Abstracts

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Oral Presentations

in chronological order of presentation
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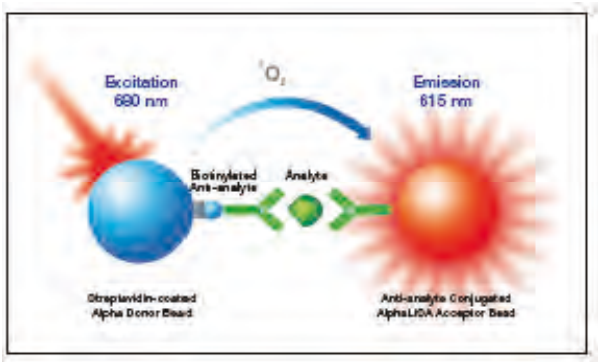

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The p63 target Sharp1 opposes breast cancer metastasis by presenting Hypoxia-Inducible-Factors to the proteasome

M. Montagner[1], E. Enzo[1], M. Forcato[2], F. Zanconato[1], A. Parenti[3], A. Rosato[4], S. Bicciato[2], M. Cordenonsi[1], S. Piccolo[1]
[1]Dept of Medical Biotechnologies, Univ. of Padua School of Medicine, Padua, Italy
[2]Center for Genome Research, Dept of Biomedical Sciences, Univ. of Modena and Reggio Emilia, Modena, Italy
[3]Dept of Medical Diagnostic Science and Special Therapies, Section of Pathology, Univ. of Padua, Padua, Italy
[4]Dep.of Oncology and Surgical Sciences and Ist. Oncologico Veneto, Padua, Italy

Identifying determinants for metastasis of very aggressive breast cancers represents an unmet medical need1. Using bioinformatic tools, we interrogated Triple Negative Breast cancer (TNBC) clinical datasets about candidate signals and molecular players mediating the malignancy of this heterogeneous class of tumors. We found that the activities of p63 and Hypoxia-Inducible-Factors (HIFs), two master regulators of the invasive and metastatic cancer cell phenotype2,3, are unanticipatedly linked in TNBC. The p63 target Sharp1 is at the center of this crosstalk. Functionally, Sharp1 inhibits invasive cell behaviors in vitro and acts as metastasis suppressor in vivo through inhibition of HIF-1α/HIF-2α. Mechanistically, Sharp1 promotes HIF-1α/HIF-2α proteasomal degradation by serving as HIFs presenting factor to the proteasome. Importantly, this process is independent of pVHL, hypoxia and the ubiquitination machinery. As such, Sharp1 is a required determinant for the intrinsic instability of HIFs proteins acting in parallel to and cooperating with oxygen levels. This work sheds light on the mechanisms and pathways by which TNBC acquires invasiveness and metastatic propensity.

Regulation of self-renewal in cancer stem cells

P.G. Pelicci
Dept of Experimental Oncology, European Institute of Oncology, Milan, Italy

Recent findings support the concept that cells with the properties of stem cells (SC) are integral to the development and perpetuation of several forms of human cancer, and that eradication of cancer stem cells (CSC) may be essential to achieve cancer cure. However, direct proof of these concepts is still lacking, mainly due the scarcity of appropriate model systems. We are characterizing the biological differences between normal and transformed SCs. SCs are defined by their abilities to generate more SCs ('self-renewal') and to produce cells that differentiate. One mechanism by which SCs accomplish these two tasks is *asymmetric cell division*, whereby each SC divides to generate one daughter with SC fate and one that differentiates. SCs, however, possess the ability to expand in number, as it occurs during development and in adulthood after injury or disease. This increase is not accounted by asymmetric divisions, in which only one daughter cell maintains SC identity. Recent findings in *C.elegans* and *Drosophila* indicate that SCs can also generate daughter cells that are destined to acquire the same fate (*symmetric cell division*). On the other hand, SC quiescence is critical to maintain tissue homeostasis after injury. We will discuss our recent findings showing increased symmetric divisions of CSCs in breast tumors (due to inactivation of the p53 tumor suppressor) and dependency of leukemia development on quiescent leukemia SCs (due to transcriptional up-regulation of the cell cycle inhibitor p21 by leukemia-associated fusion proteins). Our findings suggest that that asymmetric divisions of stem cells function as a mechanism of tumor suppression, that SC quiescence is critical to the maintenance of the transformed clone and that symmetric divisions of SCs permits its geometric expansion. Finally, I will discuss downstreeam mechanisms of regulation of SC divisions by p53 and implications of these findings for the mechanisms regulating checkpoint activation in tissue stem cells.

Targeting cancer stem cells in solid tumors

R. De Maria
Dept of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanità, Rome, Italy

Cancer stem cells (CSCs) are the rare population of undifferentiated tumorigenic cells that are thought to be responsible for tumor initiation, maintenance and spreading. Their existence might also explain why tumours are resistant to many conventional therapies, which typically target the rapidly proliferating tumor cells but spare the slow dividing tumor stem cell population.
The selective targeting of CSCs offers a potential revolutionary advance in the treatment of cancer, by attacking the roots of the disease. Such cell population should therefore represent the target of new therapies aimed at eradicating the tumor.
We developed a technology that allowed us to isolate and expand in vitro CSCs from several solid tumors, including glioblastoma, sarcomas, breast, lung, colon, thyroid and ovary cancers. We are currently characterizing these tumorigenic cell populations by high-throughput technologies at different levels, including genome-wide expression of mRNA, microRNA and proteome profiling. Such extensive characterization parallels the screening with library of pathway inhibitors and microRNAs. This strategy may provide key information on the pathways to be targeted for successful therapies. Moreover, the use of CSC-based xenografts that closely reproduce the parental tumor at morphological and molecular level may offer a unique opportunity to test new anticancer treatments and optimize individualized therapies.
Thus, although the identification of CSCs is relatively recent, this research area appears extremely promising as it may significantly contribute to the rational design of novel targeted therapies for cancer.

What's in a Picture? The Temptation of Image Manipulation

L. Williams
Rockefeller University Press, New York, NY, USA

The ease of image manipulation in powerful applications like Photoshop makes it tempting to adjust or modify digital image files. At the Rockefeller University Press, which publishes *The Journal of Cell Biology*, *The Journal of Experimental Medicine*, and *The Journal of General Physiology*, we have developed comprehensive guidelines to define the boundaries between acceptable and unacceptable manipulation, and we have a policy whereby all figures of all accepted manuscripts are examined for evidence of manipulation. These guidelines and journal practices will be discussed, along with a number of instructive examples from actual cases and tips for avoiding problems in your own work.

