Università di Firenze

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

MECHANISMS OF SIGNAL TRANSDUCTION

Villa La Stella, Fiesole (FI), 26 e 27 Marzo 2010

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Spring 2010 ABCD Meeting

Organized by:
Emilio Hirsch – University of Turin
Annarosa Arcangeli – University of Florence

Friday 26/3

Registration up to 12:30

12:30-13.45 **Lunch**

13:45–14:00 – Introduction: Emilio Hirsch and Annarosa Arcangeli

14:00-15:40 **Signaling in Cell Migration and Invasion**
Chairperson: *Livio Trusolino*

14.00-14.40 **Livio Trusolino**
"Met and adhesion"

14.40-14.55 **Cristina Cianflone**
Diacylglycerol kinase alpha regulates SDF1α-induced cell invasion by regulating atypical PKC and matrixmetallo proteinase 9

14.55-15.10 **Lina Cipolla**
Phosphatidylinositol 3-kinase beta in platelet integrin alphaIIbbeta3 signaling: a novel regulation by the focal adhesion kinase Pyk2 is essential for platelet spreading on fibrinogen

15.10-15.25 **Patrizia Dentelli**
Inhibition of beta 1 integrin and il-3r beta common subunit interaction hinders tumor angiogenesis

15.25-15.40 **Giulia Germena**
Functional role of ArhGAP15, a new RacGAP

15:40-16:00 **Coffee Break**

16:00-18:10 **Integrating Signals to Cell Proliferation and Carcinogenesis**
Chairperson: *Barbara Stecca*

16.00-16.40 **Barbara Stecca**
"HEDGEHOG signalling in cancer"

16.40-16.55 **Marco Demaria**
Metabolic switch between oxidative phosphorylation and glycolysis: a new role for Stat3 in tumours

16.55-17.10 **Roberta Ferretti**
Morgana/chp-1, a ROCK inhibitor involved in centrosome duplication and tumorigenesis

17.10-17.25 **Elisa Penna**
MicroRNA-214 promotes melanoma tumor progression

17.25-17.40 **Alessandra Petrucco**
Emilin1 controls proliferation in skin and in tumor development

17.40-17.55 **Maura Sonego**
Role of STAT3 and p70S6K in Breast Cancer recurrences

17.55-18.10 **Andrea Clocchiatti**
Does Histone Deacetylase 4 control breast cancer cell growth?

18.10-18.25 **Giusy Tornillo**
p130Cas cooperates with ErbB2 to promote invasion of MCF-10A mammary epithelial acini grown in 3D cultures

20:00 **Social dinner**

21:30 **Poster Session**

Saturday 27/3

7:30-8.30 **Breakfast**

8:30-10:10 **From DNA to Proteins**
Chairperson: *Antonio Musarò*

8.30-9.10 **Antonio Musarò**
"IGF signalling and muscle biology"

9.10-9.25 **Alessandra Calogero**
Role of protein kinase PAK4 in differentiation and survival of human keratinocytes and regulation of transcription factor p63

9.25-9.40 **Elisa Gaucci**
Involvement of the protein ERp57 in EGFR and STAT3 signaling

9.40-9.55 **Giulia Pinton**
Tumor repressive function of estrogen receptor β in malignant pleural mesothelioma

9.55-10.10 **Riccardo Serafini**
The nuclear factor BRD4 binds the nucleosomes containing both H3K9ac and H4K16ac in vivo

10:10-10:30 **Coffee break**

10:30-12:25 **From the Membrane into the Cell**
Chairperson: *Alessandra Boletta*

10.30-11.10 **Alessandra Boletta**
"Signaling from PKDs"

11.10-11.25 **Veronica Algisi**
Role of clathrin endocytic adaptors in controlling EGFR fate

11.25-11.40 **Sara Mari**
Regulation of the E3 ligase NEDD4 by EGFR

11.40-11.55 **Erika Rizzo**
NG2 null mice display a myopathic phenotype affecting skeletal muscles

11.55-12.10 **Raffaella Mercatelli**
FRET study of hERG1 channel and integrin-beta1 complex

12.10-12.25 **Gilda Nappo**
A SILAC Proteomic Approach Identified Novel Candidate Players in the Non-Clathrin Endocytic Pathway of the EGFR

Concluding remarks: Emilio Hirsch and Annarosa Arcangeli

13:00-14:00 Lunch

ABSTRACTS

AMPK inhibition induces apoptosis in pediatric B-ALL cells with MLL gene rearrangements

Benedetta Accordi[1], Virginia Espina[2], Marco Giordan[1], Amy VanMeter[2], Luisa Galla[1], Gloria Milani[1], Manuela Sciro[1], Ruggero De Maria[3] ,Geertruy te Kronnie[1] ,Emanuel Petricoin[2], Lance Liotta[2] ,Giuseppe Basso[1]
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Remarkable progress has been made in the past decade in pediatric Acute Lymphoblastic Leukemia (ALL) treatment, reaching cure rates of about 80%, but therapy is not yet effective in all cases. Infants with MLL gene rearrangements form the most striking example of patients who have not benefited from the improved treatment regimens. Consequently, current interest focuses on identifying new specific molecular targets to find new patient-tailored therapies. Thus, to identify aberrantly activated signal transduction pathways in MLL-rearranged patients, we used Reverse Phase Protein Microarrays (RPMA). This innovative technique can be used to quantify a highly multiplexed "portrait" of hundreds of signalling proteins at once from small clinical samples in a very reproducible, precise, sensitive and high-throughput manner. We further investigate RPMA results with in vitro studies testing the effects of a specific kinase inhibitor on apoptosis induction in leukemia cell lines.
We compared with RPMA the signal transduction pathways working state of 8 MLL-rearranged patients vs 41 without known genomic translocations ones. Phosphorylation status of 92 signalling proteins was analyzed. Based on RPMA results, we tested through proliferation and apoptosis assays the effect of Compound C, an AMPK inhibitor, on selected B-ALL human cell lines: 2 MLL-rearranged (SEM and RS4;11) and 2 non-translocated (MHH-CALL-2 and MHH-CALL-4). We then performed additional experiments to characterize Compound C-induced apoptosis.
MLL-rearranged patients show an hyperactivated pathway that, through AMPK phosphorylation, leads to BCL-2 activation. Selected cell lines respond very differently to AMPK inhibition. GI50 (Growth Inhibition) at 48h is 0.2μM for SEM, 3μM for RS4;11, and 26μM for non-translocated cell lines. LC50 (Lethal Concentration) at 48h is 7.5μM for SEM, 8.5μM for RS4;11 and 38μM for non-translocated cell lines. Compound C treatment induces activation of Caspase-3, mitochondrial depolarization, ROS production, PARP cleavage, and DNA fragmentation.
Our results thus demonstrate that the AMPK pathway is hyperactivated in MLL-rearranged patients, and it appears to directly contribute to the survival of MLL-rearranged cells. This study emphasizes the importance of protein pathway analysis as a route for discovery of functional derangement that may be functional, causative agents of cancer. Our data suggest AMPK as a new molecular target and encourage further studies of AMPK inhibitors as potential new drugs for treatment of MLL-rearranged leukemia patients.

Role of clathrin endocytic adaptors in controlling EGFR fate

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Epidermal Growth Factor Receptor (EGFR) can be internalized through different endocytic pathway according to ligand concentration. At low doses of ligand, the receptor (EGFR) is internalized almost exclusively through clathrin-mediated endocytosis (CME), while at higher concentrations of ligand a sizable amount of receptors becomes ubiquitinated and is endocytosed through a non-clathrin endocytosis (NCE), which targets the majority of the receptors to degradation.
EGFRs that enter through CME are subjected to different fates. CME is mainly involved in receptor recycling and allows prolonged signaling to occur from the intracellular compartments, but still one third of the receptor is delivered to degradation into the lysosomes.
Preliminary data collected in our lab suggest the existence of two distinct populations of clathrin-coated vesicles, regulated by different endocytic adaptors, which link the EGFR to distinct intracellular fates (degradation versus signaling/recycling). To gain insight into this issue, we carried out a complete characterization of the routing, fate and signaling of the EGFR upon RNA interference of the different adaptors. Biochemical assays, molecular genetics and live-imaging techniques were combined in the study in order to design a comprehensive picture. Results will be presented.

