodules is currently debated due to the potential issue of overdiagnosis, the study of molecular features of these tumors would allow for a better definition of those genetic signatures that are associated with invasiveness and metastatic potential.

In this study we explored miRNA expression profile of 28 lung tumors identified in the spiral-CT screening trial performed in our Institution on 1035 heavy smokers observed for 5 years. MicroRNA profiles of the tumors and paired normal lung tissues were analyzed using a miRNA microarray platform (Ohio State microRNA microarray version 2.0) and validation of the differentially expressed miRNAs was done using qReal Time PCR. By class comparison and class prediction analyses of paired and unpaired normal/tumor samples expression of 51 miRNAs was significantly different at the nominal 0.001 level of the univariate test. The top ten deregulated miRNAs that discriminate normal lung versus early lung cancer were: mir-7, mir-21, mir-200b, mir-210, mir-219-1, miR-324 (upregulated) mir-126, mir-451, mir-30a and mir-486 (downregulated). This list included miRNAs previously identified (i.e. mir-21, mir-200 family known to be involved in specific pathways such as survival, apoptosis, epithelial-mesenchymal transition) and unidentified (i.e. downregulation of miR-486 and miR-451) in symptomatic lung cancer patients.

Possible association of microRNA expression profiles with clinical-pathological characteristics of the patients was then investigated. Three miRNAs (mir-205, mir-21 and mir-106) significantly discriminated adenocarcinoma from squamous carcinoma histotypes. Mir-518e and mir-144 were downregulated in tumors with a faster growth rate and higher levels of mir-429, member of the mir-200 family, correlated with worst
Disease Free Survival (DFS). The miRNA expression profile of tumors detected in the first two years of the trial, including mainly Stage Ia adenocarcinoma with excellent survival, was found to be different to the profile of more advanced stage and lethal tumors appearing after the second year, with differential expression of mir-128, mir-129, mir-369-3p, mir-193, mir-339-3p, mir-185, mir-346, mir-340 and mir-206.

Interestingly, unsupervised clustering of the normal lung tissues of these patients discriminated patients identified in the first two years from those of later years suggesting a miRNAs signature in lung microenvironment affecting tumor aggressiveness. Significant associations were also found between miRNAs expression in normal lung and reduction of Forced Expiratory Volume (FEV) (mir-379, mir-485-3p and mir-29-1*), faster growth of the tumors (mir-30d*, mir-519a and mir-488) and prognosis of the patients (mir-146b-3p, mir-520a* and mir-200b).

We also analyzed, by real-time PCR using microfluidics cards, miRNAs expression profiles in plasma samples of the patients collected before the onset of the disease and compared to those of 5 different pools of disease-free heavy smokers controls enrolled in the screening trial. In this analysis 113 miRNAs were found to be representative of the plasma samples with a number of miRNAs able to identify aggressive lung cancer before its clinical appearance indicating a potential role of miRNAs in plasma as molecular predictors of high risk disease.

Modulating Epithelial-Mesenchymal Transition By Mir-205 Influences Drug Sensitivity Of Prostate Cancer Cells

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We recently showed that miR-205, which is down-regulated in prostate cancer (PCa), has the potential to revert epithelial-mesenchymal transition (EMT) in PCa cells, thus reducing their invasive potential. Given that EMT has a significant impact on the resistance of cancer cells to drugs independent of the “classical” resistance mechanisms, and that residual epithelial tumor cell populations surviving after conventional treatments seem to be enriched for subpopulations of cells with mesenchymal features, we investigated the ability of miR-205 to modulate the sensitivity of PCa cells to drugs with different mechanisms of action. The DU145 PCa cell line was stably transfected with specific vectors carrying the sequences of miR-205 and a control, and two polyclonal cell populations (DU145/miR-205 and DU145/miRVec) were selected for the study. Restoring the expression of miR-205 did not appreciably affect the growth potential of PCa cells. To test whether the basal level of miR-205 influenced the in vitro drug response, DU145/miR-205 and DU145/miRVec cells were analyzed for their clonogenic cell survival profiles after exposure to different concentrations of cisplatin. A dose-dependent reduction in cell survival was observed in both cell lines following cisplatin exposure. However, DU145/miR-205 cells showed a significantly enhanced sensitivity to the drug compared with DU145/miRVec cells.

