Associazione di **B**iologia **C**ellulare e del **D**ifferenziamento

Mechanisms of Signal Transduction

Programme & Abstracts

Florence, 16-17 March 2012

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Organisers

Annarosa Arcangeli (Chair) - University of Florence

Sara Sigismund (co-Chair) - Institute for Molecular Oncology Foundation (IFOM), Milan

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PROGRAMME

Friday, 16 March

13:30 **Registration**

14:30-17:00 **Session 1**

Cell signalling in cancer (I)

Chair: Sara Sigismund (Milan)

Federica Fusella (Turin)
Morgana: a novel player in apoptotic resistance and metastatic progression in breast cancer cells

Laura Maiorino (Milan)
The COP9 Signalosome is a new repressor of the biological barrier against liver tumorigenesis

Arcangela Gabriella Manente (Novara)
Estrogen receptor β severely impairs mitochondrial functions in human malignant pleural mesothelioma

Alessandro Zannini (Trieste)
Notch1 and notch4 require pin1 to elude the tumor suppressor barrier imposed by fbxw7α in breast cancer

Sara Chiaretti (Milan)
Regulation of tumor cell migration and invasion by the adaptor protein liprin-α1

Giuseppina Votta (Naples)
Modulation of cell migration and matrix remodeling by c-Myc expression in an immortalized human epithelial cell line

Simone Polvani (Florence)
COUP-TFII downregulation inhibits pancreatic cancer growth

17:00-17:30 **Coffee break**

17:30-18:30 **Poster Session**

18:30-19:30 **Plenary Lecture**

Chair: Annarosa Arcangeli (Florence)

Piero Crespo (Santander, Spain)
New concepts for inhibitors of Ras-ERK signals

20:00-21:30 **Dinner**

21:30-23:00 **Poster Session**

Chair: Annarosa Arcangeli (Florence)

Saturday, 17 March

8:10-9:20 **Session 2**

Cell signalling in cancer (II)

Chair: Laura Moro (Novara)

Tullio Genova (Turin)
Arachidonic Acid-mediated tumor vascularization: signal transduction and TRP channels balance

Lucia Napione (Turin)
Unraveling the influence of endothelial cell density on VEGF-A signaling

Mario Chiariello (Siena)
Signaling by the ERK8 MAP kinase in autophagy and cancer

Claudia Iavarone (Milan)
New insights into the role of the endocytic protein Epsin3 in breast cancer

9:20-10:50 **Session 3**

Cell signalling in the control of gene expression

Chair: Mauro Torti (Pavia)

Giulia Bon (Rome)
p73 sustains chemoresistance in anaplastic thyroid tumors by inducing β4 integrin-dependent PI3K/Akt survival pathway

Maurizio Risolino (Naples)
The transcription factor Prep1 triggers the epithelial to mesenchymal transition by modulating the sensitivity of A549 cells to TGF-β

Viviana di Giacomo (Chieti-Pescara)
PKC α mediated specific gene expression occurs in the inflammatory and stress response of human gingival fibroblasts to 2-hydroxyethyl methacrylate

Michela Palmisano (Milan)
Modulating the teratogenic potential of the mouse embryonic stem cells (ESC)

Mauro Prato (Turin)
Malarial pigment activates p38 MAPK and NF-kappaB pathways in human monocytes: effects on degranulation and inflammatory response

10:50-11:20 **Coffee break**

11:20-12:50 **Session 4**

Cell signalling in the control of cell differentiation and development

Chair: Guido Tarone (Turin)

Irene Franco (Turin)
Class II PI3K-C2α: a novel regulator of vesicular trafficking at the base of the primary cilium

Giusy Tornillo (Turin)
Role of p130Cas in the control of epithelial cell commitment and differentiation in the mammary gland

Gianni Guidetti (Pavia)
Regulation of the guanine nucleotide exchange factor CalDAG-GEFI by protein kinase A

Benedetta Cerruti (Candiolo, Turin)
Geometry, topology, and out-of-equilibrium dynamics in epithelial morphogenesis

Monika Pema (Milan)
The role of mTORC1 in renal cyst formation and transformation

13:15-14:15 **Lunch**

15:00 **Visit to the Benozzo Gozzoli Chapel**

(only for pre-registered particpants)

ABSTRACTS

Oral Presentations

in alphabetical order
(presenting authors are shown underlined)

p73 sustains chemoresistance in anaplastic thyroid tumors by inducing β4 integrin-dependent PI3K/Akt survival pathway

G. Bon[1], R. Loria[1], A. Prodosmo[1], S. Soddu[1], F. Moretti[2], R. Falcioni[1]
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Most epithelial thyroid carcinomas derive from the malignant transformation of follicular cells. These tumors usually present a favorable prognosis, if treated with a combined approach that associates radical surgery with radioiodine exposure. Unfortunately, some patients will develop undifferentiated anaplastic thyroid carcinoma, unresponsive to radioiodine. A combination of chemotherapeutic drugs is presently the best therapeutic approach for these patients. However, a large number of these individuals develop resistance to chemotherapy.
Others and we found that the p53-family member p73 is expressed in well-differentiated and undifferentiated thyroid carcinomas but is not present in normal thyroid tissues, suggesting that p73 might be involved in thyroid carcinogenesis. Indeed, in thyroid carcinomas, p73 is not up-regulated by DNA-damaging agents and fails to induce either cell-cycle arrest or apoptosis. Moreover, although p73 shows remarkable structural and functional similarities to p53, data from primary tumors and knockout mice argue against p73 being a classical tumor suppressor.
Here we show that p73 sustains an important survival mechanism in thyroid anaplastic tumors by regulating the expression of β4 integrin subunit. The α6β4 integrin activates several key signaling pathways in carcinoma cells. In particular this integrin promotes the activation of PI3K/Akt survival pathway by regulating the expression of ErbB-3 growth factor receptor at the translational level.
Specifically, we found that the silencing of p73 by RNA interference strongly down-regulates the expression levels of β4 integrin subunit. As a result of the abrogation of β4-dependent activation of PI3K/Akt pathway, anaplastic thyroid cancer cells are sensitized to chemotherapeutic treatments upon p73 siRNA. In this condition Adriamycin and cis-platinum treatments induce 45% to 87% apoptosis, determined by PARP cleavage, in resistant and partially resistant anaplastic cell lines.

Geometry, topology, and out-of-equilibrium dynamics in epithelial morphogenesis

B. Cerruti[1], A. Puliafito[1], A.M. Shewan[2], W.Yu[3], K.E. Mostov[3], L. Primo[1], G. Serini[1], A. Celani[4], A. Gamba[1,5,6]
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Epithelial spherical structures, such as acini, alveoli, and follicles, are widespread in metazoans. Proper functioning of these aggregates is crucially dependent on a well-ordered architecture, which is typically disrupted in tumours and often serves as a valuable prognostic indicator. The correct cell arrangement is crucially dependent on the aggregate morhodynamics, which can be studied with representative *in vitro* models, such as cysts. Mechanical constraints and cellular apico-basal polarity are the key aspects in the appearance of normal phenotypes, i.e. spheroidal monolayers delimiting a single spherical lumen. In order to characterize the geometry and topology of *in vitro* growing cysts, we
reconstructed the three-dimensional structure via computer-assisted segmentation, both in the healthy and in the aberrant case. We show that cell-cell contact statistics bears a clear signature of the out-of equilibrium character of cyst morphogenesis. Out-of-equilibrium dynamics drives the appearance of the aberrant multiluminal phenotype, unless strict control of cell division geometry is enforced. Theoretical modeling and numerical simulations allow for a quantitative comparison with experimental results and unveil the presence of multiple local equilibria associated with geometrically frustrated configurations. To falsify these novel framework, we predict and verify that the healthy single-lumen phenotype can be rescued and the quasi-equilibrium topology attained upon inducing tissue fluidization, through the inhibition of ROCK activity.

Regulation of tumor cell migration and invasion by the adaptor protein liprin-α1

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The metastatic process requires the ability of cancer cells to break the basement membrane and migrate through a complex three-dimensional environment. We have identified Liprin-α1 as a regulator of cell edge dynamics in moving cells, and demonstrated that Liprin-α1 is required to maintain the persistence of MDA-231 breast cancer cell directional migration. The impairment of directed migration following silencing of Liprin-α1 in tumor cells correlates with enhanced instability of the lamellipodia. Liprin-α1 depletion also affects the stability of invadopodia, and this effect may underlie the strong reduction of MDA-231 cell invasion and extracellular matrix degradation. The role of Liprin-α1 on invasion is supported by the immuno-histopathological analysis of samples from human breast cancers, where the expression of this protein is often enhanced with respect to normal breast tissue. In order to clarify the molecular mechanisms linking liprin-α1 to invasion, we are addressing the role of its interactors in the underlying cellular processes. Liprin-α1 partners include the ubiquitous scaffold proteins ERC1a, Liprin-β1 and β2. Our results indicate that simultaneous silencing of Liprin β1 and β2 affects the migration and invasion of MDA-MB231 cells. Moreover, we have confirmed the direct interaction and the colocalization of Liprin-α1 with ERC1a near the polarized front of these cells. Silencing of ERC1a affects not only cell invasion in vitro but also cell motility: haptotactic migration is affected, and migration in 3D-like fibrillar matrices is less efficient, with a reduction in directional motility. Accordingly, ERC1a depletion results in the reduction of the number of lamellipodia and of their halflife. Altogether our findings show that Liprin-α1 and its interactors Liprin-β and Erc1a regulate the efficiency of tumor cell motility.