Liprin-α1 regulates invasion by modulating the motility and degradative activity of breast cancer cells

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During tumor cell invasion, the mechanisms that regulate the process by which cells break the basement membrane, remodel the extracellular matrix (ECM) and migrate through the surrounding tissues remain poorly understood. We have recently identified liprin-α1 as a novel player in the regulation of focal adhesion (FA) dynamics during cell motility (Asperti et al, 2009). Liprin-α1 is an ubiquitous multi-domain adaptor protein that positively regulates cell spreading on ECM and alters the localization and the stability of β1 integrins at the cell surface (Asperti et al, 2010). Interestingly, the liprin-α1 gene (PPFIA1) is upregulated in different human cancer, but the increased levels of the protein in tumors has not been demonstrated yet. We have recently shown the high expression of this protein in different types of human tumors, using an affinity purified antibody raised against liprin-α1. We used siRNA and overexpression of liprin-α1 in MDA-MB-231 human breast adenocarcinoma cells, and showed that this protein is needed for haptotactic migration and for invasion in vitro. In addition, we found that liprin-α1 promotes tumor cell spreading, motility and activity of invadopodia, which are specialized actin-rich protrusions needed for the degradation of the ECM during invasion. Although the density of invadopodia is not affected in cells with altered levels of liprin, we observed a strong effect on ECM degradation associated to these structures. These findings indicate that liprin-α1 positively regulates the proteolytic activity of tumor cells. In fact, liprin-α1 overexpression increases the degradation activity associated to cortactin-positive invadopodia in MDA-MB-231 cells. We are currently investigating the mechanisms by which liprin-α1 modifies the proteolytic ability of these cells. Moreover, preliminary time-lapse analysis has shown a defect in the migratory behaviour of tumor cells depleted of endogenous liprin−α1. We are now addressing the signalling pathways mediating the effects of liprin-α1 on the invasive behaviour of breast cancer cells. Liprin-α1 may interact with several protein partners, including the ArfGAP protein GIT1 implicated in cytoskeletal reorganization and membrane traffic during cell migration. Still, the invasive behaviour of MDA-MB-231 cells is not perturbed by silencing of the GIT/PIX complexes in these cells. We are now investigating other interactions that could mediate the effects of liprin−α1 on cancer cell motility. Our data suggest a new role for liprin-α1 as a regulator of the invasive apparatus of tumor cells.

Role of protein kinase PAK4 in differentiation and survival of human keratinocytes and regulation of transcription factor p63

Alessandra Calogero, Francesco Galli, Luisa Guerrini, Nerina Gnesutta
Department of Biomolecular Sciences and Biotechnology, Università degli Studi di Milano, Italia

The serine/threonine kinase PAK4 belongs to the p21-Activated Kinase (PAK) family and acts as a Rho GTPases effector protein implicated in many critical biological processes, regulating cell morphology and motility, embryonic development, cell survival, response to infection and oncogenic transformation. Indeed, PAK4 shows many oncogenic features: its overexpression has been observed in many cancer cell lines, with gene locus amplification in pancreatic tumors, colon cancer and in oral squamous cell carcinoma (1). Further, its activation promotes cell migration and anchorage-independent growth, and its overexpression in fibroblast cell lines is sufficient to cause tumor formation in athymic mice. We have also previously reported that when overexpressed, PAK4 can promote survival and protect cells from apoptosis induced by different stimuli (2).
In epithelial cells, it is known that PAK4 interacts with Keratinocyte Growth Factor (KGF) Receptor, and is activated following KGF or UVB exposure (3).
To understand PAK4 role in signaling mechanisms that control survival and differentiation in human keratinocytes, we down-regulated PAK4 expression in HaCaT human keratinocyte cell line using RNA interference technique.
As already reported, PAK4 expression increases in control HaCaT cells following confluence-induced differentiation. Our data show that in this system, PAK4 expression results to be essential, in fact PAK4 knock-down HaCaT cells fail to differentiate, as we observed by loss of Keratin1 induction and impaired down-regulation of transcription factor p63, a master regulator of epithelial tissue differentiation.
The PAK4 pro-survival role is also confirmed in HaCaT cells by the higher sensitivity of silenced cells to UV induced apoptosis.
In order to understand if PAK4 could directly modulate p63 during differentiation or UV response, we evaluated activated PAK4 effects on p63 stability and transcriptional activity. Our data show that active PAK4 expression can both modulate p63 protein levels and inhibit its trancriptional activity.
Our results show that PAK4 can play an important role in survival and differentiation in human keratinocytes, and suggest a novel regulation network implicating p63 modulation. The role of this new interaction network in epithelial tumors will be investigated in future studies.
1. Molli, PM et al. (2009) Oncogene 28: 2545-55
2. Gnesutta, N and Minden A (2003) MCB 23: 7838-48
3. Lotti, LV et al. (2007) J Cell Physiol 212: 633-42

Diacylglycerol kinase alpha regulates SDF1α-induced cell invasion by regulating atypical PKC and matrixmetallo proteinase 9

Cristina Cianflone[1], Elena Rainero[1], Paolo Ettore Porporato[1], Irene Locatelli[1], Gabriella Ranaldo[1], Miriam Gaggianesi [1],Federica Chianale[1], Gianluca Baldanzi[1], Andrea Graziani[1]
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Diacylglycerol kinase enzymes (DGKs) convert diacylglycerol (DAG) into phosphatidic acid (PA). We previously showed that diacylglycerol kinase alpha (DGKα) is activated by HGF and VEGF, in epithelial and endothelial cells respectively, in a Src dependent manner and that its enzymatic activity is required for HGF and VEGF-induced endothelial cell migration and HGF-induced cell invasion of mammary breast cancer cell (1-4). More recently we unveiled a novel signalling pathway linking HGF receptor to Rac activation and formation of cell protrusion, through DGKα-mediated regulation of atypical PKCs (aPKC) and RhoGDI (5).
SDF1α, member of the chemokines CXC subfamily, and its receptor CXCR4, a G protein coupled receptor, mediate survival, proliferation, and invasion of breast carcinomas.
SDF1α stimulates invasiveness of cancer epithelial cells through extracellular matrix (ECM) by promoting pseudopods formation and activating matrixmetallo proteinase 9 (MMP9). Several evidences indicate that aPKCs activate MMP9 by stimulating its NF-κB-mediated transcription.
It has been demonstrated that during invasive migration in ECM, MDA-MB-231 cells segregate at the leading edge β1 integrin and proteases, where the latter ones drive the degradation of the surrounding matrix.
Inhere we showed that DGKα is activated and recruited to the plasma membrane in a pertussis toxin-sensitive manner. Moreover both silencing of DGKα and inhibition of its enzymatic activity impairs SDF1α-triggered invasive phenotype in breast cancer cells. In particular, DGKα activity was required for SDF1α-induced i) elongation of cell protrusion through ECM, ii) recruitment of aPKC at the plasma membrane and iii) targeting of both β1 integrin and MMP9 at the tip of cell protrusions. Finally, the expression of constitutively membrane-bound and active DGKα mutant, in absence of chemokines or growth factor stimulation, promotes formation of protrusions as well as targeting of both β1-integrin and MMP9 at the protrusion tips and activates MMP9 gelatinolytic activity in an aPKC-dependent manner.
Altogether, these data indicate that activation of DGKα stimulates a novel pathway which is essential for SDF1α-induced breast cancer cell invasion, by regulating aPKCs-mediated protrusions in a matrix plug and MMP9 targeting and activation.
1) Cutrupi et al. (2000) EMBO J. 19:4614-22
2) Chianale et al. (2007) Mol. Biol. Cell. 18:4859-71
3) Baldanzi et al. (2008) Oncogene 27:942-56
4) Filigheddu et al. (2007) Anticancer Res. 27:1489-92
5) Chianale et al. (2010) PNAS 16 [Epub ahead of print]

Phosphatidylinositol 3-kinase beta in platelet integrin alphaIIbbeta3 signaling: a novel regulation by the focal adhesion kinase Pyk2 is essential for platelet spreading on fibrinogen

Lina Cipolla[1], Ilaria Canobbio[1], Alessandra Consonni[1], Gianni Guidetti[1], Silvia Catricalà[1], Elisa Ciraolo[2], Emilio Hirsch[2], Marco Falasca[3], Mitsuhiko Okigaki[4], Cesare Balduini[1], Mauro Torti[1]
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Integrin-mediated platelet adhesion to the components of the subendothelial matrix is an essential step in haemostasis as it initiates platelet recruitment at the injured vessel wall and, subsequently, promotes the growth and stabilization of the platelet thrombus. The two main integrins on the platelet surface are integrin alphaIIbbeta3, which represents a receptor for fibrinogen, fibronectin, and von Willebrand factor, and integrin alpha2beta1, which binds collagen. Both are able to initiate outside-in signaling pathway for platelet activation, but the detailed mechanisms are still poorly characterized. By binding of von Willebrand factor, integrin alphaIIbbeta3 initiates platelet arrest on the extracellular matrix while by subsequent binding of soluble fibrinogen supports platelet-platelet interaction and thrombus formation. Platelet adhesion to immobilized fibrinogen is a useful model to investigate integrin alphaIIbbeta3-mediated outside-in signaling. Platelet adhesion and spreading on fibrinogen typically involve activation of phospholipase Cgamma2 (PLCgamma2) and the release of ADP from intracellular granules, which mediates a secondary autocrine stimulation. There is evidence that integrin alphaIIbbeta3 also mediates activation of phosphatidylinositol 3-kinase (PI-3K). In this study, we have demonstrated that platelet adhesion to immobilized fibrinogen induced a time-dependent phosphorylation of Akt, which was largely dependent on the secondary action of secreted ADP. Integrin alphaIIbbeta3-mediated Akt phosphorylation was suppressed by wortmannin and by TGX-221 a specific inhibitor of PI-3Kbeta, but not by PIK75 or AS252424, which inhibit PI-3Kalpha and PI-3Kgamma, respectively. Moreover, Akt phosphorylation occurred normally in murine platelets expressing a catalytically inactive form of PI-3Kgamma, but was totally abrogated in platelets expressing inactive PI-3Kbeta (PI-3KbetaKD). PI-3KbetaKD platelets displayed a defective ability to adhere to fibrinogen, and spreading was severely compromised. Moreover, activation of the small GTPase Rap1b was almost completely abolished. PI-3Kbeta activation in fibrinogen adherent platelets occurred normally in PLCgamma2-deficient platelets but was dramatically affected in platelets from mice lacking the focal adhesion kinase Pyk2. Moreover, fibrinogen-adherent Pyk2 deficient platelets were unable to activate Rap1b and showed defective spreading. These results indicate that PI-3Kbeta is activated downstream to integrin alphaIIbbeta3 by a mechanism involving the tyrosine kinase Pyk2, and is essential for Rap1b stimulation and platelet spreading.