In parallel, we assessed the susceptibility of PCa cells to other agents with different mechanisms of action. Specifically, we observed an increased resistance of DU145/miR-205 cells compared with DU145/miRVec cells to RAD001 exposure. In contrast, DU145/miR-205 and DU145/miRVec cells showed a comparable sensitivity to paclitaxel. Overall, these findings suggest that modulation of EMT, although influencing drug sensitivity, may result in a different response as a function of the tested drug. For only a subset of specific agents, a combination with EMT-modulators, such as miR-205, can be envisaged to improve response to treatment.

Alterations in miRNA expression profile as a possible tool to predict clinical response in advanced stage ovarian cancer patients.

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High-throughput technologies in the last decade have progressed rapidly and numerous gene expression profiles associated to epithelial ovarian cancer (EOC) have been identified. However, the still unfavorable statistics in EOC patients reflects the largely unpredictable response to first-line treatment and the occurrence of relapse after complete initial response, associated with broad cross-resistance to even structurally dissimilar drugs. Accumulating evidence of deregulation of miRNA expression in association to cancer development was reported. To evaluate whether the imbalance of the miRNAs in EOC could be involved in chemo-resistance, we profiled on human miRNA Illumina chips (1146 miR annotated on miRBase 12.0) a training set (55 samples) and an independent test set (30 cases) of EOC cases with similar clinical characteristics. The retrospective case materials with well-defined clinical history consist of surgical specimens obtained at debulking surgery of patients with advanced-stage EOC. After surgery, all patients received first-line treatment with standard platinum-based chemotherapy. Samples were grouped on the basis of debulking and time to relapse. With a supervised class comparison analysis between early and late relapsing patients, we identified 32 miRNAs differentially expressed at FDR<10% in the training set. Eighteen and fourteen miRNAs were down- and up-modulated respectively in early relapsing patients. Eleven of eighteen down-modulated miRNAs are located on the chromosome Xq27.3 region representing a highly correlated and co-expressed cluster. Among the fourteen up-regulated miRNAs, six are located on the chromosome 14q32.31 region. When the test set was challenged with the miRNA profile identified in the training set, twenty-eight out of 32 miRNAs were detected correctly classifying patients according to time to relapse. Ten of these miRNAs (8 located on Chromosome Xq27.3 region) resulted significantly differentially expressed at FDR<10% being all but one down-modulated in early relapsing patients. Expression of the 10 miRNA signature in the two clinical sets was highly correlated (R²= 0.838). The second miRNA cluster, localized at Chr14q32.31, exhibited a trend but did not reached significance cut-off in test set. To identify the Chr Xq27.3-cluster miRNA target genes we used 4 different algorithms based on different features (complementarity, seed-complementarity, thermodynamics and machine learning-based algorithm). Their intersection identified 684 genes targeted by at least one the miRNA. Those genes were further challenged with functional analysis programs such as the Ingenuity Pathway Analysis system (IPA 5.5, Ingenuity Systems, CA). Two out of ten significant identified pathways have been selected for further analyses and validation in relation to their relevance to EOC biology. Partially supported by AIRC and Health Ministry Special Project.

MiR-205 role in triple negative breast cancer

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An increasing number of experimental evidences show that microRNAs can have a causal role in breast cancer tumorigenesis as a novel class of oncogenes or tumor suppressor genes, depending on the targets they regulate. miR-205, down-modulated in breast cancer versus normal breast tissue, was originally associated to absence of vascular invasion and shown to be involved in the EMT. Moreover, we recently demonstrated that miR-205 directly targets HER3 receptor and impairs the Akt-mediated survival pathway, thus inhibiting clonogenic potential of SKBr3 cells and increasing the responsiveness to tyrosin-kinase inhibitors. Considering the striking evidences of a major role of miR-205 as oncosuppressor in breast cancer, we decided to investigate its possible involvement in another tumor subgroup, extremely aggressive and still lacking a specific therapy: triple negative breast tumors. Here we show that re-introduction
of miR-205 in the triple negative breast cancer cell line MDA-MB-231 led to a dramatic reduction of both proliferation and clonogenic potential as well as migration capability. Notably, miR-205 also significantly inhibited the in vivo growth in xenograft models obtained injecting stable clones of MDA-MB-231 cells transfected with a miR-205-encoding plasmid or the empty vector. Investigating alternative pathways potentially regulated by miR-205 in this model, we noticed that many of the predicted targets are actually interconnected and related to adhesion and migration processes. Indeed, this microRNA seems to be involved in the control of cell-cell and cell-matrix adhesion regulating the expression of extracellular matrix-related molecules. Among them, we focused on LAMC1, reported to be involved in cell adhesion, differentiation, migration, signaling and metastasis. By Western Blot and Luciferase assay, we demonstrated that miR-205 directly targets LAMC1 in MDA-MB-231 cells. Moreover, with the aim to investigate mechanisms responsible for miR-205 expression regulation, we identified the miRNA promoter region and searched for conserved transcription factor binding sites predicted according to Genome Browser (http://genome.ucsc.edu). Notably, we demonstrated that p53 is able to induce miR-205 expression by direct binding on a responsive element located upstream the miRNA transcription binding site. In conclusion, p53-induced miR-205 exerts a role as onc suppressor gene also in triple negative breast cancer: it exerts a dramatic inhibition of proliferation and migration in triple negative MDA-MB-231 cell line, at least partially through direct regulation of LAMC1. These evidences not only underline the consistent role of miR-205 in the biology of different breast cancer subgroups, but provide the rational bases for a therapeutic intervention miRs-based.