Genome Cell Biology

T. Misteli
National Cancer Institute, NIH, Bethesda, MD, USA

In higher organisms, genomes are housed and function in the cell nucleus. While we have learnt a great deal about the sequence of genomes in recent years, insights into how genomes function in the context of the architectural framework of the cell nucleus in a living cell are only now emerging. Several key cell biological concepts such as the existence of architectural proteins of the nucleus, the presence of distinct nuclear compartments, the non-random organization of genomes, and the dynamic nature of nuclear architecture are now recognized as driving genome function. Importantly, aberrations in nuclear architecture are now known to lead to various diseases ranging from cancer to pre-mature aging. An in depth elucidation of the cell biological properties of the genome will be essential to a full understanding of how genomes function.

Dissecting functions of Wnt signalling in cell reprogramming

M.P. Cosma
ICREA and Center for Genomic Regulation (CRG), Barcelona, Spain

The reprogramming of somatic nuclei, i.e. the de-differentiation of somatic cells into pluripotent stem-like cells has been induced in vitro by transferring somatic nuclei into enucleated oocytes, by fusing embryonic stem cells (ESCs) with somatic cells, and by transferring specific factors into different types of somatic cell.However, whether reprogramming can occur in vivo in higher vertebrates and what are the molecular mechanisms and genes driving reprogramming remain to be defined. We have shown that activation of the Wnt/β-catenin signalling pathway enhances reprogramming of somatic cells after their fusion with ESCs. Our main goal is to dissect gene networks and mechanisms controlling in vivo somatic cell reprogramming and to study whether cell-fusion-mediated reprogramming can enhance tissue regeneration.

Secreted noncoding RNAs and therapeutic neural stem cell plasticity

Stefano Pluchino, MD, PhD
Dept of Clinical Neurosciences, Cambridge Centre for Brain Repair and Cambridge Stem Cell Initiative, Univ. of Cambridge, UK

Compelling evidence exists that somatic stem cell-based therapies protect the central nervous system (CNS) from chronic inflammation-driven degeneration, such as that occurring in experimental autoimmune encephalomyelitis (EAE), multiple sclerosis (MS) and cerebral ischemic/hemorrhagic stroke. However, while it was first assumed that stem cells may act trough direct replacement of lost/damaged cells, it has now become clear that they are able to protect the damaged nervous system through a number of 'bystander' mechanisms other than cell replacement. In immune-mediated experimental demyelination and stroke – both in rodents and non-human primates - others and we have shown that transplanted neural stem/precursor cells (NPCs) possess a constitutive and inducible ability to mediate efficient 'bystander' myelin repair and axonal rescue. Yet, a comprehensive understanding of the multiple mechanisms by which NPCs exert their therapeutic impact is lacking. We envisage that the remarkable therapeutic plasticity of NPCs results from their capacity to engage highly sophisticated programmes of horizontal cell-to-cell communication at the level of the (micro)environment and we attribute a key role to the transfer of secreted membrane vesicles (MVs) from (donor) NPCs to (recipient) neighbouring cells. We are starting to define whether this form of communication is biologically relevant for NPCs, and look forward to establishing whether it is associated to cell-to-cell trafficking of non-coding RNAs (ncRNAs), and indeed on elucidating its molecular signature and therapeutic significance for MS. We believe that the true innovation of this approach relies in its unique peculiarity to look into an innate cellular mechanism with the visionary focus of translating the knowledge of basal stem cell functions into innovative high-impact clinical therapeutics for MS.

Cell fusion as a cell regenerative mechanism

M. Alvarez-Dolado
Dept of Cell Therapy, CABIMER-CSIC, Sevilla, Spain

Cell fusion is a natural process implicated in normal development, immune response, and tissue formation, that, more recently, it has been implicated in stem cell plasticity. The discovery that bone marrow derived cells fuse with different mature cell types, under normal condition or after an injury, introduces new possibilities in regenerative medicine and genetic repair. Cell fusion, as a cell regeneration mechanism or as a way to complement recessive mutations, has been shown in the liver, brain, muscle, lung and gut. In this talk, I will show you the current evidences about the role of cell fusion in the nervous system and its future potential as therapy for neurological pathologies.

The genetic determination of complex cell shapes

N.J. Nandanan, R. Mathew, F. Paul, S. Sigurbjörnsdottir, M. Leptin
Institute of Genetics, Univ. of Cologne, Cologne, Germany and The European Molecular Biology Laboratory, Heidelberg, Germany

The apical plasma membranes in many polarized epithelial cells show highly specialized morphological adaptations, such as villi in the gut or rhabdomeres in photoreceptors, which enable them to fulfill distinct physiological functions. The terminal cells of the Drosophila tracheal system generate an extreme plasma membrane specialization, the intracellular tubules that form the lumen of the oxygen transporting cell branches. The actin cytoskeleton has been suggested to be necessary for the formation and maintenance of the lumen, but it is not known how actin interacts with the luminal plasma membrane. We have found that the membranes forming the intracellular tubules contain lipids and proteins typical of apical plasma membranes in polarized epithelial cells. The Drosophila synaptotagmin-like protein Bitesize (Btsz) and the activated form of its interaction partner Moesin are also located at the growing luminal membrane. Depletion of either Btsz or Moesin leads to actin mis-localisation and defects in branch and lumen formation. We propose that the actin cytoskeleton, through its interaction with Btsz via Moesin directs apical membrane morphogenesis to create and maintain distinct intracellular tubules. The role of other proteins in the mechanism of tube formation will also be discussed.