Signaling by the ERK8 MAP kinase in autophagy and cancer

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ERK8 is the last identified member of the MAP kinase family. Its activity can be modulated by serum, DNA damage and activated human oncogenes such as BCR/ABL and RET/PTC3. In line with the role of all MAP kinases in controlling gene expression, ERK8 has been described to stimulate the activity of the c-Jun proto-oncogene, while reducing the activity of nuclear receptors such as androgen receptor, glucocorticoid receptor and estrogen-related receptor alpha. Interestingly, recent data suggest a role for ERK8 in cell transformation and in the maintenance of genomic integrity. ERK8 has been, in fact, involved in transformation in human colon cancer cells, and in the protection of genomic integrity by inhibiting PCNA degradation and by stimulating telomerase activity, suggesting this kinase as an important player in the mechanisms contributing to bypass replicative senescence and to immortalize tumor cells. However, its contribution to signal transduction pathways controlling cellular physiology and, possibly, human diseases is still poorly understood

We have recently identified ERK8 as a novel regulator of basal and stress-induced cellular autophagy. In addition, we have established important molecular details to understand how ERK8 stimulates autophagy, through demonstration of direct physical interaction of this kinase with LC3/GABARAP proteins. Based on the recognized participation of autophagy to regulation of cancer progression and therapy, we also suggest a new role for ERK8 in controlling BCR/ABL-dependent autophagy and cellular transformation. Altogether, our results suggest a new function for ERK8 as a regulator of autophagy and cancer, supporting the use of this MAP kinase as a potential novel therapeutic target for this disease.

New concepts for inhibitors of Ras-ERK signals

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An overwhelming body of data unquestionably links the Ras-ERK pathway to cellular transformation and to the upbringing of human malignancies. Ras is the most frequent oncogene in human cancers, being detected in approximately 30% of human tumors. If to this figure, we add the cases in which activating mutations are detected, in a non-overlapping occurrence, in other component of the pathway, in particular B-Raf, the frequency nearly reaches 50%. Thus, in the past decades colossal efforts have been devoted to the development of therapeutic agents whereby aberrant Ras signals and subsequently tumor progression, could be prevented. However, a broad clinical use of these drugs, mostly classical kinase inhibitors, has been somewhat limited by peculiarities, some still unexplained, of the Ras-ERK route, by canonical resistance acquisition and by unacceptable toxicity levels. Are there alternative ways to target the Ras-ERK pathway so as to deliver more efficient while less toxic inhibitory molecules?. In this respect, for the past ten years our laboratory has been exploring two parallel venues: the spatial specificity displayed by Ras-ERK signals as a source of potentially less toxic targets and non-catalytic protein-protein interactions among components of the route as a source of more specific, less-resistance-prone objectives. In this respect, we have recently identified ERK dimerization as a promising target for anti-tumoral therapies.

PKC α mediated specific gene expression occurs in the inflammatory and stress response of human gingival fibroblasts to 2-hydroxyethyl methacrylate

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2-hydroxyethyl methacrylate (HEMA), deriving from polymerized resinous biomaterials, can diffuse at gingival and tooth pulp level[1]. Our study aimed to investigate the human gingival fibroblasts (HGFs) response to a low HEMA concentration, in terms of inflammatory genes expression and signal transduction proteins activation.
Cultured HGFs were exposed to 3 mM HEMA for 0, 24 or 96 h. At each experimental point Reactive Oxygen Species (ROS) production were investigated by flow cytometry[2]; Tumor Necrosis Factor-alpha (TNF-α) and cyclooxygenase-2 (COX-2) gene expression were determined by RT-PCR and prostaglandin E2 (PGE2) production was detected by an enzyme immunoassay. Since HEMA treatment decreased vitality and induced apoptosis in HGFs, at the same experimental points PKCs activation, Bax and Apaf-cyt C immunoprecipitate expression were evaluated by Western Blotting analysis and NOS activity by a specific "in vitro" assay.
24h HEMA incubation induces an increase in ROS production persisting up to 96 h. 24 and 96h HEMA treatment significantly enhances TNF-α and COX-2 gene expression compared to control in a time-dependent manner. 96h HEMA incubation significantly increases PGE2 concentration in the culture medium, PKC α expression and activation, iNOS activity and Bax expression when compared to control. Interestingly, a reduced percentage of apoptotic cells and a reduced ROS production is evidenced in the presence of bisindolylmaleide VIII, a PKC α pharmacological inhibitor, giving specificity to our data.
All in all, these results suggest that 24 or 96h 3 mM HEMA treatment induces in HGFs an inflammatory response modulated by ROS production, PKC α activation and TNF-α gene expression increase. Such pathways converge on the up-regulation of COX-2 gene expression, which leads to the increase of PGE2 release, inducing cell proliferation arrest and apoptosis occurrence.

1. Schweikl et al. J Dent Res 2006;85:870-877
2. Ito Y, Lipschitz DA. Methods Mol Biol 2002;196:111-116

Class II PI3K-C2α: a novel regulator of vesicular trafficking at the base of the primary cilium

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The class II phosphoinositide 3-kinase PI3K-C2α is a protein of the early endocytic compartment and of the trans-Golgi network. Main features of this protein are the ability to bind clathrin and the catalytic activity towards different lipid substrates involved in membrane dynamics. In agreement, the involvement of PI3K-C2α in different processes of vesicular trafficking has been reported. However, the physiological relevance of these processes remains to be elucidated.
Through the generation of a Pik3c2a knock-out mouse strain, we discovered that PI3K-C2α was fundamental during embryonic development and that its loss principally affected the structure and function of a cellular organelle involved in development: the primary cilium.
Analysis of Pik3c2a deficient embryos revealed that primary cilia were shorter and swollen and displayed a defect in accumulating signaling proteins, such as Smo and Polycystin-2. Consistently, the mutation conferred features of ciliopathy: homozygous mutant embryos died at midgestation dysplaying laterality defects and impaired Hedgehog signaling, while heterozygous adults showed renal cysts susceptibility after kidney injury.
Analysis of primary mouse embryonic fibroblasts (MEFs) indicated that PI3K-C2α was excluded from the ciliary axoneme, but highly enriched at the basal body. Moreover, the absence of the protein in Pik3c2a knock-out MEFs caused a specific reduction of vesicular trafficking at the cilium base.
All these data indicate that PI3K-C2α is required for the correct exchange of structural proteins and signaling molecules between the cilium compartment and the cytoplasm and suggest PI3K-C2α as a novel regulator of vesicular trafficking at the base of the primary cilium.

Morgana: a novel player in apoptotic resistance and metastatic progression in breast cancer cells

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Morgana has been recently reported as a protein involved in centrosome duplication and genomic stability. In mammalian cells it forms a complex with Hsp90, Rho kinase I and II. Furthermore it has been described as a stress responsive protein with an intrinsic chaperone activity. Here we show that NIH3T3 overexpressing Morgana are transformed in vitro and in vivo. Furthermore NIH overexpressing Morgana are more resistant to several apoptotic stimuli (detachment, serum starvation, DNA damage induced by etoposide). Our results indicate that Morgana acts as an anti-apoptotic factor by inhibiting ROCK I kinase activity. Indeed rescue experiments demonstrate that Morgana overexpressing cells with constitutively active ROCK show a restoration of apoptosis sensitivity.
Moreover, by the analysis of Morgana expression in several tumor cell lines, we also found the up-regulation of the protein in aggressive cancer cell lines.
To assess the role of Morgana overexpression in breast carcinoma, expression of endogenous Morgana is knocked down in breast cancer cells. Silenced cells show reduced ability to grow in soft agar and a higher rate of apoptosis upon etoposide treatment. Therefore Morgana downregulation has no effect on in vitro cell proliferation but results in reduction of anoikis resistance and decrease in cell motility. Besides, lung colonization experiments reveal that Morgana is required for metastatic ability of breast cancer cells. In addition human breast tumor tissue arrays suggest a strong correlation between Morgana expression and the degree of tumor malignancy.
Taken together these results provide novel insights on the role of Morgana overexpression in conferring cells some oncogenic hallmarks.