A mix of two Micro-Rnas with Identical Seed Sequence has A Synergistic Rather Than an additive anti-cancer effect.

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Recent results show that re-establishing the expression of some microRNAs blocks tumorigenesis, suggesting that they could represent a novel class of cancer therapeutics. We have previously shown that re-expression of miR-206, a MyomiR which shares the seed sequence with miR-1, can block the growth of rhabdomyosarcoma (RMS) xenografts by inducing a major switch in the global gene expression profile toward that of mature muscle. Based on these results we proposed that these MyomiRs could represent good leads to develop a non-toxic differentiation therapy for RMS. To follow up in this direction we decided to test the effect of rescuing expression of both miR-206 and miR-1 in RMS cell lines. During myogenesis they are transcribed with a different timing, with miR-206 reaching its maximum level at the onset of differentiation and miR-1 coming up later, but remaining high in differentiated myotubes. This suggested to us that a combination of the two could have a synergistic rather than an additive effect. Here we show that inducing expression of both MyomiRs had a dramatic effect in RMS cells in culture, bringing their myogenic conversion close to 90%. Furthermore, when rescuing miR-206 alone, upon withdrawal of the inducer the tumor resumed proliferation indicating that a fraction of the RMS cells, in spite of the proliferative block, did not achieve terminal differentiation. Conversely, induction of both MyomiRs prevented tumor relapse upon removal of the inducer, allowing achievement of terminal differentiation in a higher number of tumor cells. Studies to define the molecular basis for the differential effects of mir-206 and miR-1, alone or in combination, on the expression profile of converted RMS cells are in progress.

miR-335 directly targets Rb1 in an intimate connection to p53-dependent stress response

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Loss of function mutations of Retinoblastoma family (Rb) proteins drive tumorigenesis by overcoming barriers to cellular proliferation. Consequently, factors modulating Rb function are of great clinical import. Here we show that miR-335 is differentially expressed in human cancer cells and that it tightly regulates the expression of Rb1 by specifically targeting a conserved sequence motive in its 3'UTR. We found that by altering Rb1 levels miR-335 activates the p53 tumor suppressor pathway to limit cell proliferation and neoplastic cell transformation. DNA damage elicited an increase in miR-335 expression in a p53-dependent manner. miR-335 and p53 cooperated in a positive feedback loop to drive cell cycle arrest. Together, these results indicate that miR-335 helps control proliferation by balancing the activities of the Rb and p53 tumor suppressor pathways. Further, they establish that miR-335 activation plays an important role in the induction of p53-dependent cell cycle arrest after DNA damage.

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 transcription factor that regulates the expression of many target genes linked, among others, to the control of cell cycle, apoptosis, angiogenesis and DNA repair. Recent studies identified direct p53 regulation of miRNAs and related regulatory circuits.