Does Histone Deacetylase 4 control breast cancer cell growth?

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Histone deacetylases (HDACs) are considered central players in the control of gene expression and an alteration of their function is well recognized as key step in cancer development. Moreover the use of small molecule inhibitors for these enzymes shows promising results in preclinical models. Despite this, little is known about the role employed by HDACs in solid tumors, and specifically by class IIa HDACs. In particular HDAC4 has been found significantly mutated in breast cancers. Hence, we decided to investigate HDAC4 in mammary development and breast cancer. Using primary mammary epithelial cells and tri-dimensional cell cultures we observed a correlation between HDAC4 expression and cell cycle while there is no influence of the matrix used. Intriguingly this enzyme is more expressed in most of the ER- breast cancer cell lines tested. In all the breast cancer cell lines and also in the primary mammary cells HDAC4 undergoes nuclear/cytoplasmic shuttling. In MDA-MB-231 ER- cells HDAC4 is associated with MEF2, its preferred binding partner, but not in ER+ MCF7 cells. The downregulation of this enzyme however is not linked to an increase in the expression of MEF2 target genes. Moreover HDAC4 overexpression of both the wild type and the nuclear localized mutant had a clear antiproliferative effect on both ER+ and ER- cells suggesting that promoting HDAC4 accumulation in the nucleus could be a therapeutic strategy for breast cancer.

Phosphatidylinositol 3-kinase beta in platelet integrin alpha2beta1 signaling: activation by cytosolic Ca^{2+} increase and role in integrin alphaIIbbeta3 inside-out activation

Alessandra Consonni[1], Gianni Guidetti[1], Lina Cipolla[1], Ilaria Canobbio[1], Silvia Catricalà[1], Elisa Ciraolo[2], Emilio Hirsch[2], Marco Falasca[3], Mitsuhiko Okigaki[4], Cesare Balduini[1], Mauro Torti[1]
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Integrin alpha2beta1-mediated adhesion and spreading on collagen is essential to mediate the initial arrest of circulating platelets. This event induces platelet activation, leading to the subsequent inside-out stimulation of integrin alphaIIbbeta3, which in turn binds circulating fibrinogen and captures other platelets to initiate thrombus formation. We have previously shown that this essential cross-talk between platelet integrin alpha2beta1 and alphaIIbbeta3 involves the activation of phospholipase Cgamma2 (PLCgamma2). The increase of cytosolic Ca2+ and accumulation of diacilglycerol stimulate the nucleotide exchange factor CalDAG-GEFI, which induces GTP binding to the small GTPase Rap1b, a key regulator of integrin alphaIIbbeta3 inside-out activation. In this study we investigated the role of phosphatidylinositol 3-kinase (PI-3K) in integrin alpha2beta1 outside-in signaling. We found that platelet adhesion to collagen induced a time-dependent phosphorylation of Akt. This process was only partially dependent on secretion of ADP. Studies with isoform-specific inhibitors suggested that phosphorylation of Akt was mediated by PI-3Kbeta. Accordingly, upon adhesion to collagen, Akt phosphorylation was undetectable in platelets from mice expressing a catalytically inactive form of PI-3Kbeta (PI-3KbetaKD), but occurred normally in platelets expressing a kinase dead mutant of PI-3Kgamma. We found that integrin alpha2beta1-induced PI-3Kbeta activation was mediated by intracellular Ca2+, as it was prevented by BAPTA-AM and by the IP3 receptor antagonist 2-APB. Moreover, integrin alpha2beta1 was unable to induce Akt phosphorylation in PLCgamma2 deficient platelets. Similarly to what we found for integrin alphaIIbbeta3, we observed a severely impaired activation of PI-3Kbeta upon adhesion to collagen of Pyk2-deficient platelets. It is known that Pyk2 can be activated both by Src-mediated phosphorylation and by binding of Ca2+. Interestingly, we found that, in contrast to integrin alphaIIbbeta3, recruitment of integrin alpha2beta1 induced activation of Pyk2 by a mechanism depending on PLCgamma2 and cytosolic Ca2+. Neither Pyk2 nor PI-3Kbeta were essential for integrin alpha2beta1-mediated spreading on collagen. However, they both were required for a correct activation of the small GTPase Rap1b. PI-3Kbeta activity was also required for integrin alphaIIbbeta3 inside-out activation in collagen-adherent platelets. These results indicate that integrin alpha2beta1 mediates activation of PI-3Kbeta by a mechanism requiring Ca2+-dependent stimulation of the focal adhesion kinase Pyk2 and that these events are essential in the cross-talk with integrin alphaIIbbeta3.

The PGC-1alpha-dependent pathway of mitochondrial biogenesis is upregulated in type I endometrial cancer

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Mitochondria are essential organelles that generate cellular energy (ATP) through oxidative phosphorylation and they have long been suspected to play an important role in the development and progression of cancer. In fact, since 1956, Warburg hypothesized that a key event in carcinogenesis is the development of an 'injury' to the respiratory machinery, resulting in compensatory increase in glycolytic ATP production. The master regulators of oxidative phosphorylation and mitochondrial biogenesis is the nuclear transcriptional coactivator PGC-1alpha. This factor co-activates the nuclear respiratory factor 1 (NRF-1) that stimulates the expression of a large number of nuclear genes involved in mitochondrial respiration and mtDNA replication and transcription such as the mitochondrial transcription factor A (TFAM). This factor mirrors the changing levels of mtDNA in the cell and seems to be important in mtDNA maintenance.
Endometrial carcinoma is the most frequent gynecological cancer in western country. Two different clinicopathologic subtypes are recognized: the estrogen-related (type I, endometrioid) and the non–estrogen-related types (type II, nonendometrioid). No data exist in the literature on mitochondrial biogenesis and on the existence of the PGC-1alpha-dependent mitochondrial biogenesis signalling pathway in normal and cancer endometrial tissue.
The aim of this work was to determine if there is a change of mitochondrial biogenesis in type I endometrial carcinoma and if this change can be related to alteration of PGC-1alpha-dependent signalling pathway. Therefore, we measured in samples of endometrial carcinoma and in proliferative endometrium used as control: 1) the mtDNA/nDNA content, 2) the citrate synthase activity as a measure of mitochondrial mass, 3) the expression of PGC-1 alpha, of NRF-1 and of TFAM proteins. A 2-fold increase of mtDNA content and mitochondrial mass was found in endometrial carcinoma compared to normal endometrium, indicating an activation of mitochondrial biogenesis. This increase was associated to the doubling of TFAM expression level and to a 1.6 and 1.8-fold increase, respectively, of NRF-1 and PGG-1alpha expression. This study demonstrates, for the first time, that the increased mitochondrial biogenesis in type I endometrial cancer is associated to the upregulation of PGC-1alpha signalling pathway.

Metabolic switch between oxidative phosphorylation and glycolysis: a new role for Stat3 in tumours

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The transcription factor STAT3 is considered an oncogene being constitutively activated in as many as 70% primary human tumours, which often become addicted to its activity. However, the molecular bases for its essential role in tumours of different origin are incompletely understood. In order to generate a suitable model to address this question we have generated by knock-in mice expressing only the constitutively active form STAT3-C, in which STAT3 activity is similar to the continuous but relatively low activity observed in many tumours.
$STAT3^{C/C}$ MEFs display increased proliferative potential, accelerated cell cycle, enhanced ability to grow past confluence and resistance to apoptotic stimuli and to spontaneous senescence. Surprisingly, a microarray analysis comparing $STAT3^{C/C}$ and $STAT3^{wt/wt}$ cells revealed profoundly modified expression of many genes involved in cell metabolism. $STAT3^{C/C}$ MEFs display reduced mitochondrial activity and accumulate less ROS, but activate aerobic glycolysis as shown by enhanced expression of master regulators of glycolysis such as PDK-1 and HIF-1α, and by increased lactate production, glucose avidity and sensitivity to glycolysis inhibitors. The dependence of $Stat3^{C/C}$ MEFs on glycolysis is attenuated by the silencing of HIF-1α. Moreover, $STAT3^{C/C}$ MEFs immortalized with a 3T3 protocol are transformed and able to form tumours in nude mice, and the transformation capacity is down-regulated by HIF-1α silencing.
The idea that STAT3 could contribute to cell transformation and resistance to senescence by acting on cell metabolism is confirmed by our observation that inhibition of STAT3 activity in STAT3-addicted tumour cell lines partially restores mitochondrial activity and down-regulates glycolytic metabolism.
Since most cancer cells are known to alter their glucose metabolism by activating anaerobic-like glycolysis despite available oxygen while down-regulating oxidative phosphorylation, a phenomenon known as 'Warburg effect', we propose that the essential role observed for STAT3 in so many different types of cancer may be explained by its ability to act as a molecular switch of cellular metabolism to trigger abnormal cell survival.