Arachidonic Acid-mediated tumor vascularization: signal transduction and TRP channels balance

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Tumor vascularization is a critical process that determines tumor growth and metastasis. Several studies have shown that tumor endothelial cells (EC) possess a distinct phenotype, differing from normal ECs at both molecular and functional levels. Intracellular Ca(2+) signals are involved in the regulation of the complex process of angiogenesis and tumor progression. These signals are generated by a wide variety of intrinsic and extrinsic factors. Therefore, the mechanism(s) involved in agonist-induced Ca(2+) signaling is a potentially relevant target for controlling angiogenesis and tumor growth. We had previously reported a key role for arachidonic acid (AA)-mediated Ca(2+) entry in the initial stages of tumor angiogenesis in vitro; we recently showed that the cAMP/PKA pathway is involved in this process. We also reported that AA promotes a PKA-dependent increase of migration in ECs derived from human breast carcinomas (BTEC), but not in 'normal' EC (HMVEC). Here we show that two AA-modulated TRP channels (TRPV4 and TRPM8) regulate EC migration and exert an opposite role and an opposite differential expression in BTEC and HMVEC. In particular, TRPV4 is highly expressed in BTEC but not in HMVEC; its activation by AA or 4αPDD (a selective TRPV4 agonist) is correlated with greater Ca(2+) entry and increases the migration in BTEC but not in HMVEC. Furthermore knockdown of TRPV4 expression completely abolished AA-induced BTEC migration. On the contrary TRPM8 (that is negatively modulated by AA) is highly expressed in HMVEC but not in BTEC and its activation by icilin (a selective TRPM8 agonist) decreases the migration rate of HMVEC but not of BTEC. Moreover TRPM8 silencing of this enhances the migration rate in HMVEC while its overexpression in BTEC decreases their migration, suggesting a potential anti-angiogenic role in vitro. The study shed new lights on the activity of these channels which could be strategic for understanding the process of tumor angiogenesis.

Regulation of the guanine nucleotide exchange factor CalDAG-GEFI by protein kinase A

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Rap GTPases control several cellular processes including adhesion, cell-cell junction formation, polarity, exocytosis, and proliferation, by switching between an inactive GDP-bound and an active GTP-bound conformation. In circulating platelets, the highly expressed Rap1b protein is essential for integrin inside-out activation, spreading, aggregation, and thrombus formation. All these responses are inhibited by the potent platelet antagonists prostaglandins, that induce intracellular cAMP increase and protein kinase A (PKA) activation. Rap1b is a known substrate for PKA, but phosphorylation of Rap1b alone does not prevent GTP binding. In platelets the major regulator of Rap1b activation is the Ca^{2+}- and DAG-regulated guanine nucleotide exchange factor CalDAG-GEFI. Using a platelet lysate as a source of cytosolic enzymes in a kinase assay, we demonstrated that purified GTS-tagged CalDAG-GEFI can be phosphorylated. CalDAG-GEFI phosphorylation was incremented by addition of cAMP and suppressed by the inhibitor H89, suggesting the involvement of PKA, an hypothesis that was confirmed by a binary kinase assay performed using the purified PKA catalytic domain. A rapid and sustained phosphorylation of CalDAG-GEFI also occurred in transfected 1321N1 cells treated with the cAMP-increasing agents forskolin or prostaglandin E_1. Quantification of 32P-phosphate incorporation on CalDAG-GEFI revealed two distinct phosphorylation sites, that were identified as Ser116 and Ser586 by site-specific mutagenesis. PKA activation triggered phosphorylation of endogenous CalDAG-GEFI also in platelets, as demonstrated by immunoblotting with a phospho-specific antibody. Under these conditions, the activation of Rap1b induced by Ca2+ ionophore was completely inhibited. We suggest that PKA-mediated phosphorylation of CalDAG-GEFI represents a novel mechanism for cAMP-dependent inhibition of Rap1b activation.

New insights into the role of the endocytic protein Epsin3 in breast cancer

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Epsin3 (Epn3) belongs to the Epsin family of endocytic proteins. Unlike other Epsin members which are ubiquitously expressed, Epn3 expression has been reported to be restricted to migrating keratinocytes and down-regulated following cell differentiation, suggesting that its expression may be spatially and temporally regulated. Furthermore, Epn3 has been found specifically up-regulated in pathological conditions, including human cancer.
We have analyzed the expression of EPN3 in human breast cancers by tissue microarray analysis and we have shown that it is, indeed, overexpressed in approximately 30% of the breast tumors. Moreover, western blot and Q-PCR experiments showed that several breast tumor cell lines overexpress Epn3 in comparison to normal counterparts.
Based on this, we propose now to study the function of Epn3 and its involvement in human tumors, employing both in vitro and in vivo approaches. To characterize in vitro the role of Epn3 we initially set-up stable knock down (KD) of Epn3 in BT474 cells overexpressing Epn3, and we performed a series of functional studies, including classical tumorigenic assays. Our preliminary data suggest that ablation of Epn3 impairs anchorage-independent growth, as assessed by soft-agar assays. We are planning to perform xenograft experiments by injecting subcutaneously Epn3-KD and control cells in immunodeficient mice to score for differences in tumor formation and/or growth.
As complementary experiments, we overexpressed Epn3 in MCF10A, a human normal mammary cell line, showing low levels of the protein. According to our preliminary results, it seems that Epn3 overexpression could induce transcriptional and morphological changes, which are typical of an epithelial-to-mesenchymal transition (EMT). Furthermore, Epn3 increases the capacity of these cells to form mammospheres in vitro, suggesting a possible role of Epn3 in the breast cancer stem cells. This result will be further consolidated in vivo. Furthermore, different strategies are on going in order to look into the molecular mechanisms involved.

The COP9 Signalosome is a new repressor of the biological barrier against liver tumorigenesis

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Sustained signalling by a mutated or overexpressed oncogene triggers a strong growth-arrest response mediated by the DNA damage response activation, which represents a biological barrier against progression of cancer beyond its early stages. The COP9 signalosome (CSN) is a key protein complex involved in controlling turnover of critical substrates including both oncogenes and effectors of the oncogene-induced response. The CSN positively modulates protein degradation through its catalytic subunit CSN5/JAB1 that removes the modifier Nedd8 from the cullin component of E3 ubiquitin ligases. A large body of evidence suggests that functional up-regulation of the CSN is selected early in the clonal evolution of cancer and frequently co-segregates with gains or amplifications of defined oncogenes. The overall goal of my study was to assess the impact of genetic alterations of CSN5/JAB1 function on the onset and progression of hepatic tumors and to provide a proof of principle that the CSN can be viewed as a potential therapeutic target in cancer. To this purpose, we devised a protocol to induce liver tumors in mice based on a sequential promotor-initiator treatment. At early stages of tumor development adult CSN5/JAB1$^{flox/flox}$ mice were genetically manipulated, by infection with adeno-associated viruses, to either express the dominant negative CSN5/JAB1 protein or delete the CSN5/JAB1 allele (CSN5/JAB1$^{del/del}$). Our results show that interfering with CSN5/JAB1 function in livers that have undergone tumor promotion yields a markedly reduced tumor burden. No lesions developed in CSN5/JAB1$^{del/del}$ livers. Collectively this findings suggest that targeting of CSN function during early stages of carcinogenesis relieves the complex repression on the oncogene-induced response resulting in an impairment of liver cancer progression.

Estrogen receptor β severely impairs mitochondrial functions in human malignant pleural mesothelioma

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We firstly described a positive prognostic role of Estrogen Receptor beta (ERβ) in human Malignant Pleural Mesothelioma (MMe). By in silico analysis of microarray data we generated an ERβ gene expression meta-signature, identifying 172 genes differentially expressed between 40 MMe patients with High or Low ERβ expression. Among genes down-regulated in the High ERβ group we identified the SDHB gene coding for the B subunit of the mitochondrial respiratory chain (MRC) complex II and among the up-regulated the α-ketoglutarate dependent histone H3 Lys-27 demethylase, KDM6B. The aim of this work was therefore to evaluate the relationships between these genes and assess whether ERβ acts as a tumor suppressor in MMe by interfering with mitochondrial functions. Using in vitro MMe cell models, we demonstrated that SDHB down-regulation induced a significant increase in ERβ gene expression, in a KDMB6 dependent manner. Conversely, high ERβ expression caused a reduction in the SDHB protein levels and in COX7AR gene expression, significantly compromising both MRC complexes II and IV activity and mitochondrial ATP production. In addition, high ERβ expression severely impaired the oxidative phosphorylation pathway, caused mitochondrial fragmentation and lead to an increased glucose-dependence. These data were also validated in *in vivo* experiments using mice models.

Unraveling the influence of endothelial cell density on VEGF-A signaling

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Vascular endothelial growth factor-A (VEGF) is the major determinant for the activation of the angiogenic program leading to the formation of new blood vessels. VEGF specific binding to VEGF receptor-2 (VEGFR-2) is critical for triggering different signaling pathways including phospholipase Cγ (PLCγ) and Akt cascades, crucial for endothelial proliferation, permeability and survival. We carried out a quantitative *in vitro* analysis of these pathways by studying cultures of long-confluent and sparse endothelial cells that, at different VEGF doses, mimic the *in vivo* conditions of quiescent and angiogenic endothelium. We performed accurate quantitation of the phosphorylation levels of VEGFR-2, PLCγ and Akt, and integrated the results by means of a mathematical model. We found that: (i) cell density influences VEGFR-2 protein level, as receptor number is 2-fold higher in long-confluent than in sparse cells; (ii) cell density affects VEGFR-2 activation by reducing its affinity for VEGF in long-confluent cells; (iii) despite reduced ligand-receptor affinity, high VEGF concentrations provide long-confluent cells with a larger amount of active receptors; (iv) PLCγ and Akt are not directly sensitive to cell density, but simply transduce downstream the upstream difference in VEGFR-2 protein level and activation; (v) the mathematical model correctly predicts the existence of at least one protein tyrosine phosphatase (PTP) directly targeting PLCγ and counteracting the receptor-mediated signal. The principal finding of this study is the formal demonstration of the influence of endothelial cell density on the activation of the early triggered signaling events along VEGF/VEGFR-2 axis. Our results provide a basic framework suitable for further extensions that will shed light on the complexity of the VEGF signaling, and prompt for future investigation aimed at identifying the unrecognized PTPs for the development of potential therapeutic strategies.