Using bioinformatics approaches, we 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 SNPs predicted to modulate miRNA binding. Our work aims at the validation of p53-mediated control of the newly predicted miRNA genes and related circuits that would provide additional negative and/or positive feedback loops for p53 regulation. To validate p53-responsiveness of 15 miRNA promoters not previously described to be under control of this family of transcription factors, we initially evaluated the potential for wild type p53, p63 and p73 to transactivate the predicted p53 response elements (REs) in those miRNA promoters. For these experiments we developed in the model system S. cerevisiae a panel of isogenic reporter strains harboring the chosen p53 REs upstream of the firefly luciferase reporter gene. 11 REs (including miR10b, 106a, 151, 198, 202, 221, 320) were responsive to p53 of which 9 were also inducible by p63 or p73, even though to a lower extent. Moreover, we developed RT-qPCR and ChIP assays in human cell lines where p53 proteins could be ectopically expressed or induced by genotoxic stress. In general, results confirmed p53-dependent transcriptional regulation of the studied miRNAs, although cell line differences were observed. To establish miRNA targeting of selected mRNAs and the functional impact of SNPs at the miRNA binding sites we developed 3'UTRs reporter constructs differing for the SNP status or with mutagenized miR binding sites. We also measured allele imbalance at the endogenous gene level by quantitative RT-PCR analysis in cell lines heterozygous for the SNPs and relative protein levels by western blot to evaluate the impact of the SNP allele as well as of p53-dependent or -independent miR modulation. Specific examples of p53-directed post-transcriptional circuits will be presented.
Functional analysis of CDKN2A/p16INK4a 5'UTR variants predisposing to melanoma

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The CDKN2A gene, located on 9p21, is the most common high penetrance susceptibility gene identified to date in melanoma families. Germline CDKN2A mutations are observed in 20-50% of melanoma-prone families. We identified melanoma patients that were heterozygous for non-coding germline variants in the 5'UTR of CDKN2A (c.-21C>T; c.-25C>T & c.-180G>A; c.-56G>T; c.-67G>C) and examined their impact on the p16INK4a 5'UTR activity using two luciferase-based reporter vectors, pGL3-basic and pGL3-promoter, that differ in basal transcription level and that were transfected into the melanoma-derived WM266-4 and in the breast cancer-derived MCF7 cells. The p16INK4a 5'UTR variants cloned into the pGL3-promoter plasmid type were also tested in two additional p16-null, melanoma-derived cell lines G361 and SK-Mel-5. Luciferase activity and mRNA levels were quantified to assess the impact of the mutations both at transcriptional and post-transcriptional levels. The wild type 5'UTR sequence, containing a reported SNP (c.-33G>C) and a known melanoma-predisposing mutation (c.-34G>T), were included as controls. Results revealed that the variants at -21 and -34 severely reduced the reporter activity. The variants at -56 and at -25 & -180 exhibited a milder impact, while results with c.-67G>C were dependent on the plasmid type. Quantification of the luciferase mRNA indicated that the effects of the variants were mainly post-transcriptional. Using a bicistronic dual-luciferase reporter plasmid, we confirmed that c.-21C>T and c.-34G>T had a severe negative impact in both cell lines. We also applied a polysomal profiling technique to samples heterozygous for the 5'UTR variants, including patient-derived lymphoblasts and the analysis of allelic imbalance indicated that in addition to the c.-21C>T variant, the c.-56T>G and c.-67G>C variants also reduced mRNA translation efficiency. Overall, our results suggest that the c.-21C>T sequence variant negatively impact on p16INK4a 5'UTR activity, acting mainly at a post-transcriptional level, and can thus be of clinical significance in the melanoma proneness. We propose that these variants should be considered as potential mutations.

“MicroRNA profiling of EBV associated diffuse large B-cell lymphoma and NK/T-cell lymphoma”

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The Epstein-Barr Virus (EBV) is an oncogenic human herpesvirus associated with several tumour diseases. The virus can be found in ~15% of diffuse large B-cell lymphoma (DLBCL) and in nearly all NK/T-cell lymphoma. EBV encodes for 25 viral miRNAs which are organized in three genomic clusters. The virus is also able to change the cellular miRNA profile of infected cells. miRNAs are small non-coding RNAs of about 19-24 nt length which act as posttranscriptional gene regulators by either translational arrest or mRNA degradation. The aim of our study was to understand the contribution of viral and virus regulated cellular miRNAs to tumorigenesis. The generation of miRNA profiles of DLBCLs and NK/T-cell lymphoma and combinatorial target predictions should let us to target identification and functional analysis of differential expressed miRNAs. A number of tumour samples each of EBV-positive and EBV-negative were pooled and their miRNA profiles were generated by deep sequencing. The expression of selected miRNAs was validated by quantitative Real-Time PCR in the tumour samples and by Northern blotting in tumour derived cell lines. To confirm predicted miRNA-target interactions, luciferase reporter assays and western blot analysis were used.
All known EBV miRNAs with the exception of those derived from the BHRF1 were present in both tumour entities. Apparently, the BHRF1 miRNA cluster is not expressed in primary tumours of immunocompetent patients. The viral miRNAs represented only about 2% of all miRNAs expressed in both tumour types and so the question arise whether they contribute to the tumour development. Comparing EBV-positive with EBV-negative DLBCLs, 9 miRNAs were upregulated and 7 were downregulated with a more than 1.5 fold regulation and an expression of at least 0.05%. The NK/T-cell lymphoma showed 15 induced and 16 repressed miRNAs in the EBV-positive tumours when miRNAs should show a more than 2 fold regulation and an expression of at least 0.1% in both of the libraries. A combination of published mRNA data, our miRNA profiles of EBV associated lymphomas and a combination out of three independent target programs resulted in different target predictions for deregulated, cellular miRNAs. Selected miRNAs could be shown to have an effect on their target wt 3'UTRs in luciferase assays but not after the mutation of the corresponding seed sequence in the 3'UTR. Endogenous target protein levels were also downregulated by overexpressing the corresponding miRNA in cell lines.