Inhibition of beta 1 integrin and il-3r beta common subunit interaction hinders tumor angiogenesis

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Integrin/cytokine receptor interaction provides permissive signals for neoangiogenesis and integrins are crucial for differentiation of endothelial progenitor cells (EPCs). The inflammatory cytokine interleukine-3 (IL-3) released in tumoral microenvironments contributes to both angiogenesis and vasculogenic process. Herein, IL-3 receptor beta common (IL-3Rβc) extracellular domain-derived fusion proteins (Fc) were used to dissect the molecular mechanisms regulating these processes. Three different Fc were generated, containing the entire extracellular domain of the IL-3Rβc (Fc1.4), a fragment corresponding to domain 1 to 3 (Fc1.3) and a fragment corresponding to domain 4 (Fc4) respectively. Their ability to specifically interfere with the IL-3Rβc/β1 integrin interaction was assessed both on EPCs and murine tumor-derived endothelial cells. Pull-down experiments demonstrated that, unlike Fc1.3, both Fc1.4 and Fc4 physically interacted with β1 integrin. Moreover, soluble Fc4 and Fc1.4 fragments prevented IL-3-mediated EPC expansion, arterial morphogenesis and tumor-derived endothelial cell migration, without affecting cell adhesion. Finally, in vivo, Fc4 inhibited IL-3-mediated vasculogenic process as well as inflammatory and tumor vascular growth. These data identify in the juxta-membrane IL-3Rβc extracellular domain the β1 integrin interacting domain and provide the rational for targeting this interaction to impair vascular growth.

Oxytocin receptor nuclear translocation could be a novel mechanism mediating osteoblast differentiation induced by oxytocin

Adriana Di Benedetto[1], Stefania Dell' Endice[1], G. Colaianni[1], Roberto Tamma[1], Bice Chini[2], Carlo Zambonin[3], Alberta Zallone[1]
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We demonstrated a direct anabolic effect of oxytocin (OT) on bone. OT upregulates expression of osteoblast markers and transcription factors as Osterix, Schnurri, Atf-4, Osteocalcin, Osteopontin. This effect is mediated by the seven-transmembrane G-protein coupled receptor OTR expressed by osteoblast (Tamma 2009). We investigated how OT and OTR promote bone formation and osteoblast differentiation. A previous work (Kinsey 2007) demonstrated nuclear localization of OTR in neoplastic cells and fibroblasts; accordingly we found the receptor in osteoblast nuclear extracts after OT stimulus (15-30 min), confirmed by a nuclear localization observed by confocal microscopy. MALDI-TOF analysis was performed on nuclear protein immunoprecipitated with anti-OTR and the spectra analyzed with FindPept database. Four peptydes corresponding to OTR intracellular loops were found. We further analyzed OTR trafficking and nuclear transport following OT stimulation. Exogenous OTR-EGFP fusion protein transfected in primary osteoblasts, colocalizes with β-arrestin1/2 within 2-3 min after OT treatment, thereafter the receptor dissociates from β-arrestins and localizes in endosomal acid compartment. By the time OTR-GFP is expelled from the endosome and sorted to the nucleus. Transportin-1 silencing in osteoblasts affects OTR nuclear localization, suggesting that transportin-1 mediates OTR nuclear translocation. We hypothesized a possible role for OTR in regulating transcription and/or transcription factors. By immuneprecipitation, we found physical interaction of native OTR with the osteoblast transcription factor Runx-2 and with the transcription coactivator Schnurri-2, in response to OT stimulus. Furthermore simultaneous stimulation of osteoblast with OT and BMP2, induced OTR/Smad4 interaction in the nucleus. By confocal immunofluorescence nuclear translocation of the receptor and co-localization with Runx-2 and Schnurri-2 was evident after OT stimulus.
In support of this, chromatine immuneprecipitation indicates a direct binding of OTR to the nuclear chromatine. OTR nuclear localization and association with transcription factors and DNA could be a possible mechanism for the observed increased osteoblastogenesis induced by oxytocin.

p140Cap inhibits ErbB2-induced distruption of cell polarity

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The Neu/erbB-2 gene encodes a receptor tyrosine kinase that belongs to the epidermal growth factor receptor (EGFR) family. Amplification and overexpression of ErbB2 strongly correlates with aggressive breast cancers. A deeper understanding of pathways downstream of ErbB2 signaling that are required for the transformation of human mammary epithelial cells could identify novel strategies for therapeutic intervention in breast cancer. Expression in the mammary gland of the oncogenic form of Neu (NeuT) under control of the MMTV-LTR results in the induction of mammary adenocarcinomas (NeuT-MMTV). We have previously described that the adaptor protein p140Cap/SNIP associates directly to Src and Csk kinases, and interferes with cells adhesion /growth factor-dependent Src kinase activity, thus affecting tumour properties in cancer cells. Moreover, we provided evidence that p140Cap expression in human breast tumours inversely correlates with tumour malignancy and particularly with the expression of ErbB2. To investigate the role of p140Cap in the ErbB2-dependent transformation we generated and crossed p140Cap-MMTV transgenic mice with NeuT-MMTV mice. p140Cap/NeuT-MMTV animals show a significantly later tumor onset compared to NeuT-MMTV mice, indicating that p140Cap delays NeuT oncogenic properties. Moreover, primary cells derived from p140Cap/NeuT-MMTV tumors are strongly impaired in growth as xenografts in nude mice and in lung metastasis formation. In accordance with our previous data, in these cells p140Cap affects profoundly Src and ERK signalling. Interestingly, p140Cap/NeuT primary cells show also an altered TGF beta signalling and a lower level of SMADs phosphorylation. TGF beta signalling cooperates with ErbB2 to induce distruption of apical-basal polarity of epithelial cells thus promoting transformation and epithelial-mesenchymal transition. As expected, NeuT tumors derived primary cells show distruption of cell polarity and Zo-1 mislocalization both in 2D and 3D cultures. Consistently, p140Cap/NeuT cells rescue the correct apical-basal polarity and Zo-1 localization. Therefore, p140Cap mechanistically behaves as a tumour suppressor that inhibits signalling pathways leading to aggressive phenotype induced by NeuT oncogene

Morgana/chp-1, a ROCK inhibitor involved in centrosome duplication and tumorigenesis

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Centrosome abnormalities lead to genomic instability and are a common feature of many cancer cells. Here we show that mutations in *morgana/chp-1* result in centrosome amplification and lethality in mouse. In mammalian cells, morgana forms a complex with Hsp90, ROCK I and II, and directly binds ROCK II. Morgana downregulation promotes the interaction between ROCK II and nucleophosmin (NPM), leading to an increased ROCK II kinase activity, which results in centrosome amplification. *morgana +/-* primary cells and mice display an increased susceptibility to neoplastic transformation. In addition, tumor tissue array histochemical analysis revealed that morgana is underexpressed in a large fraction of breast and lung human cancers. Thus, morgana/chp-1 appears to prevent both centrosome amplification and tumorigenesis.

Negative regulation of diacylglycerol kinase theta mediates adenosine-dependent hepatocyte preconditioning

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A tissue becomes ischemic upon the imbalance between the need and the availability of oxygen and metabolic substrates. Both in vitro and in vivo, short periods of no-lethal ischemia followed by reperfusion, induce pre-conditioning of cell from either heart, liver and brain , i.e. protection of the tissue from prolonged ischemic damage. In vivo and in vitro studies indicate that preconditioning is mediated by extracellular release of adenosine and activation of A2a receptors (A2aR). We previously showed that in liver, A2aR-mediated ischemic preconditioning(IP) prevents ischemia/reperfusion injury by promoting diacylglycerol-mediated sustained activation of novel protein kinase C delta (PKC-delta). Diacylglycerol kinases (DGKs) convert diacylglycerol (DAG) into phosphatidic acid, acting as terminator of diacylglycerol signalling. The aim of this study is to investigate the hypothesis that negative regulation of a DGK isoform contributes to sustained DAG-mediated signalling leading to PKC-delta activation and protection from ischemia-induced cell death. We first showed that, tightly associated with the onset of hepatocyte tolerance to hypoxia, total DGK activity is negatively regulated in primary rat hepatocytes preconditioned either by 10 min hypoxia followed by 10 min re-oxygenation or by cell treatment with CGS21680, an A2aR agonist, followed by prolonged hypoxia. Based on previous finding that GPCR- and Rho-mediated negative regulation of DGK-theta orthologue in C.elegans upregulates DAG-mediated signalling, we verified the hypothesis that indeed negative regulation of DGK-theta mediates IP- and A2aR-triggered cyto-protection. Indeed we showed that CGS21680-induced stimulation of A2aR specifically inhibits DGK-theta through a Rho-mediated mechanism. Consistently, both siRNA-mediated downregulation of DGK-theta and pretreatment with the DGK inhibitor R59949 induce hepatocytes tolerance to hypoxia. Pharmacological inhibition of DGK causes DAG-dependent activation of novel PKC-delta and epsilon and of their downstream target p38 MAPK.
In conclusion, we unveil a novel signalling pathway contributing to the hepatocyte preconditioning onset, which through RhoA-GTPase, couples A2aR to the downregulation of DGK. This inhibition is essential for the sustained accumulation of diacylglycerol required for triggering DAG-dependent novel PKCs-mediated survival signals