Modulating the teratogenic potential of the mouse embryonic stem cells (ESC)

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ESCs have the potential to differentiate into all cell types, but the immunological rejection and the teratoma formation stand as obstacles in the path of ESC-based therapy.
Our proposal aims at investigating and developing ESCs as a therapeutic tool by ESC genetic manipulation, in order to preserve their differentiation potential, while escaping a development into teratoma.
We established a mouse ESC culture protocol enabling ESCs to grow in absence of both FBS and feeder cells, allowing easier manipulation and usage of ESCs. The ESCs cultured in suspension were validated for the maintenance of stemness and pluripotency, and to confirm their differentiation potential we performed an in vivo teratoma formation assay.
To inhibit teratoma formation we modulated the self-renewal and/or differentiation patterns, by modulating ESC-specific miRNAs that control gene expression patterns associated with pluripotency and cell cycle control. We demonstrated in vitro, by transient transfection of antago-miR294 and confirmed by stable transduction of lentiviral sponge vector, that the inhibition of miR294 blocks the downregulation of p21, and the indirect upregulation of c-Myc, leading to a slight reduction of proliferation, as assessed by CFSE staining, without interfering with the pluripotency and differentiation marker expression. On this basis, to analyze the teratogenic potential, we performed the teratoma formation assay, by s.c. injection of ESC in NSG mice.
At day 21 post injection the tumor formed by ESC/scramble are palpable in all mice, whereas the engineered ESC failed at all to form teratoma, suggesting the implications of miR294 in the tumorigenicity of ESC.
To confirm this results, we will perform the teratoma formation assay with newly engineered ESC, we will perform a differentiation ability assay, the transcriptome analysis and the apoptosis assay in stress conditions, to exclude the possibility that the cells undergo apoptosis upon injection in vivo.

The role of mTORC1 in renal cyst formation and transformation

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The mTOR pathway has been implicated in the evolution of several different types of cancer, including renal cell carcinoma (RCC). In several syndromes, the kidney is affected by cyst formation, considered benign initial lesions of the renal tubule which progressively evolve to cystadenomas and RCCs. The molecular determinants of these manifestations are currenty unknown.
We have developed a mouse model recapitulating this progressive formation of cysts followed by transformation into cystadenomas by homozygous inactivation of the *Tsc1* gene in the collecting ducts of the kidney. *Tsc1*$^{flox/-}$:KspCre mice display cyst formation after P9, papillary projections arising from the wall of the cysts by P28 and cystadenoma formation by P56.
Intriguingly, we have found that cells lacking the *Tsc1* gene downregulate the epression levels of Polycystin-1 (PC1). Rapamycin treatment restores PC1 expression levels, suggesting an mTORC1 dependent regulation of the protein. PC1 is the protein mutated in ADPKD, a genetic disorder characterized by massive renal cysts formation. Our hypothesis is that downregulation of PC1 following mTORC1 activation in the kidneys of *Tsc1*$^{flox/-}$:KspCre mice might be the step that initiates renal cyst formation and then something else accounts later for their transformation.
Morphological analysis of kidneys from *Tsc1*$^{flox/-}$:KspCre mice at P56 revealed that cystadenomas appear like acinous formations characterized by round-shaped cells that lack correct localization of E-cadherin at the cell-cell junctions. The expression levels are however unaltered. We hypothesize that trafficking of E-cadherin might be regulated by mTORC1 and that its mislocalization might account for the transformation of cysts into cystadenomas. We are currently testing this hypothesis.

COUP-TFII downregulation inhibits pancreatic cancer growth

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Background and aim: Alterations in molecular pathways regulating cell survival, proliferation, metabolism, and migration have been identified in pancreatic cancer. However, no substantial improvements in the clinical prognosis have been made and pancreatic cancer continues to be a leading cause of cancer death in the Western world.
The orphan nuclear receptor COUP-TFII is an important regulator of a wide range of biological processes. Interestingly, COUP-TFII may exert a pro-oncogenic role regulating tumor vascularization, and the proliferative and metastatization behavior of cancer cells. Although there are not direct evidences linking COUP-TFII to pancreatic cancer, indirect evidences suggest that the receptor could be potentially involved in this disease. In fact, COUP-TFII is a downstream effector of hedgehog, Wnt/β-catenin, and RAS-MAPKs pathways that are constitutively activated in pancreatic cancer. Furthermore, activation of PPARγ suppresses pancreatic cancer growth in vitro and in vivo and COUP-TFII is a negative regulator of PPARγ. The aim of this study is to evaluate the expression of COUP-TFII in primary human pancreatic tumors and to examine its role in the regulation of tumor growth in nude mice.
Methods: COUP-TFII expression in human pancreatic tumor samples was evaluated by immunohistochemistry. Pancreatic cancer cell lines expressing shRNA against COUP-TFII in an inducible manner were produced and injected in nude mice.
Results: COUP-TFII is expressed in primary samples and correlates with overall survival. Silencing of COUP-TFII determines a reduction in the proliferation, migration, anchorage independent growth and it strongly inhibits tubule formation, whereas in nude mice the silencing reduces tumor growth by 50%.
Conclusions: Our results indicate that COUP-TFII is an important regulator of the behavior of pancreatic adenocarcinoma, thus representing a possible new target for pancreatic cancer therapy.

Malarial pigment activates p38 MAPK and NF-kappaB pathways in human monocytes: effects on degranulation and inflammatory response

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INTRODUCTION. Phagocytosis of malarial pigment (HZ, hemozoin) impairs several functions of human monocytes by enhancing release of pro-inflammatory molecules (i.e. TNFalpha, IL-1beta, MIP-1alpha) and of enzymes stored in gelatinase granules (i.e. MMP-9, TIMP-1, lysozyme). Here the mechanisms underlying HZ-dependent enhanced release of these molecules were investigated. METHODS. Human monocytes were fed with HZ and treated with/without SB203580 (inhibitor of p38 MAPK phosphorylation) or quercetin, artemisinin and parthenolide (inhibitors of cytosolic I-kappaBalpha phosphorylation/degradation, p50/p65 NF-kappaB subunits nuclear translocation, and p65/DNA binding, respectively). Thereafter, we studied in cell lysates the following parameters: cytosolic phospho-p38 MAPK, phospho-I-kappaB and I-kappaB or nuclear p50/p65 protein levels by WB and p65/DNA binding by EMSA. Meanwhile, in cell supernatants we studied the release of the following proteins: TNFalpha, IL-1beta, and MIP-1alpha by ELISA; MMP-9 by gelatin zymography; TIMP-1 by WB; lysozyme by spectrometric assay. RESULTS. HZ promoted p38 MAPK phosphorylation, I-kappaB phosphorylation/degradation, p50/p65 nuclear translocation, p65/DNA binding. As expected, p38 MAPK signalling was inhibited by SB203580, while quercetin, artemisinin and parthenolide blocked NF-kappaB pathway activation. All these inhibitors abrogated the HZ-enhanced release of TNFalpha, IL-1beta, MIP-1alpha, MMP-9, TIMP-1 and lysozyme. CONCLUSION. In human monocytes, HZ promotes release of pro-inflammatory factors and degranulation of enzymes stored in gelatinase granules by activating p38MAPK and NF-kappaB pathways. These findings provide new information useful to clarify mechanisms of malaria pathogenesis, and to design specifically targeted adjuvant therapy in complicated malaria.