The role of the polycystins in promoting and maintaining renal tubular structure to prevent cystogenesis

A. Boletta
Dulbecco Telethon Institute (DTI) at Dibit San Raffaele Scientific Institute

The main interest of our group is to study the normal function of Polycystin-1 (PC-1), the *PKD1* gene product, in the believe that this will help elucidating the molecular basis of the disease and will explain why in the absence of PC-1 normal function renal epithelial cells degenerate and give rise to cysts.
PC-1 is a large (520kDa) non-tyrosine kinase receptor postulated to be involved in cell-cell/matrix interactions. Consistent with this, the protein localizes at the cell-cell junctions in polarized renal epithelial cells. Recently a role for both *PKD1* and *PKD2* genes in mechanosensation has been proposed as both PC-1 and –2 localize also in the primary cilium on the apical side of epithelial cells.
In previous studies we have shown that PC-1 is involved in inducing renal tubulogenesis in vitro by showing that stable expression of full-length *PKD1* in MDCK cells induces reduced growth rates, resistance from apoptosis and spontaneous tubulogenesis when cells are cultured in 3D collagen gels. Furthermore, we have shown that PC-1 is able to regulate the actin cytoskeleteon and cell migration through PI3kinase, processes necessary for in vitro tubulogenesis.
Now we find that PC-1 is able to coordinate the microtubular and the actin cytoskeleton to achieve a polarized migration and that it does so by interacting with and regulating the aPKC/Par3 complex.
Finally, we find that this property of PC-1 might be important for proper renal development and tube morphogenesis in vivo.

Lysosomal enhancement as a therapeutic strategy for Neuronal Ceroid Lipofuscinoses

M. Sardiello, M. Palmieri, D. Sanagasetti, V. Mauri, A. di Ronza, A. Schiano
Molecular and Human Genetics Dept, Baylor College of Medicine, Jan and Dan Duncan Neurological Research Institute at the Texas Children's Hospital, Houston, TX, USA

Neuronal ceroid lipofuscinoses (NCLs) are a subgroup of lysosomal storage disorders characterized by CNS involvement, including loss of vision, motor deterioration, seizures, dementia and early death. Collectively, NCLs have an incidence of 1:12,500 live births and are currently incurable, with the only treatments being supportive and aimed at managing psychotic and affective features. NCLs are characterized by the ubiquitous intralysosomal accumulation of autofluorescent lipopigments (lipofuscin). This storage material has heterogeneous composition and, in most NCL variants, is immunoreactive to antibodies against the subunit c of mitochondrial ATP synthase (SCMAS), which is a lipophilic protein normally immersed in the inner mitochondrial membrane. This observation suggests that some specific autophagic pathway (for example, mitophagy) may be defective in NCLs, leading to the incomplete digestion of mitochondrial membranes by autophagy. The central hypothesis of our project is that genetically or chemically induced lysosomal enhancement may help restore intracellular clearance pathways in lysosomal storage disorders, thus improving disease progression. We discovered that the transcription factor EB (TFEB) is a master regulator of lysosomal biogenesis and function in mammalian cells. We found that TFEB coordinates the expression of genes involved in the synthesis, modification, import and activation of lysosomal hydrolases. It also controls the expression of autophagy genes. We demonstrated that TFEB overexpression results in an increased number of lysosomes in the cell and subsequently improved degradative capabilities against lysosomal/autophagic substrates, such as glycosaminoglycans and polyQ-expanded huntingtin. Therefore, we propose that TFEB is a genetic modifier of cellular clearance and that the modulation of its function can be the basis for the development of therapies for storage disorders. Our results in cellular and mouse models of Batten disease show that genetic or chemical modulation of TFEB function can improve the clearance of storage molecules by enhancing lysosomal pathways.

Cell biology and pharmacology of proximal tubular dysfunction in Lowe syndrome

M. Vicinanza[1], A. Di Campli[2], E. Polishchuk[2], M. Santoro[1], G. Di Tullio[3], E. Levcthenko[4], M.P. Marzolo[5], M.A. De Matteis[1]
[1]Telethon Institute of Genetics and Medicine, Naples, Italy
[2]Institute of Protein Biochemistry, CNR, Naples, Italy
[3]Consorzio Mario Negri Sud, Santa Maria Imbaro, Italy
[4]Dept of Paediatrics, Univ. Hospitals Leuven, Leuven, Belgiu
[5]Dept of Cellular and Molecular Biology, Pontificia Universidad Católica de Chile, Santiago, Chile

Oculo-cerebro-renal Lowe syndrome is a rare genetic disease that is characterised by congenital cataracts, central hypotonia and renal Fanconi syndrome, with urinary loss of protein, salt and nutrients. Lowe syndrome is caused by mutations in OCRL, a multidomain protein with a phosphatidylinositol 4,5-bisphosphate (PtdIns4,5P2) 5-phosphatase catalytic activity that is localized in the endosomal system and the trans-Golgi network. Our knowledge of the functional role of OCRL lags well behind that of its molecular structure. To gain insight into its function, into its PtdIns4,5P2 5-phosphatase activity in membrane trafficking, and into the pathogenetic mechanisms of Lowe syndrome, we have analysed the endocytic compartments in kidney proximal tubular cells obtained from Lowe syndrome patients. Similarly, in kidney cell lines, we have studied the functional and ultrastructural consequences of the knock-down of OCRL on multiple endocytic pathways, through RNA interference. Our data indicate that through its PtdIns4,5P2 5-phosphatase activity, OCRL maintains the identity and function of early endosomes, the structure of early endosomes as a compartment, and the efficiency of trafficking pathways that traverse this compartment. This includes megalin, the multiligand receptor that drives uptake and absorption of protein in proximal tubular cells, and that undergoes mistrafficking in cells devoid of functional OCRL. We have exploited the endocytic defects of cells deprived of OCRL to set up assays for high-content screening of small-molecule libraries to identify active compounds for drug development for the treatment of patients with Lowe syndrome.

Microglia: the guardians of the developing brain

F. Peri
EMBL, Heideberg, Germany

A significant proportion of neurons in the brain undergo programmed cell death. In order to prevent the diffusion of damaging degradation products, dying neurons are quickly collected by microglia, specialised phagocytes that are resident in the brain. Despite the importance of these cells in several neuronal pathologies, many fundamental questions concerning microglial-neuronal interactions remain unaddressed. How these cells collectively ensure that the entire brain is surveyed and how they react to damage with high precision is still entirely unknown. Recent findings suggest that diffusible molecules such as lipids and nucleotides could attract microglia in response to neuronal apoptosis and injury, respectively. While these molecules can trigger dynamic changes in microglia motility in vitro, elucidating how their activity is controlled within the intact brain, both in space and time, remains the most important challenge in understanding this fascinating biological problem.
We exploit the imaging potential of the transparent zebrafish embryo for studying microglial biology *in vivo*. By combining forward and reverse genetic approaches with quantitative imaging technology, we address the mechanisms underlying the attraction of microglia towards apoptotic, sick and injured neurons.