Involvement of the protein ERp57 in EGFR and STAT3 signaling

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ERp57, a member of the disulfide isomerase family (PDIs), is a soluble protein that is mainly located in the lumen of the endoplasmic reticulum (ER), but that has also been found in the nucleus, cytoplasm and cell surface membrane. Little is known regarding its role in these other cellular compartments. The first suggestion of an involvement of ERp57 in signal transduction has come from the detection of its interaction with STAT3 at the level of cell membrane (Guo G.G. et al. J. Interf. Cytok. Res. 22 (2002) 555), cytosol (Ndubuisi M.I. JBC 274 (1999) 25499) and nucleus (Eufemi M. et al. BBRC 323 (2004) 1306). To verify the importance of ERp57 in this process of signal transduction, we have investigated the involvement of ERp57 on the expression of a subset of STAT3-dependent genes. Upon depletion of ERp57 by RNA interference, the phosphorylation of STAT3 on tyrosine 705 was decreased, and the IL-6-induced activation of CRP (C-Reactive Protein) expression was completely suppressed. In vitro experiments showed that ERp57 is also required for the binding of STAT3 to its consensus sequence on DNA. Therefore the probable sites of action of ERp57 reside both in the activation reaction of STAT3 and in the DNA binding of STAT3 at the nuclear level. We also found that, unexpectedly, the protein ERp57 is involved in the function of the epidermal growth factor receptor (EGFR), being necessary for the proper phosphorylation, internalization and nuclear import of the receptor after the stimulation by EGF. A full understanding of EGFR activity is complicated by the different internalization and signal transduction pathways for which it is responsible. Our experiments were carried out on the breast cancer cell line MDA-MB-468, which is not only highly enriched in EGFR, but also displays ERp57 on its cell surface. The internalization of EGFR brought up by EGF, which is accompanied by the internalization of ERp57, was drastically decreased when ERp57 was silenced by RNA interference, indicating that ERp57 is a required cofactor for this process. Moreover, the silencing of ERp57 was accompanied by a strong decrease in the amount of phosphorylated EGFR upon EGF binding. Everything considered, our studies indicate a role of the ERp57 in the trafficking from membrane to nucleus and its role in the signal transduction. The mechanism by which ERp57 contributes to the phosphorylation of tyrosines of STAT3 and of EGFR remains to be ascertained.

Functional role of ArhGAP15, a new RacGAP

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Rho GTPases activation is regulated by cycling between inactive GDP-bound and active GTP-bound states. Guanine nucleotide exchange factors (GEFs) promote the activation of GTPases by stimulating the exchange of GDP to GTP, while GTPase-activating proteins (GAPs) accelerate hydrolysis of GTP, returning the GTPase to an inactive form. The fact that about 0.5% of all predicted human genes encode putative GAPs suggests that these proteins have widespread and important roles in GTPase regulation (Bernards A. and Settleman J., 2004). Although, the role of Rac isoforms in different cell types has been well established, how RacGAPs regulate leukocytes functions is still obscure.
ArhGAP15 is a RacGAP highly expressed in macrophages (Costa et al., 2007) constituted by a pleckstrin homology (PH) domain at C-terminus required for its localization on plasma membrane and a RhoGAP domain at N-terminus (Seoh et al., 2003). To explore the role of ArhGAP15 in vivo, we generated ArhGAP15-deficient mice. Here we report that ArhGAP15 functions as a RacGAPs in vivo, regulating multiple aspects of innate immunity. ArhGAP15-null macrophages displayed an altered morphology with no effect on migration. On the contrary, ArhGAP15-null neutrophils showed increased directional migration correlating with higher Rac activity which triggers enhanced ROS production. These distinctive features protect ArhGAP15-deficient mice upon CLP (cecal ligation and puncture) treatment. Taken together, these data provides evidence for the involvement of ArhGAP15 in cytoskeleton remodelling, polarization, migration and ROS production in leukocytes.

Laminin and neuregulin signaling in Schwann cells

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During peripheral nervous system (PNS) development, Schwann cells (SC) contact large caliber axons and adopt a 1:1 relationship with them, in order to initiate myelination. This process depends on the cross talking between axons, Schwann cells and the extracellular matrix (ECM).
It has been demonstrated that neuregulin 1 (NRG1) type III, which is present on neuronal membranes, induces myelination through a juxtacrine mechanism that causes activation of ErbB2/ErbB3 receptors and the Akt pathway in Schwann cells (Taveggia et al. 2005). Other effects of neuregulins in development (such as survival and proliferation) are instead caused by soluble neuregulins. Laminin 211, which is produced by SC and released in ECM, also favours myelination using various SC receptors, including β1 integrins (Feltri et al., 2002).
It is known that laminins enhance NRG1 signalling in the brain (Colognato et al. 2002), suggesting that a functional interaction between laminin211 and NRG1 exist in SC myelination. To explore this hypothesis, we treated primary rat Schwann cells with laminin 211, soluble NRG1 or both. We observed an activation of Akt after treatment with soluble NRG1 and a little activation of Akt after treatment with laminin 211. However, there was no enhancement in Akt activation after treatment with both. To test if NRG type III transmembrane form has the same effect on Akt activation, we performed the same experiment using neurite membranes isolated from primary Dorsal Root Ganglion neurons. As shown before, we observed an activation of Akt after NRG1 treatment, a little Akt activation after Laminin 211 treatment and no enhancement in Akt activation after treatment with both. These data suggests that laminin 211 and NRG1 regulate Akt using parallel pathways.
We also analyzed sciatic nerves from Schwann cells specific β1 integrins null mice and we found a reduced Akt activation. These observations suggest a defective NRG1 signalling in the absence of β1 integrins. This could be due to a defective capability of β1 integrin null SC to respond to NRG1 stimulation or to an unsuccessful access of β1 null SC to axonal NRG1 type III, caused by the impaired contact between SC and axons observed in these mice . In order to clarify if β1 integrins deficient SC are unresponsive to NRG1 stimulation, we are treating rat SC with siRNAs against β1 integrins and we will stimulate these cells directly with axonal membranes containing NRG1 type III. These experiments will be useful to clarify how signals from the ECM and axons are integrated in SC.

Sumoylation affects EGF induced Egr-1 expression and stability

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Human early growth response-1 (Egr-1) is a member of the zing-finger family of transcription factors induced by a range of molecular and environmental stimuli including epidermal growth factor (EGF). Previously we demonstrated that integrin/EGFR cross-talk is required for expression of Egr-1 through activation of Erk1/2 and PI3K/Akt/Forkhead pathways. The aim of this work was to assess the influence of sumoylation, a more recently described post-translational modification, on EGF induced Egr-1 expression and stability. We demonstrated that in basal conditions and after EGF treatment a fraction of endogenous Egr-1 was mono-sumoylated. Quantitative Real Time PCR experiments were performed to investigate whether sumoylation could affect EGF induced EGR-1 gene transcription. Here, we report that EGF induced Egr-1 mRNA levels were increased by SUMO-1/Ubc9 over-expression. Conversely, Egr-1 protein levels were strongly reduced in SUMO-1/Ubc9 transfected cells. Data obtained from protein expression and ubiquitination analysis in the presence of the proteaosome inhibitor MG132, suggested that sumoylation increased Egr-1 ubiquitination enhancing its degradation.

Signaling modulation at G2/M transition: old and new players

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The cell cycle progression is an extremely complex process, which fidelity is monitored by a variety of control systems: the checkpoint mechanisms. Signaling events coming from different routes (i.e. growth factors, cytokines, cell-to-cell contacts or DNA damage) are concerted for ensuring normal cell division by regulating the cell-cycle checkpoints. Among those, the G2/M checkpoint is the most involved in the control of genome integrity, ensuring its preservation before undertaking mitosis; thereby it has a considerable impact on cellular transformation and cancer.
Recently we identified DEPDC-1A and DEPDC-1B proteins as new players of the pathway modulating G2/M transition. Depletion of the two proteins inhibits proliferation and causes a delay in mitotic entry in normal and cancer cell lines, suggesting that DEPDC-1A/1B are necessary for a correct G2/M transition.
DEPDCs hold a predicted Rho-GAP domain and biochemically interact with RhoA and Rac1, in their active (GTP loaded) conformation, arguing for a role of RhoGTPases in this pathway. Indeed, Rho-GTPases depletion completely rescues the mitotic defect of DEPDC silenced cells, thus revealing a genetic interaction between the two genes and the aforementioned Rho-GTPases.
As downstream event, we also identified the MAPK-Erk cascade, that is active from late S to G2/M transition for a correct onset of mitosis. Upon DEPDCs depletion MAPK activity is increased, while pharmacological modulation (by U0126) is sufficient to fully rescue the mitotic phenotype, reinforcing the idea that coordinated signaling events control the G2/M transition.
In conclusion, our study proposes a new intriguing role of Rho-GTPases in the modulation of cell cycle progression and identifies DEPDCs, together with RhoA/Rac1 and MAPK, as major players of this G2/M signaling pathway.