Comprehensive characterization of microRNA deregulation in colorectal cancer

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Altered expression of microRNAs (miRNAs) is associated with development and progression of various human cancers, where they mainly regulate the translation of oncogenes and of tumor suppressor genes. In colorectal cancer (CRC), these regulators complement the Vogelstein multi-step model of pathogenesis and have the potential of becoming a novel class of tumor biomarkers and of therapeutic targets. To identify miRNAs deregulated in CRC, we examined by Quantitative Real-Time PCR the global expression of 632 mature miRNAs in 40 CRCs and their paired non-tumor tissues. Simultaneous confidence Intervals (SCIs) for each miRNA were calculated and twenty-three miRNAs whose expression is significantly regulated were identified. Many of them have previously been reported in CRC, others, such as miR-139-5p, miR-204, miR-512-3p and miR-517c, miR-885-5p and miR-30a* were found for the first time involved in CRC. We subsequently evaluated the association of the 23 miRNAs with different clinical characteristics of the samples and presence of alterations in the molecular markers of CRC progression (APC, TP53, KRAS and loss of 18q arm). Expression levels of miR-31 were correlated with CA199 and miR-18a, miR-21 and miR-31 were associated with mutations in APC gene. To investigate the downstream regulation of the differentially expressed miRNA identified we integrated putative mRNA target predictions with the results of a meta-analysis on eight public gene expression datasets of Normal and Tumor samples of CRC patients and extracted miRNA-RNA pairs with opposite expression patterns. The list of possible miRNA gene targets was further filtered by focusing on hub and bottleneck genes in a protein-protein interaction network. Many of these genes belong to key pathways related to CRC progression such as Wnt/TGF-beta, MAPK and TP53 signaling which are currently being validated functionally. This study has identified promising miRNAs to be used as biomarkers (for example in plasma) in diagnostic and prognostic settings of CRC. And, in addition it shows their direct involvement in the regulation of driving events of CRC carcinogenesis which should be explored further in biological models.

microRNAs as key regulators of osteoblast differentiation

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Osteogenesis, or the formation of bone tissue,
depends on the differentiative potential of mesenchymal stem cells (MSCs). These multipotent stem cells can differentiate into a variety of cell types, which include osteoblasts, chondrocytes and adipocytes. Differentiation into any of the lineages is tightly regulated by distinct transcription factors, which is why dysfunctional regulation can have pathological consequences. One of the key transcription factors required for osteoblast differentiation is osterix.

Over the past two decades, the importance of microRNAs (miRNAs) in many regulatory pathways and related pathologies has been established. In the present study we aim to find a role for miRNAs in the regulation of osterix. We demonstrate that one miRNA controls osterix expression and provide evidence for direct binding of the miRNA to the 3’ untranslated region of this transcription factor. In addition, the miRNA-target interaction affects, at least in part, osterix mRNA stability.

Currently, we are investigating the biological and pathological relevance of the regulation of osterix by this miRNA. It is in our interest to acquire knowledge about the contribution of the miRNA to bone cancers, in which the tumorigenic potential can be related to the inhibition of MSC differentiation.

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Regulatory interactions between AP-1 transcription factors and microRNAs in tumorigenesis.