The transcription factor Prep1 triggers the epithelial to mesenchymal transition by modulating the sensitivity of A549 cells to TGF-β

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Prep1 is a transcription factor belonging to the TALE (Three-Aminoacid-Loop-Extension) family of homeoproteins, along with the MEIS and PBX family members. Prep1 stands for Pbx-Regulatory-protein-1, on the basis of its ability to form tripartite complexes with PBX and HOX proteins. It modulates the expression, nucleo-cytoplasmic distribution, target site specificity and transcriptional activation of Pbx. Recently, Prep1(Pknox1) has been characterized as a novel haploinsufficient tumor suppressor, able to antagonize the B-cell-lymphomagenesis in the Eμ-MYC transgenic mouse model. Accordingly, Prep1 is undetectable or strongly downregulated in many human tumors, including a significant fraction of non-small cell lung cancer (NSCLC).
To dissect the Prep1-dependent mechanisms implicated in lung tumorigenesis, we have investigated the function of Prep1 in NSCLC. By both gain- and loss- of function analysis of Prep1 in the NSCLC-derived A549 cell line, we have found that Prep1 is a novel regulator of EMT (Epithelial Mesenchymal Transition). TGF-β is a well characterized regulator of EMT in advanced carcinoma cells, which are insensitive to the cytostatic effect of TGF-β. We have found that Prep1 expression is induced in response to TGF-β and modulates the mesenchymal transition, motility, invasiveness and proliferation rate of A549 cells. Functional analysis of the Prep1-dependent control of EMT revealed that Prep1 increases the TGF-β sensitivity of A549 cells by controlling the activity of Smad3, which is essential for the Prep1-induced changes.
In summary, these results allow to propose a new role of the transcription factor Prep1 in lung cancer progression, by demonstrating for the first time that Prep1 is able to modulate the response to the TGF-β pathway and the Epithelial to Mesenchymal Transition.

Role of p130Cas in the control of epithelial cell commitment and differentiation in the mammary gland

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Like most adult tissues, the mammary epithelium has a hierarchical organization: stem cells via a series of progenitors give rise to two major cell types, the basal and the luminal cells, which include ductal and alveolar cells. However, signaling pathways that specify mammary lineages are not well known. We previously reported that the adaptor protein p130Cas is upregulated in breast cancers and that its overexpression promotes mammary tumorigenesis. Here we describe how high levels of p130Cas affect cell fate decisions and normal homeostasis in the mammary epithelium. By a deep analysis of MMTV-LTR-p130Cas transgenic mice, which overexpress p130Cas in the mammary gland, we found that MMTV promoter-driven p130Cas overexpression is mainly addressed to the mammary progenitors and profoundly alters the cell composition of the mammary epithelium. Indeed, MMTV-p130Cas mammary cells display a strong upregulation of genes peculiar of basal cells. Moreover, progenitors from MMTV-p130Cas glands preferentially differentiate into basal rather than luminal cells in vitro. In addition, during pregnancy transgenic glands show an aberrant alveolar development as well as an accumulation of immature alveolar progenitors and reduced levels of milk proteins. It has been recently reported that mammary progenitor cells, the major target for the MMTV-LTR in our mouse model, highly express the tyrosine kinase receptor c-kit. Interestingly mouse mammary epithelial cells (MMECs) from MMTV-p130Cas animals exhibit hyperactivation of c-kit in the presence and even in the absence of its ligand. Notably, wt MMECs transduced to express a constitutively active c-kit result biased towards a basal differentiation route and refractory to mature into alveolar cells, thus clearly mimicking p130Cas overxpressing cells. Together these data shed light on a novel function for p130Cas and c-kit, whereby p130Cas seems to act in the control of mammary cell differentiation.

Modulation of cell migration and matrix remodeling by c-Myc expression in an immortalized human epithelial cell line

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The serine protease urokinase (uPA) and its receptor (uPAR) play a central role in tumor development because of their ability to regulate cytoskeleton dynamics and matrix integrity. Moreover, uPAR signalling prevents apoptosis of RPE retinal pigment epithelial cells1.
Among the gene products regulating cell proliferation and survival, the oncoprotein c-Myc is one of the most relevant in tumorigenesis. In an attempt to characterise the effect of c-Myc activation on uPA/uPAR expression as well as on the cell survival/apoptosis balance, we took advantage of a 4-hydroxitamoxifene activatable form of c-Myc (MycER) in the RPE cell line. We found that c-Myc activation induces sensitisation to apoptosis and reduction in cellular motility as consequence of a negative regulation of uPA and uPAR mRNAs and proteins2.
These effects are counteracted by further expression of V12Ras Furthermore, conditioned media of hT-RPE/MycER following c-Myc activation modulate the motility of parental hT-RPE cells and 3T3 fibroblasts. Analysis of secretoma of c-Myc expressing hT-RPE cells reveals changes in the expression of many proteases, matrix-remodeling factors and factors associated to growth inhibition and cellular senescence3.
These findings may provide insights into the multiple activites of c-Myc during oncogenic transformation, highlighting its ability to overcome senescence and to induce changes in cell motility properties.

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Notch1 and notch4 require pin1 to elude the tumor suppressor barrier imposed by fbxw7α in breast cancer

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Notch signaling controls different cellular processes during normal breast development and in breast cancer progression. Although molecular details of Notch pathway alteration in other tumors are known, it is not clear how Notch-dependent oncogenesis is elicited in the mammary gland. Proteasome-mediated degradation induced by the E3 ubiquitin-ligase Fbxw7α is a key event in blocking the Notch signaling cascade, as witnessed by T-ALLs that loose this control due to mutations hitting NOTCH1 or FBXW7. In human breast cancers, where genetic alterations of these genes do not occur, we observed that Notch1 signaling is activated despite presence of Fbxw7α. Here we demonstrate that in this kind of tumors Notch1 and also Notch4, another Notch family member and conserved target of Fbxw7α, escape from proteasomal degradation following a conformational switch elicited by interaction with the prolyl-isomerase Pin1, an enzyme frequently over-expressed in breast cancer. As a consequence, Pin1 uncouples Fbxw7α from intracellular Notch1 and Notch4 that accumulate, endowed with enhanced transcriptional and oncogenic activitiy. Fbxw7α displayed a strong negative effect on Notch-dependent transformation and self-renewal of putative breast cancer stem cells, but simultaneous over-expression of Pin1 rescued these phenotypes. Being Pin1 a direct target gene of both Notch1 and Notch4, a selfsustaining circuitry might be established to promote breast cancer aggressiveness eluding Fbxw7α negative regulation.

ABSTRACTS

Poster Presentations

in alphabetical order
(presenting authors are shown underlined)

Hydrogen sulfide as a signaling molecule in vascular physiopathology

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Hydrogen sulfide (H2S) is now considered the third member of the gasotransmitter family (together with NO and CO)with functions in different tissues and systems. In particular in vascular endothelium H2S may act as vasorelaxant and antiinflammatory agent as well as a stimulator of angiogenesis. Here we investigate the effects of this molecule in vascular physiopathology considering tumor vascularization and the possible protective role in heart injury and consequent endothelial cell dysfunction.
Ca2+ imaging experiments show that NaHS, a H2S donor, activates calcium signals both in normal endothelial cells (HMEC) as well as tumor-derived endothelial cells (BTECs), with differences in peak amplitude and sensitivity between the two cell lines. While NaHS fails to promote either migration and proliferation on HMECs, BTEC migration was enhanced at low H2S concentrations. The involvement of hydrogen sulfide in the response of a proangiogenic factor was studied using a Cystathionine γ-lyase inhibitor (a H2S producing enzyme), which reduces the percentage of VEGF-responding cells in Ca2+ imaging, and inhibits B-TEC migration.
In the second part of the research we evaluated the protective role of H2S on heart injury and endothelial cell dysfunction by the use of a rat cardiomyoblast cell line (H9C2) as well as the HMEC. The protective role of NaHS for oxidative stress and myocardial injury was studied preconditioning HMEC and H9C2 with NaHS followed by the incubation with H2O2 or in hypoxic chamber. In both experiments H2S protect cells from death. Moreover Ca2+ imaging in H9C2 reveals a reduction of calcium fluxes by H2S release: this effect is mimicked by nifedipine, a L-type calcium channel inhibitor. Interestingly nifedipine preconditioning protects H9C2 from H2C2 or hypoxia.
These data suggest an important role of H2S as a signaling molecule involved in vascular functions as both proangiogenic and protective agent, through direct or indirect calcium channels regulation.

The tyrosine kinase Abl is a component of macrophage podosomes and is required for podosome formation and function

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Background: Activation of the tyrosine kinases of the Abl family is a key step in signal transduction to the cytoskeleton in both hematopoietic e non hematopoietic cells. Our own recent work (Baruzzi et al. FEBS Lett. 584:15-21, 2010) implicated Abl and Src kinases in macrophage migration and here we investigated the role of Abl in podosome formation and function.
Materials and methods: Murine and human macrophages and murine fibroblasts were assayed for cell migration, gelatin degradation and podosome formation by standard assays. Murine macrophages were transfected by electroporation with a mixture of four siRNA designed to silence Abl expression (Dharmacon).
Results: In this study we show that tyrosine kinases of the Abl family are present in podosomes formed by murine and human macrophages and murine fibroblasts. Silencing of Abl expression by siRNA or Abl enzymatic inhibition with imatinib results in disassembly of podosomes and reduction of matrix degradation and migratory capacity of macrophages.
Conclusions: These findings suggest that podosome disassembly induced by Abl targeting may inhibit podosome-dependent leukocyte recruitment into inflammatory sites. Together with the recent evidence that Abl kinases localize to invadopodia and regulate matrix degradation in carcinoma cells our findings highlight drug targeting of Abl as a possible strategy to inhibit neoplastic cell invasive capacity and cancer-related inflammation at the same time.