Regulation of the E3 ligase NEDD4 by EGFR

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Stimulation of cells with epidermal growth factor (EGF) results in the activation of the intrinsic tyrosine kinase of the EGFR and the establishment of a complex signalling network where both ubiquitination and phosphorylation regulate protein interactions. Eps15 is one of the endocytic adaptor proteins that undergo coupled monoubiquitination upon EGF stimulation through the action of the E3 ligase Nedd4 [1,2.]
How the signal from EGF is delivered to the Ubiquitination machinery was unknown. We have now evidences that Nedd4 is both ubiquitinated and tyrosine phosphorylated upon EGF stimulation. Using an integrated approach based on pharmacological inhibitors and molecular genetics tools we found that the kinase activity of both, EGFR and Src are required for Nedd4 phosphorylation as well as for eps15 monoubiquitination. To gain insight on a possible regulative role exerted by these post-translation modifications on the ligase activity we set up mass spectrometry approach to identify phosphorylation and ubiquitination sites present in Nedd4 protein upon EGF stimulation. Results will be presented.

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FRET study of hERG1 channel and integrin-beta1 complex

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hERG1 (human ether-a-gò-gò-related gene 1) channels, a peculiar type of K+ channels belonging to the EAG family, are often aberrantly expressed in primary human cancers. hERG1 channels exert pleiotropic effects in cancer cells, in turn regulating cell proliferation, cell motility and invasiveness or stimulating the process of neo-angiogenesis. hERG1 can induce such diverse effects since it triggers and modulates intracellular signaling cascades. This role depends on the formation, on the plasma membrane of tumor cells, of macromolecular complexes with integrin receptors. Inside the complex, the link between hERG1 and integrins is twofold: integrins, mainly the beta1 subunit, can activate hERG1. Conversely, the channel, once activated by integrins, can modulate signaling pathways downstream to integrin receptors.
Then the characterization of the interaction hERG1/integrin is a very useful task.
Unfortunately, the resolution of the optical microscope is not sufficient to observe molecule interactions and the electronic microscope doesn't allow the observation of living samples. These limitations can be overcome by using Fluorescence Resonance Energy Transfer (FRET) microscopy technique. When the two proteins, stained with appropriate fluorescent dyes, interact an energy transfer among the excited donor dye and the acceptor one occurs and the fluorescent emission characteristics change. This is a well tested spectroscopic technique for measuring distances in the range 1–10nm.
We have used the FRET technique in living HEK cells to characterize the interaction between the potassium channel hERG1 and integrin-beta1, stained, respectively, with Cyan Fluorescence Protein (CFP) and Yellow Fluorescent Protein (YFP).
Such experiments have clearly indicated that CFP-labeled hERG channels and YFP-labeled beta 1 integrins directly interact to form a membrane complex in living HEK cells resulting in a detectable intensification of the measured YFP signal and a decrease in CFP lifetime. This result, besides providing a useful confirmation of the biochemical characterization of this complex , represents an important validation of the in vivo, fluorescent protein-based approach to FRET imaging that we have used to perform experiments in several conditions.

A SILAC Proteomic Approach Identified Novel Candidate Players in the Non-Clathrin Endocytic Pathway of the EGFR

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Epidermal Growth Factor Receptor (EGFR) can be endocytosed through different entry routes depending on ligand concentration. At low doses of EGF, the receptor is not ubiquitinated and is internalized exclusively through clathrin-mediated endocytosis (CME). At higher concentrations of ligand, however, a substantial fraction of the receptor is endocytosed through a non-clathrin endocytosis (NCE), as the receptor becomes ubiquitinated [1]. Importantly, the two pathways couple with distinct receptor functions. CME is mainly involved in receptor recycling and allows prolonged signalling to occur from the intracellular compartments. On the contrary, NCE targets the majority of the receptors to degradation [2].
The molecular mechanisms involved in NCE of the EGFR are mainly unknown. Essentially, NCE is defined by its insensitivity to functional ablation (KD) of clathrin and for its sensitivity to cholesterol-interfering drugs, hence its definition as a "raft-dependent pathway".
In order to identify the molecular components of NCE, we are employing a large-scale proteomic approach. To this end, pure preparation of EGFR-containing vesicles in condition of clathrin KD has been obtained by differential centrifugation technique followed by an immunopurification step using phosphospecific anti-EGFR antibodies. This method, coupled with quantitative proteomics, allowed us to select a list of candidate players, particularly enriched for specific raft-mediated endocytic proteins, which are currently being validated by RNA interference.
Given the degradative function of NCE, this approach can be useful to identify negative regulators of EGFR, which might represent novel potential targets for cancer therapies.

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Dissecting the role of Cbl in EGFR endocytosis

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Epidermal Growth Factor Receptor (EGFR) can be internalized through two different entry routes, clathrin-mediated endocytosis (CME) and non-clathrin endocytosis (NCE). EGFR ubiquitination, triggered by the E3 ligase c-Cbl, is required for this latter pathway, while it is dispensable for CME. A dual role for c-Cbl has been shown: it acts as a major E3 ligase in the ubiquitination of different plasma membrane receptors, targeting them to lysosomal degradation, and it functions also as an adaptor, by recruiting several proteins involved in the early phase of CME. In order to understand the molecular details of Cbl activity, we plan to systematically knock-down (KD) c-Cbl and its related proteins Cbl-b and Cbl-c in HeLa cells. This will be instrumental to i) characterize their effects on EGFR ubiquitination (if they act at different steps of the endocytic cascade or through different type of ubiquitin signals), ii) dissect their involvement in CME vs NCE and iii) split their roles as adaptors and E3 ligases through reconstitution of KD cells with different sets of mutants. We initially set-up both transient and stable KD of Cbl proteins in HeLa cells. Our preliminary data suggest that Cbl-b acts similarly to c-Cbl in the process of EGFR CME. Yet, Cbl-b has a minor impact on EGFR ubiquitylation compared to c-Cbl, suggesting distinct roles of the two proteins in EGFR NCE. Generation and characterization of Cbl mutants (affecting ligase vs adaptor function) is on going. Results will be presented.

MicroRNA-214 promotes melanoma tumor progression

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MicroRNAs are endogenous non-coding RNAs, proven to regulate tumor progression by binding to the 3'UTRs of specific target genes and suppress their expression by translational repression or mRNA degradation. We identified a microRNA, miR-214, whose expression correlated directly with the metastatic potential of a human melanoma progression model, represented by the poorly metastatic A375P parental cells and its metastatic variants MA-1, MA-2, MC-1 and MC-2, derived by repeated passages *in vivo*. miR-214 was also found to be up-regulated in samples of human spreading melanomas (n=57) and cutaneous metastases (n=18) compared with non malignant *in situ* melanomas (n=13). Modulation (over-expression or down-modulation) of miR-214 in melanoma cells strongly altered motility, migration and matrigel invasion *in vitro* as well as lung metastasis formation *in vivo*. In addition, it influenced *in vivo* extravasation from blood vessels and survival to *anoikis*, suggesting an essential role in the establishment of distant metastases. By combining bioinformatic predictions and microarray gene expression analyses we identified a panel of miR-214 target genes involved in tumor progression. Via biological validations we proved that the transcription factor TFAP2C, a well known melanoma tumor suppressor and the integrin ITGA3, a cell adhesion receptor, are direct miR-214 targets. Our results indicate a direct role of miR-214 in metastasis dissemination.

Emilin1 controls proliferation in skin and in tumor development

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EMILIN1 is an ECM glycoprotein which interacts with the integrin α4β1 through the gC1q1 domain and negatively regulates pro-TGF-β maturation via the EMI domain. It is well known that TGF-β has a dual role on carcinogenesis: it behaves as a tumor suppressor in the early stages, but it can also drive malignant progression invasion and metastasis. Moreover, TGF-β family members are known to be potent cell growth inhibitors of mouse keratinocytes. In this study we investigated the role of EMILIN1 in a two step skin carcinogenesis model. Emilin1-/- mice showed significantly accelerated tumor development and an increased in the number and size of skin tumors compared to WT mice. Since these evidences were in contrast with the known suppressive role of TGF-β in early skin tumor development, we hypothesized that EMILIN1 might play a dual role in cell proliferation in skin. As already known TGF-β is more abundant in Emilin1-/- mice. Accordingly to this, we measured TGF-β and its major signalling molecules in normal skin. We found that TGF-β was more expressed in KO mice. However, pSmad 2/3 was strongly downregulated, whereas Erk was dramatically upregulated. Keratinocytes proliferation in mice genetically haplodeficient for TGF-β was not affected by the different levels of TGF-β but by EMILIN1 expression suggesting that EMILIN1 exerts a major control in cell proliferation. In fact, Emilin1-/- mice displayed thicker dermis and epidermis accompanied by a higher number of Ki67-positive cells compared to WT mice. Moreover, we showed that EMILIN1 expression was abundant in the dermal stroma, with a peculiar distribution up to the basal layers of epidermis and hair follicles. Accordingly we demonstrated that the growth of both WT and Emilin1-/- keratinocytes, in vitro, was significantly inhibited only when they were co-cultured with WT fibroblasts, which produce EMILIN1. Moreover, the proliferation rate of WT keratinocytes, which do not produce EMILIN1, was significantly higher when cultured on plastic compared on gC1q1 suggesting that the direct contact with EMILIN1 inhibited cell proliferation. The EMILIN1 dependent cell proliferation is linked to fibroblasts via α4β1 and to epithelial cells via α9β1 interaction as demonstrated by the rescue of proliferation levels in the presence of integrin specific function blocking antibodies. All these evidences suggest that EMILIN1 plays could exert a direct role in cell proliferation both under normal conditions and possible in the early phases of tumour growth. Further studies will be addressed to confirm this hypothesis also in papillomas.