Chromatin and behavioral plasticity: Toward an understanding of the role of chromatin plasticity in long-term memory formation

C. Margulies[1,2], T. Schauer[1,2], P. Schwalie[4], A. Handley[2,3], P. Flicek[4], A. Ladurner[1,2]
[1]European Molecular Biology Laboratory (EMBL) Heidelberg, Germany
[2]Present address: Butenandt Institute for Physiological Chemistry, Medical Faculty, Ludwig Maximilians Universitity Munich, Germany
[3]IMPRS Graduate Program Munich, Germany
[4]European Bioformatics Institute (EMBL-EBI) Cambridge, UK

Misregulation of transcription and chromatin has serious consequences for neuronal development and behavior. Mutations in genes regulating chromatin structure and gene expression result in retarded growth and impaired cognitive function, as seen in Retts, Coffin-Lowry, Rubinstein-Taybi, Fragile X and Kleefstra Syndrome.
To better understand how neuronal and behavioral plasticity regulate gene function, we use behavioral paradigms and novel genomics approaches to probe the role of chromatin architecture and gene expression. The central nervous system is a complex network of different cell-types that respond to different stimuli. To obtain a molecular correlate of how animal behavior regulates chromatin and transcriptional dynamics, it is therefore necessary to profile gene expression and chromatin states within the specific cell-types that are required for a given animal behavior. We have established cell-type specific methods to monitor gene expression and chromatin states within intact, behaving animals, the fruit fly Drosophila melanogaster. By combining cell-type specific approaches and genome wide approaches, we have characterized the gene expression states of several different cell types and brain regions vital for the formation of olfactory memory. We have evaluated the role of histone variants and other histone modifications in tissue specific gene expression in the central nervous system. Our approaches will provide us novel insight into tissue- and cell-type specific chromatin states and gene expression patterns, which will allow us to dissect how complex animal behaviors dynamically regulate gene expression.

Roles of the RNA binding proteins Staufen and Pumilio in neuronal morphogenesis.

J.P. Vessey[1], E. Luzi[2], M. Kiebler[3], P. Macchi[4]
[1]Development and Stem Cell Biology, The Hospital for Sick Children - MaRS Centre, Toronto Medical Discovery East Tower, Toronto, Canada
[2]Center on Endocrine Hereditary Tumors, AOUC, Dept of Internal Medicine, Univ. of Firenze, Italy
[3]Center for Brain Research, Medical Univ. of Vienna, Vienna, Austria
4 Centre for Integrative Biology, Lab of Molecular and Cellular Neurobiology, Univ. of Trento, Italy

Eukaryotic cells adopt different mechanisms to control gene expression: transcriptional regulation, post-transcriptional regulation of mRNA translation and turnover, and post-translational regulation of proteins turnover. Another mechanism, the localization of RNAs, is emerging as an important process to restrict certain messages and proteins to specific subcellular domains and thus spatially control the expression of genes within cells. In neurons, local translation of localized mRNAs in dendrites allows for de novo synapse formation, the morphological rearrangement of dendritic spines and for the regulation of the efficacy in the strength of existing synapses.
One key feature of mRNA localization is that this event should precede translation. As a consequence, mRNAs have to be kept translationally silent during their transport towards the proper target compartment. RNA localization has been studied in many organisms and cell types and research over the last decade has shown that homologs of key components of the mRNA localization machinery exist from yeast to humans. Members of the Staufen (Stau) and Pumilio (Pum) gene family are important regulators of intracellular mRNA transport and translation that have been conserved in all eukaryotic organisms during evolution. The role of both Stau2 and Pum2 in developing and mature neurons has been recently determined. In immature neurons, loss of these RNA-binding proteins led to impaired dendritic outgrowth and arborization. In mature neurons, theirs down-regulation resulted in a significant reduction in dendritic spines and an increase in elongated dendritic filopodia. These observations reveal novel roles for Stau2 and Pum2 in the formation and the maintenance of the neuronal polarity.

Dynamics of endocytosis

T. Kirchhausen
Dept Of Cell Biology, Harvard Medical School, Boston, MA, USA

This talk will focus on the use of cutting-edge high resolution structural visualization combined with dynamic single molecule and live cell fluorescence imaging techniques to understand clathrin mediated endocytic processes involved in communication of cells with its environment, in pathogen invasion and viral infection, in cell growth control and cancer, and in the biogenesis of organelles. The goal is to generate molecular-resolution movies describing the function of machineries responsible for the control of these types of carefully choreographed interactions in cells. We will explore the regulation of the clathrin machinery engaged in classical endocytosis by presenting an integrated view based on recent data from snapshots (cryoEM tomography and 3-D single-particle reconstruction, x-ray crystallography) and from dynamic imaging (live cell single-object tracking and single molecule fluorescence microscopy) to show how they are used to inform cell, biochemical and mechanistic studies.

Systems Biology approaches to understand gene function and drug mode of action

D. di Bernardo
Telethon Institute of Genetics and Medicine & Dept Computer Science and Systems, Univ. of Naples "Federico II", Naple, Italy

In this presentation, I will describe our recent results on reverse-engineering gene networks, which allow to identify putative regulatory and physical interactions among genes from measurements of thousands of Gene Expression Profiles. The resulting networks can be used to make hypotheses on regulators of specific pathways or on the function of a gene of interest. In addition, using gene expression profiles obtained only from specific tissues, it is possible to identify tissue specific pathways. Application of this methods to elucidate the function of the Granulin gene and to identify tissue specific Transcription Factors regulating the expression of enzymes in specific metabolic pathways will be presented.
Finally, a recent extension of this work to identify the drug mode of action of a small molecule of interest from gene expression profiles obtained by treating multiple cell lines with more than 1300 compounds will be presented.