Urokinase plasminogen activator receptor (uPAR) interacts with vitronectin to influence cancer growth

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uPAR and vitronectin (VN) are two interacting proteins that are have been correlated with the malignant progression and metastasis of cancer. However the direct uPAR-VN interaction in the cancer process has never been investigated to date.
A complete functional alanine scan of human uPAR, pinpointed the extracellular matrix (ECM) protein VN as the critical uPAR-interactor required to induce cell adhesion, migration, and signaling in vitro. To determine if the direct uPAR/VN-interaction is important in tumor formation and progression, we exploited a xenograft mouse model of tumorigenesis. For this purpose muPAR, muPAR mutant unable to interact with VN (muPARW32A) or a muPAR variant lacking of the Domain1 (D1), required for both VN and uPA binding (muPARΔD1), were expressed in HEK293 Flp-In GFP positive cells and injected in the fourth mammary fat pad of immunodeficient mice. Cells expressing the wt receptor significantly reduced the time of palpable tumor formation, whereas palpable tumor formation induced by muPARW32A or muPARΔD1 were comparable to mock cells. Moreover uPAR-VN interaction is required to accelerate cancer growth rate, since the W32A point mutation negatively regulated the tumor growth. The obtained in vivo data are completely in agreement with in vitro results emphasizing uPAR-VN interaction as a key element to promote cell proliferation and to prevent programmed cell death.
Moreover, requirement of uPAR-VN interaction to promote cell spreading and migration suggested a possible role of this interaction in cancer progression, identifying this molecular interaction as a possible target for anti-cancer therapy.

A model for EGFR ubiquitination and endocytosis

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Epidermal growth factor receptor (EGFR)-dependent signaling is involved many physiological processes, and its deregulation leads to many cellular disfunctions and pathologies, commonly related to cancer.
Endocytosis has a crucial impact on downstream EGFR signaling response and it is regulated by ligand concentration. Indeed, depending on the EGF dose, the EGFR can be internalized through clathrin-mediated endocytosis (CME) or non-clathrin endocytosis (NCE). The switch between CME and NCE occurs over a narrow range of EGF concentrations (1-10 ng/ml). Importantly, receptor ubiquitination shows a threshold response over the same range of EGF doses and it indeed is responsible for the commitment of EGFR to NCE. Importantly, the switch between CME and NCE has a crucial impact on signaling, since CME is mainly for recycling and sustaining of signals, while NCE cause signal extinction through EGFR degradation.
In collaboration with system biology group in our Institute, we have designed a mathematical model of EGFR activation that quantitatively accounts for the ubiquitination threshold observed in vivo. Importantly, the model is also able to predict the behavior of the receptor upon different perturbations. A crucial aspect for the biological validation of the model was to obtain quantitative data to be integrated in mathematical formalism. To this aim we have set-up a quantitative ELISA-based assay to follow EGFR ubiquitination and phosphorylation. With this tool, we have started the validation of some predicted responses of the system to perturbations relevant to cancer, such as variation in EGFRs number on the cell surface. Finally, the impact of these perturbations on signaling and downstream biological response will be also investigated. Data will be presented.

Molecular characterization of the Meis1 oncogenic activity; possible competition with Prep1

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Meis1 and Prep1 are members of the diverged TALE class homeobax gene family essential during early mouse development. They act as vertebrate Hox cofactors and regulate the subcellular localization and stability of Pbx proteins.
Meis1 was originally identified at the sites of retroviral insertion leading to acute myeloid leukemia in BXH-2 mice. It is a well known oncogenic collaborator of HoxA9 in a significant proportion of human leukemias. Meis1 accelerates the onset of HoxA9-induced AML.
In contrast to Meis1, Prep1 does not accelerate the onset of HoxA9-induced AML. TMA data on a set of different human tumors indicate that Prep1 is not (or under) expressed in many tumors. Furthermore Prep1i/i mice develop spontaneous tumors, mostly lymphomas, but also carcinomas. Altogether these findings indicate that Prep1 might have tumor suppressor activity.
So far the oncogenic pathway of Meis1 is mainly unknown. Also no information is available on the possible oncogenic role of Meis1 in solid tumors. Moreover no study has been done concerning the possible competition between Prep1 and Meis1, since they both act by binding to Pbx1.
Therefore the aims of the present study are to unravel the Meis1 oncogenic activity and try to figure out the possible competition between Meis1 and Prep1 in tumorigenicity. Our preliminary data shows that, unlike Prep1+/+ MEFs in which Meis1 alone is not sufficient for transformation, Prep1i/i MEFs are susceptible to neoplastic transformation by Meis1a overexpression. The tumor formation is partially inhibited by overexpression of Prep1. We are currently exploring the region of Prep1 capable of neoplastic inhibition.
In conclusion, the absence of Prep1 makes cells susceptible to transformation by the single oncogene Meis1. This may be due to either a specific Prep1-Meis1 competition, or to the absence of the tumor suppressive function of Prep1.

Non-integrin cell adhesion triggers ligand-independent integrin signaling

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IIntegrins are the major family of adhesion receptors responsible for the physical contact and biochemical communication between cells and the extracellular matrix (ECM). The engagement of integrins with ECM triggers "outside-in" signaling, resulting in context-dependent changes in cell morphology, migration and proliferation.
We now demonstrate that integrins conducts outside-in signaling independently of their ligand binding activity as long as firm cell binding to the matrix is sustained by other adhesion receptors. The urokinase plasminogen activator receptor (uPAR) is a non-integrin vitronectin (VN) adhesion receptor linked to the outer membrane leaflet by a (GPI)-anchor. Through a structure-function analysis of uPAR, VN, β1 and β3 integrins, we document that uPAR-mediated VN adhesion triggers integrin-mediated, but ligand independent, cell spreading and signaling. Ligand-independent integrin signaling is not restricted to uPAR as it poses no identifiable constraints to the adhesion receptor with respect to ternary-structure and ligand type. Consistently, we show that cell adhesion mechanically supported by a signaling-incompetent β3 integrin is effectively translated into β1 integrin-dependent cell spreading and signaling.

Angiotensin-converting-enzyme (ACE) insertion/deletion (I/D) polymorphism and pterygium. A case-control study in Sardinian population

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Pterygium is a common ocular surface disorder characterized by proliferation, inflammatory infiltrates, fibrosis, angiogenesis and extracellular matrix breakdown. Epidemiological studies indicate exposure chronic to UVB light as the most important risk factor for the development of pterygium. The Angiotensin Converting Enzyme (ACE) is the major component of the Renin-angiotensin system (RAS). It converts the inactive decapeptide Angiotensin I (Ang I) to the active octapeptide Angiotensin II (Ang II). Ang II is the most potent vasoconstrictor and stimulant of the aldosterone release. Recent discoveries have demonstrated that Ang II is also involved in cell proliferation, apoptosis, angiogenesis and tissue fibrosis. Moreover, it acts as growth factor and participates in inflammatory responses. The gene encoding ACE is mapped on chromosome 17q23; it contains 25 introns and 26 exons and shows a polymorphism characterized by the presence (insertion, I) or absence (deletion, D) of a 287-bp Alu sequence of DNA in intron 16. The presence or absence of Alu sequence in the ACE gene leads to the D/D, I/D and I/I genotypes. Novel studies have reported that the absence or presence of specific ACE I/D polymorphisms within ACE gene in several illnesses, such as cardiovascular diseases and breast cancer, can confer increased risk to develop the pathologies. Due to these evidences and the pterygium features, the aim of our study is to evaluate the ACE I/D gene polymorphism in relation to pterygium risk in a case-control study within a group of Sardinian population.

Novel extraribosomal function of human ribosomal protein rpL3

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Surveillance of ribosome assembly plays an important role in the regulation of cell growth and defects in ribosome biogenesis can lead to cell cycle arrest or apoptosis. Mounting evidences show that several ribosomal proteins may be involved in this regulation through extraribosomal function. A main outcome of the impairment of the ribosome synthesis is the nucleolus destruction and the p53 stabilization through the inhibitory interaction between some ribosomal proteins and MDM2. The effects of p53 activation are mainly mediated by upregulation of p21 which promotes cell cycle arrest or apoptosis, depending on the cellular context. In addition to p53, several other proteins are able to activate p21 expression. It has been demonstrated that various protein factors involved in ribosome biogenesis such as nucleophosmin (NPM) can regulate p21 expression p53-independently at transcriptional and post-translational levels. In a previous study, we have reported a direct protein-protein interaction between NPM and the ribosomal protein rpL3, required for the autoregulatory circuit of the rpL3 expression. We wondered whether this interaction could occur also in another context and whether rpL3 could be involved in the regulation of p21 expression. In order to verify this hypothesis, we first analyzed changes in p21 protein levels in p53-null Calu-6 cells upon alteration of rpL3 production. We performed transient transfection experiments of a construct encoding for the recombinant protein HA-rpL3 in the aforementioned cell lines. We observed that the enforced expression of the HA-rpL3 protein resulted in a dose-dependent increasing of p21 protein amount. In addition, the rpL3-mediated p21 upregulation was associated with cell cycle arrest in Calu-6 cells. The results of these experiments will be discussed.