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MicroRNA (miRNA) dysregulation has recently emerged as a major determinant of tumorigenesis. Nearly every hallmark of cancer is affected by both oncogenic and oncosuppressor miRNAs, which control cell cycle progression, apoptosis, invasiveness, metastasis and angiogenesis.

Several tumor-associated miRNAs have been characterized as components of regulatory feedback loops involving nuclear oncoproteins and tumor suppressors. One paradigmatic example is represented by the regulatory network formed by the Myc and E2F oncoproteins and the miR-17-92 onco-miRNA cluster. On the other hand, a well characterized oncosuppressor miRNA, miR-34a/b/c, is a p53 transcriptional target and key executor of the p53 growth-inhibitory and pro-apoptotic functions.

The AP-1 transcription factors are critical targets of multiple tumorigenic pathways. The oncogenic role of AP-1 is complex, because of the large number of homo- and hetero-dimers formed by JUN (c-Jun, JunB, JunD) and FOS (c-Fos, FosB, Fra-1, Fra-2) family members, which play distinct, sometimes antagonistic functions, depending on the cell-context. Each JUN and FOS member is regulated at multiple levels, by transcriptional, posttranscriptional and posttranslational mechanisms, but very little is known about the miRNA-mediated control of AP-1 components in tumorigenesis.

MiR-21, representing one of the best-characterized oncogenic miRNAs, is overexpressed in the majority of solid and hematopoietic tumors, where miR-21 exhibits mainly antiapoptotic and pro-invasive roles.

In an in vitro system of thyroid cell transformation, we have recently reported that the miR-21 precursor (pri-miR-21) is transcriptionally regulated by AP-1 in response to the RAS oncprotein. We have characterized a novel autoregulatory mechanism, in which the AP-1-induced downregulation of the proapoptotic factor PDCD4 is essential for the full stimulation of AP-1 activity in response to RAS (Talotta et al., Oncogene 2009).

More recently, we have identified new miR-21 targets in thyroid cells, including the transcript encoding for a novel oncosuppressor homeoprotein, that we have found downregulated by RAS in a miR-21-mediated fashion. In addition, given the prognostic relevance of miR-21 in lung cancer, we have studied miR-21 in a NSCLC cell line, in which we have investigated the role of miR-21 in cell-matrix adhesion and TGF-beta-mediated EMT (Epithelial-Mesenchymal-Transition).

In addition to investigating the functions of AP-1 as transcriptional regulator of miRNAs, we have also analyzed the miRNA-dependent
regulation of AP-1 during tumorigenesis. Our recent results, showing that two AP-1 components frequently overexpressed in neoplastically transformed cells are targeted by two major oncosuppressor miRNAs, point to novel regulatory mechanisms of AP-1 activity during tumorigenesis and in response to chemotherapeutic drugs.

MiR-205 expression levels in non-small cell lung cancer do not always distinguish adenocarcinomas from squamous cell carcinomas.

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Background: Classification and therapy of lung tumours is based on accurate histological diagnosis. As most cases of lung cancer are detected when tumours are unresectable, the great majority of lesions are diagnosed on very small cytological or biotic samples, which are frequently a diagnostic challenge for pathologists. A panel of immunohistochemical markers, which are differentially expressed in adenocarcinomas (ADC) and in squamous cell carcinomas (SQC), greatly improves the reliability of cytohistological diagnostic activity. However, there is still a relevant proportion of cases which may escape precise classification. Recent data suggest that a different diagnostic approach based on the quantification of micro RNA expression may be more much more reliable than histology in distinguishing ADC from SQC. A panel of immunohistochemical markers, which are differentially expressed in adenocarcinomas (ADC) and in squamous cell carcinomas (SQC), greatly improves the reliability of cytohistological diagnostic activity. However, there is still a relevant proportion of cases which may escape precise classification. Recent data suggest that a different diagnostic approach based on the quantification of micro RNA expression may be more much more reliable than histology in distinguishing ADC from SQC.

Aim: Aim of the present study is to analyse a series of well characterized surgically removed lung tumours using real time PCR to quantify the expression of mir 205 in relation to histotype.