Why it is so difficult to make a model of the Spindle Assembly Checkpoint

R. Manzoni[1], F. Gross[1], L. Mariani[1], G. Varetti[2], A. Musacchio[2,3] A. Ciliberto[1]
[1]IFOM, The FIRC Institute of Molecular Oncology, Milan
[2]IEO, European Institute of Oncology, Milan
[3]Max Planck Institute of Molecular Physiology, Dortmund

The spindle assembly checkpoint is a surveillance mechanism that arrests cells in pro-metaphase in the presence of unattached kinetochores. While it is accepted that kinetochores originate the 'wait anaphase signal', it is not yet clear whether they can support the checkpoint alone, or if they are backed by SAC activators away from kinetochores. In particular, it was proposed that a positive feedback loop could be involved in checkpoint maintenance, an hypothesis criticized for the possibility of the checkpoint to remain active despite of kinetochore-microtubule attachment.
We have developed a mathematical model based on data of mammalian cells, where we have analyzed the implications of the presence of foci of checkpoint activation away from kinetochores. We show that the presence of a positive feedback loop greatly helps to maintain the checkpoint active with few unattached kinetochores, and that it can be fully switched off as a consequence of checkpoint attachment. Our data also suggest that the checkpoint is much more robust when we hypothesize that unattached kinetochores both originate the checkpoint signal and concur to stabilize it. Finally, by constraining our model with published biochemical parameters we reproduce time courses of checkpoint activation and release experiments.

Between systems and data-driven modeling for computational biology: target identification with Gaussian processes

N. Lawrence
Depts of Computer Science and Neuroscience, Univ. of Sheffield, Sheffield, United Kingdom

A simple approach to target identification through gene expression studies has been to cluster the expression profiles and look for coregulated genes within clusters. Within systems biology mechanistic models of gene expression are typically constructed through differential equations. mRNA's production is taken to be proportional to transcription factor activity (with the proportionality given by the sensitivity) and the mRNA is assumed to decay at a particular rate. The assumption that coregulated genes have similar profiles is equivalent to assuming both the decay and the sensitivity are high.
Typically researchers either use a data driven approach (such as clustering) or a model based approach (such as differential equations). In this talk we advocate hybrid techniques which have aspects of the mechanistic and data driven models. We combine simple differential equation models with Gaussian process priors to make probabilistic models with mechanistic underpinnings. We show applications in target identification from mRNA measurements.

S1P- $S1P_1$ signaling – a critical negative regulator of tip cell formation and angiogenic sprouting

C. Betsholtz
Karolinska Institutet, Stockholm, Sweden

Several signaling pathways have been implicated in endothelial tip cell formation and guidance. Dll4/Notch signaling, for example, has an essential role as a negative regulator of angiogenic sprouting. Dll4 is released by tip cells in response to VEGF-A and suppresses the tip cell phenotype in neighboring stalk cells by down-regulating the expression of VEGF receptors. Likely, additional negative regulators exist in order to inhibit sprouting to occur illegitimately at different locations. One such location is the dorsal aorta. Here, VEGF-A drives formation of the vessel by promoting condensation of dispersed progenitors, however once the vessel has formed and carries flow, the migratory and mitogenic activities in its endothelium are rapidly shut down. Yet, VEGF-A produced at nearby locations (i.e. the somites) drive growth and branching of the inter-somitic vessels. Why the aorta itself remains quiescent to these signals remains unknown. We have postulated that molecules present in plasma might provide angiogenesis-inhibitory signals. Sphingosine-1-phosphate is a plasma-borne bioactive lipid that binds to several G-protein-coupled receptors ($S1P_{1-5}$). Knockout of the endothelial-specific $S1P_1$ receptor leads to endothelial hyper-sprouting in the developing central nervous system and at other analyzed locations. Ectopic formation of numerous aortic branches in the knockouts leads to progressive disintegration of the aortic wall and embryonic death at E14. $S1P_1$-specific antagonists promote hyper-sprouting in vitro and in vivo, whereas agonists have the opposite effect. In vitro experiments demonstrate that $S1P_1$ inhibition enhances the angiogenic sprout-inducing effect of VEGF-A, and also leads to detachment of endothelial cells from each other, whereas $S1P_1$ activation in the presence of VEGF inhibits the formation of new sprouts and promotes endothelial cell-cell adhesion and stability of already formed sprouts. Biochemical experiments point to a role of $S1P_1$ signaling in regulating the activity of VEGFR2. These results identify S1P/ $S1P_1$ signaling as a new fundamental regulator of angiogenic sprouting, potentially coupling cessation of angiogenic sprouting to the onset of blood flow.

Special Interest Groups • Parallel Sessions
Mechanisms of Signal Transduction

Genetic modeling of PI3K inhibition

E. Hirsch
Molecular Biotechnology Center, Univ. of Torino, Torino, Italy

Phosphoinositide 3-kinases (PI3K) are crucial elements needed for receptor-mediated signal transduction and modification of PI3K signaling is emerging as a key element in cancer, inflammation, metabolic disorders and cardiovascular diseases. PI3K consist of heterodimers of a 110 kD catalytic (p110) as well as a regulatory/adapter subunit and are required for the production of a membrane bound phosphorylated lipid (PIP3) that acts as a critical secondary messenger molecule. Class I p110s (p110α, β, γ and δ) share significant homology but studies using genetically engineered mice show that they all play non-redundant roles. Interestingly, modeling by genetic means of PI3K inhibition revealed that different isoforms can be distinctly involved in different pathologies. For example, we recently demonstrated that, while PI3Kγ is crucially involved in the establishment of inflammatory responses, PI3Kβ is a key determinant in the development of Erbb2-driven mammary gland cancer. In addition, we also recently found that PI3Kγ signaling also occurs in the heart where it can modulate the contractile response and contribute to the development of heart failure. While these genetic studies recently provided support for PI3K catalytic activity as a promising drug target they also unexpectedly revealed that these proteins not only work as kinases but also as scaffolds for protein-protein interactions. Despite this complex regulation, genetic modeling of PI3K inhibition clearly supports that selective targeting of different PI3K isoforms can represent a promising strategy to improve efficacy and reduce side effects. Efforts to produce and test such drugs are under way and clinical trials are foreseen for the next future.