SAP-mediated inhibition of diacylglycerol kinase alpha regulates TCR-induced diacylglycerol signaling

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Diacylglycerol (DAG) kinases (DGKs) metabolize DAG to phosphatidic acid and therefore terminate DAG signalling. In T lymphocytes, two DGK isoforms, DGKα and DGKζ act as negative regulators of T cell receptor (TCR) signalling by decreasing DAG levels and inducing anergy. Here we show that upon co-stimulation of the TCR with CD28 or SLAM DGKα undergoes rapid negative regulation of its enzymatic activity and is recruited to the plasma membrane.
Surprisingly, SLAM-associated protein (SAP), whose reduced function causes XLP, is required for the inhibition of DGKα induced by either TCR/SLAM or TCR/CD28 stimulation. Consistently, SAP over-expression results both in the inhibition of DGKα and its membrane recruitment. These findings suggest that SAP-mediated inhibition of DGKα may allow DAG accumulation upon TCR stimulation. Thus, we may hypothesize that lack of DGKα negative regulation may contribute to the defective DAG-mediated TCR signalling observed in absence of SAP in both XLP patients and SAP deficient mice. Indeed, we show that shRNA-mediated SAP down-regulation impairs TCR/CD28-induced DAG-mediated PKCΘ membrane recruitment, Ras and NFAT activation, as well as IL-2 secretion. Moreover, activation of these pathways is rescued by either pharmacological or siRNA-mediated inhibition of DGKα in SAP-deficient T cells.
Together, these findings unveil a novel TCR-triggered signalling circuit, by which SAP mediates negative regulation of DGKα, thereby sustaining DAG-mediated signalling and regulating T cell activation. Therefore our observations suggest that inhibition of DGKα may constitute a potential pharmacological strategy for the treatment of XLP.

Tumor repressive function of estrogen receptor β in malignant pleural mesothelioma

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Malignant pleural mesothelioma is an asbestos-related neoplasm with poor prognosis, refractory to current therapies, the incidence of which is expected to increase in the next decades. Female gender was identified as a positive prognostic factor among other clinical and biological prognostic markers for malignant mesothelioma, yet a role of estrogen receptors (ERs)has not been studied. Our goal was to investigate ERs expression in malignant mesothelioma and to assess whether their expression correlates with prognosis. Immunohistochemical analysis revealed intense nuclear ERβ staining in normal pleura that was reduced in tumor tissues. Conversely, neither tumors nor normal pleura stained positive for ERα. Multivariate analysis of 78 malignant mesothelioma patients with pathologic stage, histologic type, therapy, sex, and age at diagnosis indicated that ERβ expression is an independent prognostic factor of better survival. Moreover, studies in vitro confirmed that treatment with 17β-estradiol led to an ERβ-mediated inhibition of malignant mesothelioma cell proliferation as well as p21CIP1 and p27KIP1 up-regulation.
Consistently cell growth was suppressed by ERβ overexpression, causing a G2-M-phase cell cycle arrest, paralleled by cyclin B1 and survivin down-regulation. Our data support the notion that ERβ acting as a tumor suppressor is of high potential relevance to prediction of disease progression and to therapeutic response of malignant mesothelioma patient.
Conversely ERβ silencing in MMe positive cells confers a more invasive phenotype, increases anchorage independent cell proliferation and induces the basal activation of PI3K/Akt and Erk 1/2 MAP kinase signal transduction pathways.

Acyl ghrelin and des-acyl ghrelin protect from skeletal muscle atrophy

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Skeletal muscle atrophy is a debilitating response to several diseases resulting in a massive loss of muscle mass and function. Ghrelin (GHR) and des-acyl ghrelin (D-GHR) are, respectively, a circulating peptidyl hormone acylated on Ser3, and its un-acylated precursor. Through binding to its receptor GHSR-1a, GHR stimulates growth hormone (GH) release. D-GHR does not bind GHSR-1a, however D-GHR and GHR share some biological activities, such as inhibition of apoptosis through activation of PI-3-k/Akt, and differentiation of skeletal myoblasts.
Here we demonstrate a direct anti-atrophic activity of D-GHR both in vitro and in vivo, without inducing ipertrophic effect. In vitro D-GHR and GHR prevent dexamethasone-induced atrophy in C2C12 myotubes by activating the PI-3-k/Akt/mTOR pathway. In vivo, by either using a transgenic mouse model (TG) over-expressing D-GHR, or injecting D-GHR in wild type (WT) mice, we show that D-GHR protects skeletal muscle from fasting- and denervation-induced atrophy.
In both models of atrophy, TG mice show reduced skeletal muscle loss and reduced atrogenes expression. Moreover, subcutaneous injection of D-GHR in WT mice induces Akt and mTOR phosphorylation in muscles, indicating that in vivo D-GHR activates Akt/mTOR as it does in C2C12 myotubes. Indeed, D-GHR pharmacological treatment protects skeletal muscle from atrophy similarly to what observed in D-GHR TG mice.
Although the receptor through which des-acyl ghrelin exerts this activity remains unknown, we ruled out the involvement of GHSR-1a and the GH-IGF-1 axis, as treatment of GHSR-1a-/- mice with des-acyl ghrelin results in the activation of Akt/mTOR pathway in skeletal muscle.
Altogether these findings provide the first demonstration that D-GHR directly inhibits atrophy induced by different atrophic stimuli. Moreover, as D-GHR, differently from GHR, does not activate the GH/IGF-1 axis, this finding provides a new therapeutic strategy alternative to IGF-1 for the treatment of muscle wasting.

NG2 null mice display a myopathic phenotype affecting skeletal muscles

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Collagen VI (ColVI) is large ECM protein with a widespread distribution in several tissues.Mutations of genes encoding for ColVI cause various forms of human muscular dystrophies,including Bethlem Myopathy and Ullrich Congenital Muscular Dystrophy.We have shown that ColVI null (Col6a1$^{-/-}$) mice display an early onset myopathic phenotype affecting skeletal muscles,characterized by mitochondrial dysfunction and spontaneous apoptosis (3, 1). Molecular pathways transducing ColVI signals are largely unknown.NG2 is a transmembrane proteoglycan acting as a cell surface receptor for ColVI (6).Originally discovered on oligodendrocytes progenitors,NG2 is expressed by developing tissues and tumors.Notably,it is expressed by postnatal skeletal muscles and it is selectively downregulated in myofibers from Ullrich patients and Col6a1$^{-/-}$ mice (5).
This study was aimed to investigate the role of NG2 and its interplay with ColVI in skeletal muscle.Although NG2 null (Cspg4$^{-/-}$) mice appear phenotypically normal (2),careful investigation of muscles showed that they are affected by a myopathic syndrome.In particular,Cspg4$^{-/-}$ myofibers showed increased incidence of apoptosis and mitochondrial dysfunction.When Cspg4$^{-/-}$ animals were subjected to exercise,they showed muscle weakness and decreased resistance to fatigue.Electron microscopy revealed increased thickness of basement membrane and proliferation of pericytes in the capillary vessels of skeletal muscles.These ultrastructural alterations are remarkably similar to those detected in Congenital Myosclerosis,a rare human disorder recently linked to a peculiar COL6A2 mutation producing a truncated α2(VI) chain (4). These findings suggest that NG2 may influence pericyte phenotype and contribute to muscle fiber degeneration in ColVI-related diseases.
In conclusion,the phenotype of NG2 null mice is partially similar to that of ColVI ones,strongly pointing at an interaction between these two molecules in muscle.Future studies will allow elucidating the role of NG2 in normal and diseased muscle. 1) Bernardi P, Bonaldo P (2008). Dysfunction of mitochondria and sarcoplasmic reticulum in the pathogenesis of collagen VI muscular dystrophies. ANYAS, 1147: 303-311. 2) Grako KA et al. (1999). PDGFα-receptor is unresponsive to PDGF-AA in aortic smooth muscle cells from the NG2 knockout mouse. JCS, 112: 905-915. 3) Irwin WA et al. (2003). Mitochondrial dysfunction and apoptosis in myopathic mice with collagen VI deficiency. NG, 35: 367-371. 4) Merlini L et al (2008). Autosomal recessive myosclerosis myopathy is a collagen VI disorder. Neurology, 71: 1245-1253. 5) Petrini S et al (2005). Altered expression of the MCSP/NG2 chondroitin sulfate proteoglycan in collagen VI deficiency. MCN 30: 408-417. 6) Stallcup WB, Huang FJ (2008). A role for the NG2 proteoglycan in glioma progression. CAM, 2(3): 192-201.