Nestin and vimentin intermediate filaments expression related to cytoplasmic anchoring of glucocorticoid receptor in cutaneous melanoma

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Nestin, a class VI intermediate filament (IF), is considered as a cancer stem cell marker of malignancies of neuroectodermal origin and as a prognostic factor in several tumors. It may play a role in connecting the components of cytoskeleton and in coordinating changes in cell dynamics. It is well known that nestin copolymerizes into heteromeric filaments with class III IF-proteins, mostly vimentin, contributing to his disassembly during mitosis. Vimentin is ubiquitously expressed in normal mesenchymal cells and its overexpression in cancer well correlates with poor prognosis, invasion and accelerated tumor growth. Recently, vimentin has also been recognized as a marker for epithelial-mesenchymal transition, a process which enhances cell migration and invasion. It is known that nestin, when copolymerized with vimentin, modulates glucocorticoid receptor (GR) function by cytoplasmic ancoring. GR is a nuclear receptor that, when activated by his specific ligand, can act as a transcription factor that binds to GRE (glucocorticoid response elements). It affects inflammatory responses, differentiation and cellular proliferation.
The cancer stem cell hypothesis suggests that mutated melanocyte stem cells are present in skin as precursors of melanoma cells. In fact, genetic and/or epigenic alterations occurring in the multipotent tissue-specific adult stem cells may lead to their malignant transformation. It was shown that nestin expression in both tumoral and endothelial cells is an important early prognostic marker in melanoma.
Based on these considerations, the aim of our study was to investigate the colocalization of nestin, vimentin and GR in cutaneous melanoma by immunofluorescence and immunohistochemistry methods. Furthermore, we evaluated if intermediate filaments composed of nestin and vimentin mediate cytoplasmic anchoring of the unliganded GR, impeding negative regulation of growth by GR. In addition we correlated these data to clinical-pathological variables.

Role of miR-361 in the regulation of VEGF in HUVEC

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Angiogenesis, the formation of new blood vessels from existing ones, is a process tightly regulated by pro-angiogenic and anti-angiogenic factors. Hypoxia stimulates angiogenesis by inducing the expression of several pro-angiogenic factors, such as VEGF. Recent evidence indicate that small non-coding RNA molecules, called microRNAs (miRNAs), can bind the 3'UTRs of mRNAs and affect their translation, regulating several processes, including angiogenesis. Limited information is available regarding the function and target genes of miRNAs in endothelial cells. In order to identify new miRNAs involved in the post-transcriptional modulation of angiogenesis-related genes, we used specific algorithms to select five putative miRNAs for HIF1α, STAT3, and VEGF genes. The expression of selected miRNAs was evaluated in Human Umbilical Vein Endothelial Cells (HUVEC) cultured in normoxic and hypoxic conditions. We found that the expression of the three miRNAs including miR-361, was reduced by hypoxia. In a previous study, we demonstrated that hypoxia increses VEGF and that Somatostatin (SRIF), a widely distributed polypeptide with antiangiogenic activity, prevented the hypoxia-induced up-regulation of VEGF through the activity of two transcription factors, HIF-1 and STAT3 (Dal Monte et al., 2011). In the present study, we found that SRIF prevented the hypoxia-induced reduction of miR-361 indicating that miR-361 plays a key role in the SRIF-mediated regulation of VEGF. To explore this possibility, we transfected hypoxic HUVEC with miR-361 either in the absence or in the presence of SRIF and the expression of HIF1α, STAT3 and.VEGF was evaluated. We found that STAT3 and HIF1α were unaffected by miR-361, which, in contrast regulated the expression of VEGF mRNA and protein. Our results are the first demonstration that SRIF controls miRNA expression and suggest that miR-361 plays a key role in the antiangiogenic activity of SRIF.

Role of the endocytic adaptor proteins Eps15 and Eps15L1 in the regulation of Notch signaling

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Eps15 and Eps15L1 are two endocytic adaptors involved in both clathrin-dependent and clathrin-independent internalization via their AP2 and ubiquitin binding sites. Double Knockout (DKO) mice for Eps15/Eps15L1 die between 9.5 and 11.5 dpc. The morphological analysis suggested a Notch loss of function phenotype, confirmed by QPCR of Notch target genes. Genetic evidence in Drosophila melanogaster supports a model whereby endocytic control of the ligand Delta is essential for Notch signaling. This process is dependent on the Ubiquitin E3 ligase mindbomb and the fly homolog of Epsin, liquid facet. Also in mammals the Epsins appear to be essential for Notch signaling as Epsin1/2 DKO mice have a Notch loss of function phenotype similar to the one of Eps15/L1 DKO mice. At present it is unknown whether in mammals Eps15/L1 or Epsin1/2 affect Notch signaling by affecting ligand endocytosis. To study this, we set up an in vitro coculture system using as signal sending cell the murine cell line OP expressing the ligand Dll1 (OP9-Dll1) and as signal receiving cell the C2C12 cell line expressing the Notch1 receptor (C2C12-N1). As a read-out we use a Luciferase Notch reporter assay by transiently transfecting a Notch-responsive Luciferase reporter into C2C12-N1. After validating the system we used it to evaluate the effect of silencing Eps15 and Eps15L1 in the signal sending cell. Surprisingly, we observed a ca. 40-50% reduction in Notch activity after KD of Eps15 or Eps15L1, but no further increase after the combined KD. Similarly, the KD of either Epsin1 or Epsin2 lead to a significant reduction in Notch signaling that was not increased after the combined KD. Instead, the combination of Eps15/Epn1 KD leads to a complete block of Notch signaling. These findings suggest an unexpected complexity in the regulation of endocytosis by Eps15/L1/Epsin1/2 network that we hope to at least partially untangle with a Dll1 internalization assay that we are setting up at the moment

New therapy for malignant pleural mesothelioma: a preclinical study

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Malignant mesothelioma (MMe) is an asbestos-related, lethal cancer arising from mesothelial cells of the pleura, lacking a standard therapeutic approach. Among alternative remedies to cancer, a growing interest has been directed to the preventive action of active nutrients.
We have previously shown that ascorbate is more cytotoxic to MMe cells than to non-neoplastic mesothelial cells, due to a redox mechanism. These results seem to encourage the use of ascorbate in MMe chemotherapy. In this preclinical study, we have first showed in vitro synergistic interactions of ascorbate with epigallocatechin-3-gallate (EGCG), gemcitabine, or with the complex of EGCG and gemcitabine. Thereafter, we have studied the effects of ip injections of the above combination on MMe cells engrafted into the peritoneum of immunodeficient mice. Animals have been examined at the end of treatments by survival curves, measurements of tumor weight and number, histochemical analyses, angiogenesis antibody array, and protein multiplex analysis. Data have shown that the combined therapies increase mouse survival, limit tumor growth and invasiveness, reduce the activation of cell growth signaling pathways and increase apoptosis rates in tumor growing regions. These results strongly indicates potentials for the use of this therapy in MMe clinical treatments.

Non-canonical RhoU GTPase as a crossroad between Wnt and Stat3 signaling

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RhoU is a Cdc42-like GTPase originally identified as a Wnt1 target gene and implicated in Wnt-mediated cellular migration and proliferation. Atypically, RhoU expression is not constitutive but inducible. We have demonstrated that Wnt1-mediated RhoU transcriptional induction does not involve the canonical β-catenin-dependent pathway but requires JNK activity instead, which is typical of the non-canonical Wnt/Planar Cell Polarity pathway (Schiavone et al, Biochem. J.,2010). We found that also Wnt4 and Wnt5a, respectively involved in canonical and non-canonical Wnt signaling, induce JNK-dependent RhoU transcription.
We analyzed the transcriptional mechanisms leading to Wnt1-mediated induction of the RhoU promoter as a mean to characterize transcription factors involved in the still ill-defined PCP pathway. Since bioinformatic analysis of the RhoU promoter did not disclose any obvious candidate(s), we analyzed progressive 5' deletions of the 750 bp-long Wnt1-responsive region of the RhoU promoter. We found that the region between -366 and -200 was required both for basal and inducible promoter activity but could not identify any specific Wnt1-responsive element. Likewise, detailed linker scanning mutagenesis did not reveal any specific region responsible for Wnt1-mediated induction, suggesting that this requires the cooperation of many different elements. Work is ongoing to identify the molecular mechanisms involved.
Interestingly, we have shown that RhoU is also a transcriptional target of STAT3 and contributes to its pro-migratory functions. Although Wnt1-mediated RhoU induction does not require STAT3, and is thus likely to occur via completely independent mechanisms, RhoU clearly represents a point of convergence between the STAT3 and Wnt oncogenic pathways. Further studies will address the role of RhoU in mediating specific features of Stat3 and Wnt1-mediated cell transformation and potential synergy between these two important pro-oncogenic pathways.