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Exploring liprin-α1-mediated cell migration and invasion

V. Astro[1], S. Chiaretti[1], F. Capriotti[1], D. Tonoli[1], M.G. Cangi[2], C. Doglioni[2], I. de Curtis[1]
[1]Cell Adhesion Unit, San Raffaele Univ., Milan, Italy
[2]Pathology Unit, San Raffaele Univ., Milan, Italy

The metastatic process requires the ability of cancer cells to break the basement membrane and migrate through a complex three-dimensional environment. We have recently identified liprin-α1 as a regulator of cell edge dynamics in moving cells. We demonstrated that liprin-α1 is required to maintain the persistence of MDA-231 breast cancer cell directional migration. This effect correlates with the enhanced instability of the lamellipodia, and the consequent decrease of their lifetime in cells depleted of endogenous liprin–α1. Liprin-depletion also affects the stability of invadopodia, and this effect may underlie the strong reduction of MDA-231 cell invasion and extracellular matrix degradation observed after liprin-α1 silencing. The role of liprin-α1 on invasiveness is supported by the immuno-histopathological analysis of samples from human breast cancers, where the expression of the liprin-α1 protein was often enhanced with respect to normal tissue. In order to clarify the molecular mechanisms linking liprin-α1 to invasion, we are addressing the role of its interactors in the cellular processes relevant to invasion. Liprin-α1 binds a number of partners, including the scaffold proteins ELKS, the LAR protein tyrosine phosphatase receptors, and the ArfGAPs of the GIT family. Liprin-α1-mediated MDA-231 cell spreading and active β1 integrins redistribution are dependent on the interaction of liprin-α1 with LAR. LAR and GIT proteins are not involved in liprin-α1-mediated cell invasion. On the other hand our results show the direct interaction and the colocalization of liprin-α1 with ELKS, and inhibition of MDA-231 invasion following ELKS silencing. Our findings indicate that liprin-α1 is an essential player during cancer cell migration and invasion, and are leading to the identification of a novel molecular pathway needed to coordinate cellular events required for directed malignant cell migration.

STAT3 enhances migration and invasion in mammary tumor cells: a potential role for microRNAs -143 and -145

L. Avalle, I. Barbieri, S. Pensa, P. Provero, V. Poli
Molecular Biotechnology Center, Dept of Genetics, Biology and Biochemistry, Univ. of Turin, Turin, Italy

The transcription factor STAT3 is constitutively activated in many tumors, where it contributes to several processes such as proliferation and tissue invasion. We have generated knock-in mice expressing STAT3C, a constitutively active form of STAT3, and shown that it can cooperate with HER2 in mammary tumorigenesis (Barbieri et al., 2010). The development of more aggressive tumors in Stat3C/MMTV-NeuT mice correlates with an increased migratory and invasive ability of tumor-derived cells, which display disrupted distribution of cell junction markers. We showed that Cten, known to be required for EGF-induced cell migration, is a novel Stat3 target that contributes to the aggressive phenotype of Stat3C cells. We found that all STAT3C cell lines express high levels of microRNAs-143 and -145, which are known to be required for the reprogramming of adult fibroblasts into smooth muscle cells and other processes involving an epithelial to mesenchymal transition (EMT). Interestingly, one STAT3C cell line generates a subpopulation of spindle-shaped cells displaying a mesenchymal-like phenotype, express high levels of EMT markers, and further up-regulate microRNAs-143 and -145. The potential contribution of these two miRNAs to the highly migratory and invasive phenotype of the Stat3C cells, as well as to their ability to undergo this EMT-like transition, is being explored.

HERG1 potassium channels are able to influence VEGF secretion through a macromolecular complex involving the beta1 integrin and the PI3K subunit p85

O. Crociani[1], E. Lastraioli[1], S. Pillozzi[1], A. Fiore[1], A. Morabito[1], F. Zanieri[1], A. Fortunato[1], M. D'Amico[1], E. De Lorenzo[1], A. Becchetti[2], A. Arcangeli
[1]Dept of Experimental Pathology and Oncology, Univ. of Firenze and Istituto Toscano Tumori (ITT), Firenze, Italy
[2]Dept of Biotechnology and Biosciences, Univ. of Milano Bicocca, Milano, Italy

Tumour growth greatly depends on angiogenesis. To sustain tumour growth, cancer cells begin to promote angiogenesis early in tumorigenesis, mainly through the secretion of angiogenic factors, the most potent of them being the vascular endothelial growth factor (VEGF)-A.
We studied the relationships between K+ channels of the hERG1 family and VEGF-A expression in cancers of the gastrointestinal tract. We provide evidence that hERG1 channels are functionally linked to the VEGF-A pathway in both colorectal (CRC) and gastric cancer (GC) cells. In fact, in such models, hERG1 channels regulate the secretion of VEGF-A through the transcriptional control of the vegf-a gene. In these cells the level of hERG1 expression is significantly related to VEGF-A expression and secretion. Moreover, the quantity of the secreted protein is negatively affected by treatment with specific hERG1 channel blockers and by transfection of specific anti-hERG1 siRNAs. Such mechanisms can be ascribed to the presence of a macromolecular complex on the plasma membrane of CRC and GC cells, in which hERG1 and the beta1 integrin interact and are able to trigger downstream signaling cascades. Such mechanism has Akt/PKB (and its activation) as leading actor. The Akt activation is due to a phosphorylation elicited by the regulatory subunit of PI3 Kinase, p85. Akt phosphorylation is also significantly reduced in the presence of specific hERG1 blockers. It is worth noting that p85 is directly linked to the hERG1/beta1 complex and the use of specific hERG1 inhibitors, as well as beta1 blockers, lead to a decrease of its phosphorylation. In the same way, beta1 activation can elicit p85 phosphorylation.
These data, together with the fact that Hypoxia Inducible Factors seem to be involved in this complicated protein network, lead us to suppose a novel molecular device which controls VEGF-A. Such regulation has been demonstrated in vitro, as well as in different in vivo models.