M&M: A series of 40 consecutive lung carcinomas (20 Adenocarcinomas and 20 squamous cell carcinomas) resected between 2002 and 2005 were retrieved from the archives of the Unit of Surgical Pathology of the S.Chiara Hospital in Trento, Italy. All cases have been routinely formalin-fixed and paraffin-embedded. Histological slides of all cases have been reviewed and only clear-cut well- to medium-differentiated carcinomas were selected. Poorly differentiated tumours whose classification was not straightforward were excluded. Heterogeneous tumours were also excluded. Particular attention was paid to exclude adeno-squamous carcinomas. For each case one representative paraffin block was selected and 6 sections were cut and used for total RNA extraction. Quantification of microRNA expression was carried out using TaqMan MicroRNA Assay kits according to manufacturer’s protocol (Applied Biosystems, Foster City, CA, USA). Real-time RT-PCR assay is based on a stem-loop RT primer design. U6snRNA is a widely used endogenous reference RNA in microRNA quantification experiment. Hsa-miR-205, hsa-miR-21, and U6snRNA were measured by qRT-PCR in triplicate. Normalized Ct of hsa-miR-205 and hsa-miR-21 were calculated by subtracting AvgCtU6 from AvgCtmir205 or AvgCtmir21, respectively, Ct205 = AvgCtmir205 - AvgCtU6 and Ct21 = AvgCtmir21 - AvgCtU6. Results: RNA has been successfully extracted from all samples and amplification of miR-21, U6 and miR-205 has been possible in all cases. The relative level of miR-205 was generally lower in AC as compared with SQC. Most SQC showed high levels of miR-205 and their “sample score” was below the proposed cut-off value of 1.5 to classify tumors in the two broad categories of AC and SQC. Interestingly a relevant number of ADC would have been classified as SQC (4 out of 20) and 3 out of 20 SQC would have been misclassified as ADC. Discussion: Recently Bishop et al. reported that the relative quantification of miR-205, in comparison to two other small non-coding RNAs (hsa-miR-21 considered an “oncomiR” and U6, an housekeeping sn-RNA) is a very reliable diagnostic tool. In their hands miRNA analysis was able to correctly classify all 50 ADC and 52 SQC on resected specimens and all but one of the 20 biotic specimens. Our present results, although confirming high miR-205 expressions in lung SQC as compared with ADC, clearly show that this approach may misclassify a non negligible percentage of cases. Which are the possible causes of this discrepancy?
A first hypothesis could relate to tumour heterogeneity. In fact lung tumours, although frequently subdivided for adjuvant treatment selection in ADC and SQC, are much more complex. ADC can be subdivided in several different entities based on morphology alone or immunophenotype and molecular profiling, possibly underlining histogenetic or molecular differences. Even SQC, which seem a more homogeneous group of tumours, may show differences in location (peripheral versus central), morphology and immunophenotype suggesting that we are still missing their possible heterogeneity. Alternatively, it is possible that slight technical differences in the procedure might have influenced our results. If this is the case, a test bases on quantification of miR-205 might be less robust than expected for diagnostic use.

A deeper insight into bladder cancer: identification of new micro-RNA as tumor markers

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Bladder cancer is a common malignant cancer in economically advanced nations. About the 95% of bladder cancer (transitional cell carcinoma) originates from the urothelial epithelium. The disease presents two forms: non-muscle invasive tumors and muscle-invasive cancers. The 5-year survival rate of advanced bladder cancer is approximately 20-40%. There is an obvious urgent need for novel diagnostic and prognostic tools for this disease and effective therapies against bladder cancer. Recent findings indicate miRNAs as a class of 21-25 nucleotides small non-coding RNAs negatively regulating gene expression at post-transcriptional level and involved in temporal and tissue-specific eukaryotic regulation. Comparison between human cancers and their normal counterparts revealed that miRNAs exhibit differential expression profiles in normal versus cancer tissues and unique miRNAs expression signatures could be used to define biological or clinical features of human cancers. We investigated the expression signature and the involvement of miRNAs in bladder cancer by miRNA microarray profiling using TaqMan Human MicroRNA Array A and B (Applied Biosystem) in normal and cancer tissues. We identified 23 up and 19 down-regulated miRNAs in the malignant versus normal tissues. We also carried out a bioinformatic analysis of genes targeted by the aberrantly expressed miRNAs using PicTar (http://pictar.mdc-berlin.de), TargetScan (http://www.microrna.org) and DIANA microT (http://diana.pcbi.upenn.edu) web tools. We identified a number of genes potentially targeted by one or more miRNAs. Our potential target genes were investigated, by using GEO database of gene expression data from NCBI, in their differences in mRNA expression in bladder tumor compared with healthy tissue. We found a set of putative target genes with inverse expression pattern to that of their targeting miRNAs, suggesting they play a regulatory role in expression.