Dissecting the role of Cbl in EGFR endocytosis

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Cbl is the major E3 ligase involved in ubiquitination of Epidermal Growth Factor Receptor (EGFR). Ubiquitination by Cbl has a critical role in EGFR endocytosis by targeting receptors to lysosomal degradation. In addition to its role as an E3 ligase, Cbl functions also as an adaptor, by recruiting several proteins involved in the early phases of clathrin endocytosis. Importantly, Cbl has been found mutated in different disorders, from myeloproliferative disease to Noonan syndrome and non-small lung cancer (NSCLC). Most of these mutations are located within the Ring finger domain and in the regulatory linker region, and are therefore predicted to affect E3 ligase activity. However, a series of mutations have been found also outside this region, suggesting that they might affect the adaptor function without altering E3 ligase activity. None of these mutations were characterized in detail at the mechanistic level. In order to have a more precise molecular picture of Cbl activity in EGFR ubiquitination and endocytosis, we plan to study different set of cancer-relevant mutants, combining two distinct approaches: 1) RNAi-based functional assays and 2) in vitro ubiquitination assays.
1) First, we knocked-down (KD) c-Cbl and its related protein Cblb in HeLa cells and we initially studied their effects on EGFR ubiquitination and endocytosis, showing a major role of c-Cbl in both processes. Reconstitution experiments with different sets of mutants are ongoing and preliminary data will be presented.
2) We were able to reconstitute in vitro the EGFR ubiquitination reaction, and now we can use this tool to test some peculiar behaviours of the system and to study the molecular details of c-Cbl and Cblb. Moreover, this assay will be helpful to characterize Cbl mutations found in cancer, in order to dissect the role of Cbl between the adaptor function and E3 ligase activity.

Bilirubin-mediated oxidative stress involves activation of the Nrf2 pathway in SH-SY5Y cells

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Background: Unconjugated bilirubin (UCB) is responsible for neonatal jaundice. However, UCB at highly elevated level is neurotoxic and can eventually cause kernicterus or death. A growing body of evidence suggests that oxidative stress as a hallmark of UCB induced neurotoxicity. Cells have developed specific mechanisms to overcome cellular stress. The transcription factor Nrf2 is a master cellular regulator of endogenous cytoprotective enzymes and a powerful sensor for cellular redox state. Upon activation, Nrf2 binds to Antioxidant Response Element and enhances the coordinated upregulation of different cytoprotective enzymes, such as, those involved in glutathione homeostasis (GCL, Gpx, xCT, and SLC6A9) and antioxidant/detoxification enzymes (HO1 and NQO1).
Materials: SH-SY5Y neuroblastoma cells were incubated with high concentration of UCB (free bilirubin concentration = 140 nM) for 1,4,8,16,and 24 hrs. Cells viability were determined by MTT assays and intracellular ROS were monitored by using H2DCFDA. Nrf2 proteins were detected in the nuclear fractions by Western blot and mRNA of Nrf2 target genes were analyzed by qRT-PCR.
Results: SH-SY5Y cells showed a 40% loss of cell viability starting 1 hour after UCB exposure. Treated cells showed an increased level of intracellular ROS at 1hr. Nrf2 proteins showed nuclear accumulation between 3 and 6 hours. This was paralleled by an upregulation of Nrf2 target genes in treated cells. Gpx and GCL mRNA expression were 2 fold higher at 8 and 16hrs, respectively. At 16hrs, SLC7A11, SLC6A9 and HO-1 mRNA were 16, 20 and 100 fold higher, respectively while NQO-1 was increased 6 fold at 24hrs.
Conclusion: In SH-SY5Y cells, exposure to UCB is followed by an increased intracellular ROS and decreased cell viability. Survival cells are able to develop an adaptive response to UCB treatment by enhancing the nuclear accumulation of Nrf2 and an upregulation of cytoprotective enzymes.

Epithelial mesenchymal transition traits in honey-driven keratinocyte wound healing

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Wound healing is of great relevance for skin medicine and a particular focus is set on natural compounds. Honey has been renowned since ancient times for its wound-repair properties but the underlying mechanisms are almost unknown. We have tried to elucidate the modulatory role of honey in an in vitro model of HaCaT keratinocyte re-epithelialization, by using acacia, buckwheat, and manuka honeys. Scratch wound and migration assays showed significant increases of wound closure rates and chemoattractant effects in the presence of honey, with similar activities among different honey types. However, the use of kinase and calcium inhibitors suggested the occurrence of different mechanisms. All honeys increased the expression of syndecan-4, while regulators of cell proliferation and cytoskeletal rearrangement showed variable activation among honeys. Re-epithelialization recapitulates traits of epithelial mesenchymal transition (EMT) and the induction of this process was evaluated by a PCR array. Data showed that honey-driven wound repair goes through the activation of keratinocyte re-epithelialization, but the ability of inducing EMT varies sensibly among honeys, according to their botanical origin.

Role of second messenger pathways in bFGF-induced calcium signals in parasympathetic neurons

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Basic Fibroblast Growth Factor (bFGF or FGF-2) has been shown to promote neuronal survival and neurite outgrowth in dissociated neurons from embryonic (E7-E8) chick ciliary ganglion (CG). The three main signal transduction pathways downstream the activated FGFR receptor are those mediated by MAPK, PI3-K and PLCγ. All of them are differentially involved in these bFGF-induced growth effects, but, while it has been shown that bFGF can elicit long lasting elevations in intracellular calcium concentration, $[Ca^{2+}]_i$, the role of the three pathways in this process has not been elucidated. Here we show, by means of pharmacological inhibitors, that all three are involved, at a different extent, in the generation of the $[Ca^{2+}]_i$ increase induced by bFGF. In particular, inhibition of the PLCγ pathway, in addition to reducing the number of responsive cells, induces, in a significant population of cells, basal calcium oscillations even in the absence of the growth factor and downregulates voltage-dependent calcium channels. This complex behaviour can be due to a perturbation in PIP_2 levels at the plasmamembrane.

Melatonin prevents chemical-induced apoptosis in u937 cells

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Melatonin, the main hormone secreted by pineal gland, has a wide range of functions which include oncostatic, anti-inflammatory and immune-stimulatory effects. Due to its capacity in eliminating free radicals, melatonin provides protection against oxidative stress (Carpentieri et al., Pharmacol Res, 2012). Several authors demonstrated that melatonin exerts anti-apoptotic actions in several cell models (Ferreira et al., Rev Assoc Med Bras, 2010). Moreover, our previous studies evidenced that melatonin prevents apoptosis induced by UV-B irradiation in hemopoietic U937 cells (Luchetti et al., J Pineal Research, 2006). In this work, melatonin activity has been investigated in the same cell line after various apoptotic chemical treatments, chosen for their different mechanisms of action, which finally determine a radical oxygen species (ROS) increase (Salucci et al., Micron, 2010). U937 cells were pretreated with melatonin and then exposed to hydrogen peroxide, etoposide and staurosporine.
Morphological techniques and statistical analysis were used to verify melatonin anti-apoptotic effect. Data obtained show that all triggers induce apoptotic death and evidence melatonin protection in all conditions. In particular, melatonin prevents apoptosis induced by hydrogen peroxide and, to a lesser extent, by etoposide and staurosporine. In addition, in cells pretreated with melatonin and then exposed to etoposide or staurosporine, another type of death or survival mechanism appeared: autophagy.
In conclusion, we found that melatonin prevents apoptosis induced also by chemical triggers, exerting its major protection against hydrogen peroxide treatment. Probably, this is due to the fact that etoposide and staurosporine have a dual mechanism of action and, besides ROS increase, they induce p53-dependent apoptosis. Furthermore, these findings reveal that melatonin induces protective autophagy preventing apoptotic cell death.

Relationship between transcription factor AP-1 and miRNAs in tumorigenesis

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The transcription factor AP-1, resulting mainly from dimerization between members of Jun and Fos families, has been implicated in multiple hallmarks of cancer, including altered cell proliferation, evasion from apoptosis, tumor invasiveness and angiogenesis.
Oncogenic-miRNAs have been discovered among the miRNAs overexpressed in human tumors, while various downregulated miRNAs have been characterized as tumor-suppressors in neoplastic cells.
Previously, we identified one of the most relevant oncomiRs, miR-21, as a transcriptional target of the AP-1 complex in response to the RAS oncoprotein. MiR-21 in turn, is able to potentiate the AP-1 activity by a positive feedback loop mechanism. Our model has been validated in (RAS -/+miR-21) transgenic mice, in which miR-21 drives the tumorigenesis by inhibiting negative regulators of the RAS/MEK/ERK pathway.
More recently, I have focussed my efforts on the study of miRNAs as negative regulators of the AP-1 complex. In particular, I have analyzed the post-transcriptional control of the oncoprotein Fra-1, which represents a key regulator of tumor cell proliferation, apoptosis, motility and invasion.
I will present data concerning the control of Fra-1 by two important oncosuppressor miRNAs, functionally relevant in colon, prostate and lung cancer. These miRNAs affect Fra-1 expression by both direct and indirect mechanisms. Functional inhibition of Fra-1 recapitulates the effects of the overexpression of these two miRNAs. Accordingly, the ectopic expression of the Fra-1 transcript lacking the 3'-UTR antagonized, at least in part, the biological function of both miRNAs.

Design and layout

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