Human immunodeficiency virus-1 Tat activates NF-κB through direct interaction with IκB-α and p65

G. Fiume, F. Trimboli, C. Palmieri, E. Vecchio, C. Falcone, M. Pontoriero, A. Pisano, A. Rossi, A. de Laurentiis, G. Scala and I. Quinto
Dept of Experimental and Clinical Medicine, Univ. of Catanzaro "Magna Graecia"

The NF-κB activity is deregulated in HIV-1-infected cells leading to up-regulation of NF-κB-dependent expression of pro-inflammatory genes; the mechanisms underlying the NF-κB deregulation by HIV-1 are relevant for immune dysfunction in AIDS. We have investigated whether the HIV-1-encoded Tat transactivator sustains the NF-κB activity by interacting with the IκB-α repressor of NF-κB and the p65 transcriptional subunit of NF-κB. Upon single round infection of Jurkat cells with HXB2env-pseudotyped NL4-3 R-E- Luc virions, Tat associated with IκB-α competing its inhibitory binding to the NF-κB complex, and bound to p65 increasing its DNA-binding affinity and transcriptional activity. The arginine rich region of HIV-1 Tat (R49,52,53,55,56,57) was required for the interaction with IκB-α, while the Cys-rich region of Tat (C22, 25, 27) and the Rel homology domain of p65 (1-121 aa) were involved in physical interaction of these proteins. In HIV-1 pseudotyped infected-Jurkat cells as well as Tat-transfected Hela cells, Tat enhanced the expression of several NF-κB-dependent genes as measured by Real time PCR. In particular, the expression of the MIP-1α gene, coding for a chemokine activated in neuronal glia cells in AIDS patients, was mostly induced by Tat. By chromatin immunoprecipitation assay, Tat increased the recruitment of p65 at the MIP-1α NF-κB enhancer and was present within the p65 complex bound to the DNA. Tat also promoted the removal of IκB-α from the same NF-κB enhancer while increasing the recruitment of Ser5-phosphorylated RNA polymerase II. The evidence of direct interaction of Tat with IκB-α and p65 for NF-κB activation suggests a new mechanism of deregulated inflammatory response in AIDS.

Sam68 defines tumorigenic and metastatic capacity of colon cancer stem cells via CD44v6 and met

M. Gaggianesi[1,2] ,A. Turdo[2], A. Benfante[2], V. Catalano[2], F. Iovino[2], M. Todaro[2], A. Graziani[3], G. Stassi[1,2]
[1]Dept of Cellular and Molecolar Oncology IRCCS Fondazione Salvatore Maugeri, Pavia (PV) Italy
[2]Dept of Surgical and Oncological Sciences, Univ. of Palermo, Italy
[3]Dept of clinical and exsperimental medicine, Univ. del Piemonte Orientale "A. Avogadro", Novara, Italy

Until few years ago, all neoplastic cells within a tumor were suggested to contain tumorigenic growth capacity, but recent evidence hints to the possibility that such feature is confined to a small subset of cancer-initiating cells, also called cancer stem cells (CSCs). Thus, malignant tumors are organized in a hierarchical fashion in which CSCs give rise to the more differentiated tumor cells.
CSCs possess high levels of ABC transporters and anti-apoptotic molecules and slow replication capacities, that confer refractoriness to antineoplastic treatments.
Sam68 (Src-asociated in mitosis 68 kDa) is a multifunctional RNA-binding protein involved in multiple steps of RNA metabolism, such as alternative splicing, whose expression and activity are often altered in several cancer, including colon cancer.
We find that Sam68 plays a pivotal role in the tumorigenic potential of CSCs, interfering simultaneously with CSCs growth and migration. Sam68 downregulation reduces colon CSCs metastatic capacity regulating CD44 and Met splicing and the expression of Snail and Twist.
CD44 is a cell-surface glycoprotein involved in cell-cell interactions, cell adhesion and migration.
CD44 gene is composed of two groups of exons: one group forms the standard isoform, while the second group can alternatively be spliced and transcribed in alternative variant proteins (CD44v). CD44v6 acts as co-receptor of tyrosine kinase receptor Met. The formation of this complex induces Met activation promoting cell proliferation, migration and invasion. Interestingly, CSCs express CD44v6 and the CD44v6/Met overlapped population is limited to a small number of cells, that are the unique subpopulation showing both in vitro and in vivo metastatic potential.
These results establish an accurate roadmap of the signaling pathways that control CSCs tumorigenicity and metastatic potential, whose investigation will allow the identification of specific molecular targets for innovative selective cancer therapy.

Non-integrin cell adhesion triggers ligand-independent integrin signaling

G. M. Sarra Ferraris[1], C. Schulte[1], C.D. Madsen[1,2], N. Sidenius[1]
[1]IFOM, The FIRC Institute for Molecular Oncology Foundation, Milan, Italy
[2]Cancer Research UK London Research Institute, London, UK

Integrins 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 find out that most, if not all, adhesion receptors trigger integrin signaling independently of direct contacts between integrins and the ECM. 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. This signaling is fully active on VN lacking the integrin binding site and sustained by integrin mutants deficient in ligand binding, but is dependent on an "active" conformation of the receptor as well as its binding to intracellular adaptors such as talin
and kindlin. Ligand-independent integrin signaling is not restricted to uPAR as it poses no identifiable constraints to the adhesion receptor with respect to ternary-structure, ligand type, or means of membrane anchorage.
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.

Pin1 and notch1: a molecular circuitry fueling tumor progression

A. Zannini[1], A. Rustighi[1], L. Tiberi[1,2], G. Sorrentino[1], I. Aifantis[5], G. Del Sal[1]
[1]National Laboratory CIB, Trieste, Italy. and Dept. of B.B.C.M. Univ. of Trieste, Trieste, Italy
[2]present address: Laboratory of Neurophysiology, Institute for Interdisciplinary Research (IRIBHM), Free Univ. of Brussels, Belgium. 5 NYU Cancer Institute, NYU School of Medicine, USA

Emerging evidence point to a crucial role of the prolyl-isomerase Pin1 as an important modulator of many cellular processes. This enzyme catalyzes conformational changes on its substrate following prolyl-directed phosphorylation that profoundly affect their functions. Recently we have demonstrated that Pin1 affects the Notch1 receptor pathway1, that plays fundamental roles in breast cancer and in the maintenance of breast cancer stem cells. Notch1 encodes a transmembrane receptor that is cleaved to release an intracellular domain (N1-ICD) that is directly involved in transcriptional control. Several findings suggest that the stability of N1-ICD could be an important regulator of intracellular signalling thresholds and that abrogation of the Notch1 degradation machinery could predispose cells for transformation. An important E3 ubiquitin-ligase that targets nuclear N1-ICD for proteasomal degradation is Fbxw7. This ligase has been shown to cause suppression of the Notch signal, acting as a potent tumour suppressor. Owing to the strong correlation between high nuclear accumulation of N1-ICD and Pin1 protein levels, that we observed in a breast cancer tissue array, we investigated the role of Pin1 in the Fbxw7 dependent downregulation of N1-ICD. We have found that Pin1 contributes to sustained high levels of N1-ICD by interfering with proteasomal degradation mediated by the Fbw7. Our results furthermore indicate that Pin1 and N1-ICD cooperate in N1-ICD dependent transcription and tumorigenic activities in breast cancer cells. Notably, same results have been obtained for Notch4, that has an emerging role in breast cancer stem cells' properties. Moreover, we have found Pin1 to be transcriptionally upregulated by both, N1- and N4-ICD, highlighting a self-sustaing molecular circuitry with potential impact in tumor progression.
1) Rustighi, A. et al. The prolyl-isomerase Pin1 is a Notch1 target that enhances Notch1 activation in cancer. Nat Cell Biol 11, 133-142 (2009).