Our data reveal new insights into the disease biology of bladder cancer because provide innovative information on putative targeting miRNAs as regulators of the genes already known to be differentially expressed in bladder cancer. Therefore our study can have an important implications regarding tumor diagnosis, prognosis and therapy in bladder cancer.

MiRNA expression in Osteosarcoma

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Osteosarcoma (OS) is made up of mesenchymal osteoblastic cells producing bone substance. Although rare in the general population, this tumour is the most common primary malignancy of bone and the fifth most common primary malignancy of adolescence. In 75% of cases it is manifested between 10 and 30 years of age with a preference for the male sex at a ratio of 1.5-2:1. The skeletal areas more frequently involved are the distal femur and the proximal tibia, followed by the proximal humerus. In the long bones, OS principally arises in the metaphysis and the metaphysis.

Conventional therapy for OS has reached a plateau of 60% - 70%, a 5-year survival rate that has changed little in two decades, highlighting the need for new approaches as the introduction of new targets for therapy.

MicroRNAs (miRNAs) are approximately 22 nucleotide-long noncoding RNAs involved in several biological processes including development, differentiation and proliferation. Recent studies suggest that knowledge of miRNA expression patterns in cancer may have substantial value for diagnostic and prognostic determinations as well as for eventual therapeutic intervention.

We first performed comprehensive analysis of miRNA expression profiles in 3 OS cells lines (MG63, 143b and U2OS) by TLDA cards to identify potential miRNAs involved in this tumour. In all cell lines we found overexpression of miRNA miR-484, miR-196a, miR-9 and miR-183 as compared with osteoblast cells.

Then, we evaluated their expression in 23 OS samples, 10 low grade and 13 high grade, and paired normal tissue by using Real Time PCR analysis. The results showed a significant lower expression of all miRNAs in tumour samples as compared with paired normal tissue, when we compared high grade with low grade OS, we found that miR-484 and miR-196a were more expressed in the first group. These results are in accordance with previous data reported in literature that describe miR-196a with oncogenic potential promoting cell proliferation, cancer cell detachment, migration and invasion.

Our data suggest that miR-196a expression could be relevant in the biological and clinical behaviour of OS, and in vitro functional studies are on-going to better investigate its role as potential prognostic marker and drug target.

Non coding RNAs as new biomarkers in the assessment of prostate cancer diagnosis: a pilot study


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Prostate cancer (PCa) is the most frequent male cancer and is the second worldwide cause of death in men. The mean age of patients with this disorder is 72-74 years, and about 85% of patients are diagnosed after age 65 years. Apart from age and ethnic origin,
a positive family history is probably the strongest known risk factor. The recorded incidence of prostate cancer has substantially increased in the past two decades, probably because of the introduction of screening with prostate-specific antigen (PSA), the use of improved biopsy techniques for diagnosis, and increased public awareness. Unfortunately, PSA is characterized by low sensitivity and specificity for acceptable false-positive rates and so the discovery of new PCa biomarkers characterized by a higher specificity and sensitivity is mandatory. Several authors have recently shown that non coding RNAs, such as Prostate Cancer Antigen 3 (PCA3) and miRNAs could represent new promising tumor biomarkers in PCA.

The purpose of this pilot study is to develop a highly specific molecular test using new biomarkers in urine and plasma, for early detection of prostate cancer and for its use as an alternative method to the traditional biopsy.

Forty-seven consecutively admitted patients to the INRCA Hospital Urology Center were recruited. After prostate massage, urine and blood samples were drawn from each patient and RNA was extracted by standard method. PCA3 mRNA/PSA score and miRNAs (miR
16 and miR 141) assay were analyzed by means of two semi-quantitative Real-time PCR methodologies. From preliminary analysis, positive results from biopsies are associated with higher levels of PCA3 mRNA/PSA score and miRNA 141 in respect to patients in which the biopsies tests resulted negative.
Call for: 2011 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;
- 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 25,000 laboratory, translational, clinical and epidemiological scientists engaged in all areas of cancer research in the United States and in more than 60 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 (April 2011), and the
memorial Korsmeyer lecture at the VIMM in Padua and Tecce lecture at the Regina Elena Cancer Institute in Rome, and will receive the award in a ceremony at the Foundation’s headquarters in Trento, Italy (May, 2011). The award consists of a prize of € 75.000 and a commemorative plaque.

Nomination Deadline: September 15, 2010
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