The nuclear factor BRD4 binds the nucleosomes containing both H3K9ac and H4K16ac *in vivo*

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BRD4 is a double bromodomain protein that binds acetylated histones. We here attempted to identify the histone acetylation pattern that is recognized and bound by BRD4. By co-immunoprecipitation experiments we observed that BRD4 associates preferentially with nucleosomes that are acetylated on H3K9, but not with H3 acetylated on K14, as well as on nucleosomes that are acetylated on H4K16 but not with other forms. Analysis of in vivo mobility of BRD4 by fluorescence recovery after photobleaching (FRAP) demonstrated that BRD4 exchange more rapidly in nuclei expressing H3K9G and H4K16G mutant with respect to nuclei expressing wild type H3 and H4. Acceptor photobleaching fluorescence resonance energy transfer (apFRET) assay demonstrated a direct interaction of BRD4 with wild type H3 and H4 that is reduced with H3K9G and H4K16G mutants. To test whether the interaction of BRD4 with acetylated histones was via its bromodomain, we analyzed BRD4 mutant deleted for either bromodomain 1 (ΔBD1), bromodomain 2 (ΔBD2) or both (ΔBD1,2). Each mutation resulted in a reduction of the interaction between BRD4 histones H3 and H4. Moreover, recombinant BRD4 binds more efficiently to in vitro recostituited nucleosome acetylated in H3K9 and H4K16. Taken together these results demonstrate that BRD4 interacts with chromatin by recognizing and binding to nucleosome acetylated at H3K9 and H4K16.

An analogical to digital ubiquitin-based switch controls EGFR fate

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Depending on ligand concentration, epidermal growth factor receptor (EGFR) is targeted to different endocytic pathways, clathrin-dependent or -independent, which ultimately lead to distinct intracellular fates, signalling vs degradation, respectively [1]. Endocytosis has therefore a complex impact on the final biological outcome, which is the result of the integration of signals originating from different entry routes. How do the cells sense the ligand concentration and translate a quantitative input in qualitative distinct biological responses? We showed here that this is achieved through the establishment of a threshold for receptor ubiquitination over a range of EGF concentrations. To gain insight into this phenomenon we used a multidisciplinary approach, involving both molecular biology experiments and mathematical modelling. With these studies, we were able to give a mechanistic explanation of this threshold response, which is achieved through a avidity-based mechanism between two phosphotyrosine sites involved in Cbl recruitment. Finally, we developed a mathematical model of the EGFR early signalling, including EGF binding, EGFR phosphorylation and ubiquitination. Remarkably, the model reproduces – with quantitative resolution – not only the threshold effect for EGFR ubiquitination, but also its occurrence in the exact range of EGF doses where it is observed in vivo and where it exerts its biological relevance.

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Role of STAT3 and p70S6K in Breast Cancer recurrences

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Breast cancer is the most common malignancy in women worldwide. Surgery coupled with external beam radiotherapy (EBRT) represents the current standard treatment for breast cancer patients. EBRT is necessary since it reduces of about 30% the rate of local recurrences. Targeted intraoperative radiotherapy (TARGIT) is the first technique that enables delivery of radiotherapy to the tumor bed immediately after surgical excision of the tumor achieving a lower than expected recurrence rate.
Several clinical and experimental observations suggest that modifications of the local microenvironment by surgery may alter the growth of breast cancer cells.
In our work we tested the hypothesis that the act of surgery may stimulate cancer cell growth and that TARGIT may impair its effects not only by killing residual tumor cells but also by altering the local microenvironment after lumpectomy. To this aim wound fluids (WF) from women underwent lumpectomy for early breast cancer treated or not with TARGIT were collected and used to stimulate normal and tumor derived breast epithelial cells proliferation and motility.
No differences in normal breast epithelial cells proliferation and motility were observed when the cells were stimulated with WF from control or TARGIT treated patients. Conversely, TARGIT treatment significantly impaired the ability of WF to stimulate 3D cell growth and motility of breast cancer cells.
A phosphoproteomic screening of breast cancer cells stimulated with WF from TARGIT or control treated patients, revealed that the activation of STAT3 and p70S6K pathways were significantly impaired. To clarify the role of these two pathways, we introduced constitutively active or dominant negative mutants of both STAT3 or p70S6K in mammary carcinoma cell lines with high or a low invasive phenotype.
Proliferation and motility experiments demonstrated that increased p70S6K or STAT3 led to an increased proliferation or motility rate of the cells. On the contrary, the inhibition of their activity by the presence of dominant negative mutant resulted in a reduced proliferation rate and a reduced migration in response of WF used as chemo-attractants.
Overall our work suggest that p70S6K and STAT3 plays an important role in the ability of breast cancer cells to move toward and proliferate in response to attractant stimuli produced in women after lumpectomy and imply that impairing their activity could provide new specific treatment aimed to prevent local relapses in patients with early breast cancer.

p130Cas cooperates with ErbB2 to promote invasion of MCF-10A mammary epithelial acini grown in 3D cultures

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p130Cas is an adaptor protein that regulates cell survival, migration and cell cycle in normal and pathological cells. Our previous work demonstrated that p130Cas cooperates with ErbB2 in breast tumourigenesis. ErbB2 is a member of the epidermal growth factor receptor family and is over-expressed in approximately 25% of invasive breast cancers and in a large percentage of non invasive carcinomas suggesting that its over-expression alone is not sufficient to drive metastasis. Here, we explored whether p130Cas over-expression may act as a second hit that synergizes with ErbB2 to gain invasiveness. The non transformed human mammary epithelial MCF-10A cells are an excellent in vitro model for studying breast cancer progression as they form, in 3D culture, polarized, growth arrested acini, that recapitulate several aspects of the mammary gland architecture. In particular, we used the MCF-10A.B2 cells expressing a chimeric ErbB2 receptor that can homodimerize with the synthetic ligand AP1510. Activation of ErbB2 in preformed acini induces re-initiation of proliferation and disruption of cell polarity giving rise to non-invasive multiacini mimicking the early stages of breast cancer. Interestingly, MCF-10A.B2 cells overexpressing p130Cas (10A.B2 Cas cells) form multiacinar structures even in the absence of ErbB2 activation. Moreover, upon treatment with AP1510, 10A.B2 Cas cell-derived multiacinar structures showed invasive protrusions, indicating that p130Cas overexpression coupled to the activation of ErbB2 leads to the acquisition of invasive properties. Interestingly, upon ErbB2 activation higher levels of Erk1\2, Akt and p70 S6 kinase phosphorylation were found in 10A.B2 Cas invasive acini compared to controls. Consistently, inhibition of mTOR\p70S6K with rapamycin, as well as treatment with PD98059 or LY294002, inhibitors of Mek\Erk and PI3k\Akt pathways respectively, severely impaired the formation of invasive protrusions from the AP1510 stimulated 10A.B2 Cas multiacini. In addition, in silico analysis of gene expression profiles of human breast cancers revealed a significant correlation between ErbB2\p130Cas co-overexpression and tumor invasiveness. Therefore, these results suggest a role for p130Cas in conferring invasive properties to ErbB2 over-expressing tumors.

MEK/ERK/Myc axis disruption reduces growth of *in vivo* and *in vitro* rhabdomyosarcoma cells

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Myc stability and turnover depends on ERKs and GSK3-beta phosphorylations respectively, and GSK3-beta-dependent Myc degradation requires the prior phosphorylation by ERKs. This implies that the Ras-dependent PI3K pathway, through the control of GSK3-beta, also regulates Myc accumulation in transformation process.
We previously reported that the disruption of Myc pathway through MEK/ERK inhibition, blocks the expression of transformed phenotype in embryonal rhabdomyosarcoma-derived cell line (RD), inducing myogenic differentiation. In this work we address the issue on the possibilities for interventions targeting c-Myc in RD cell line by down regulating MEK/ERK pathways with the specific MEK/ERK inhibitor, U0126, in xenograft tumor model. U0126 significantly reduced tumor growth of immunodeficient mice eterotransplanted with human RD cell line. Analysis of tumor extracts showed that levels of phospho-active ERKs are sensibly reduced after prolonged period of the therapy (up to 5 weeks) demonstrating that tumors did not become refractory to the drug. Phospho- and c-Myc accumulation underwent to dramatic down regulation in tumors paralleling phospho-ERK inhibition. By contrast, normal tissues were not responsive to the drug. Proliferation and endothelial markers expression (Ki67 and CD31), highly expressed in tumors from untreated mice, were also significantly reduced in U0126-treated mice, thus indicating that MEK/ERK inhibition affects growth and angiogenic signals.
TE671, an alternative embryonal rhabdomyosarcoma cell line, and a cell line derived from untreated eterotransplanted tumor (RD-M1) were tested for responsiveness to antigrowth and differentiative action of U0126. Likewise RD cells, U0126 inhibited growth and anchorage independent growth, down regulated Myc and induced myogenic differentiation in both TE671 and RD-M1. These results suggest that during rhabdomyosarcoma development cells retain the addiction of responding to growth and transformation inhibitory effects of MEK/ERK inhibitor.
Transient and stably transfection and co-transfection with GSK3-beta and c-Myc mutants expressing vectors have been performed in order to investigate the mechanism by which c-Myc degradation occurs in active-ERK-depleted cells.
We found that a pathway other than GSK3-beta could be involved in c-Myc down regulation in U0126-treated rhabdomyosarcoma cells. On the basis of these results we suggest that MEK/ERK inhibitor might be used in a signal transduction-based Myc targeting for combating rhabdomyosarcoma.