Special Interest Groups • Parallel Sessions
Stem Cells, Development and Regenerative Medicine

The multifaceted essence of CNS stem cells in vitro: developmental properties and physiological relevance

L. Conti
Dept Pharmacological Sciences and Center for Stem Cell Research, Univ. degli Studi di Milano, Milano, Italy

Neural stem cells (NSCs) are self-renewing multipotent populations present in the mammalian CNS. They generate the neurons and glia of the developing and adult brain. NSCs can be experimentally derived or induced from different sources, and the systems generated so far are promising tools for basic research and biomedical applications.
In the last years, it has become clear that during brain development, predetermined developmental programmes give rise to different NSC populations in specific locations and at specific times. In my presentation, I will provide an overview of how this temporal development is partially recapitulated in vitro during embryonic stem cell neuralization processes. In particular, I will focus on monolayer NSC systems that have allowed the generation of pure cultures of NSCs that can be stably maintained in vitro. These NSCs can divide and differentiate in vitro in the absence of any regulatory niche and represent useful tools to investigate aspects of the NSCs biology in physiological and pathological conditions.

Tbx1 is required in endothelial cells to establish vascular patterning in the brain

S. Cioffi[1,2], M. Bilio[2], S. Martucciello[3], S. Arbucci[2], E. Nusco[1], E. Illingworth[2, 3]
[1]Telethon Institute of Genetics and Medicine, Naples, Italy and European School of Molecular Medicine (SEMM)
[2]CNR Institute of Genetics and Biophysics "Adriano Buzzati Traverso", Naples, Italy
[3] Universita' degli Studi di Salerno, Fisciano, Italy

Angiogenesis, the growth of new blood vessels from pre-existing ones, is acrucial force for shaping the nervous system and protecting it from disease. Blood vessel abnormalities and blood-brain barrier disruption have been documented inseveral brain diseases.
TBX1 mutation causes most of the physical features associated with 22q11.2 deletionsyndrome (22q11.2DS) in humans and mice. Behavioral disorders are found in most 22q11.2DS patients and an estimated 35-40% of patients develops psychiatricdisorders, mainly schizophrenia. Currently, it is not known whether TBX1 mutation contributes to these disorders.In the mouse brain, Tbx1 expression is restricted to endothelial cells (EC) during development and postnatal life. Here we show for the first time that loss of Tbx1 inmice causes brain vascular abnormalities consisting mainly of a widespread increase in vessel number, enhanced angiogenic sprouting and branching and disorganizationof the entire brain vessel network. This phenotypic picture is reproducible in a 3D matrigel culture of HUVECs after Tbx1 knockdown by siRNA, in which there is anincrease in the number of microtubule branch points, suggesting that the vascular phenotype is cell-autonomous.At the molecular level, we show in ECs, Tbx1 regulates Delta-like ligand 4 (Dll4), a transmembrane ligand for the Notch family of receptors. Dll4-Notch1 signalingnegatively regulates angiogenesis during development by inhibiting endothelial tip cell formation and reducing vessel branching. Accordingly, heterozygous deletion ofDll4 in mice greatly increases the number of filopodia-extending endothelial tip cells in several tissues, including brain, a phenotype that is similar to that seen Tbx1 nullembryos. Our findings lead us to hypothesize that Tbx1 regulates brain angiogenesis through the Dll4-Notch pathway.

Stemness in complex epithelial/mesenchymal organ: the liver specific derivatives common origin

A. Conigliaro[1], F. Garibaldi[1], C. Cicchini[1], V. Costa[1], M. De Santis[1], B. Sacchetti[2], M. Riminucci[2], P. Bianco[2], L. Amicone[1], M. Tripodi[1]
[1]Dept Cellular Biotechnologies and Hematology, Univ. Sapienza of Rome, Italy
[2]Dept Experimental Medicine,Univ. Sapienza of Rome, Italy

Our work focuses on liver stem cell plasticity: we gathered evidences for a common origin of liver specific epithelial/mesenchymal derivatives.
We previously characterized a number of stable stem cell lines named RLSCs (from Resident Liver Stem Cells) that spontaneously differentiate into hepatocyte RLSCdH (from RLSC derived Hepatocytes) (Conigliaro et al.CDD 2008 Jan). Notably RLSCs were proved also to recapitulate the hepatocitic post-differenziative patterning defined "zonation"; their spontaneous differentiation, in fact, generates periportal hepatocytes that may be induced to switch into perivenular hepatocytes by means of the convergence of Wnt signalling on the HNF4a-driven transcription (Colletti et al. Gastroenterology 2009 Aug).
Recently we gathered data on: 1) the RLSC capacity to give rise to both epithelial and mesenchymal liver specific derivatives; 2) the niche factors influencing their plasticity; 3) the molecular mechanisms allowing the mesenchymal/epithelial diffentiations.
In summary we found that:
1) in orthotopic transplants RLSCs give rise to both parenchymal (hepatocyte and colangiocyte) and stromal cells (Hepatic Stellate Cells, HSC). The morphology and anatomical localization of exogenous cells match the expected one: while polarized HNF4-positive cells are arranged in hepatic cords within the lobuli architecture, mesenchymal GFAP-positive cells were found scattered throughout the organ and under the vessel (Disse space). This evidences are of particular relevance considering HSCs involvement in liver homeostasis and fibrogenesis;
2) in heterotopic co-transplants of RLSCs and HUVEC, the endothelium provides a pivotal instruction allowing RLSCs to execute four different destinies: self renewal, hepatocitic, cholangiocytic and HSC differentiation;
3) the RLSCs plasticity is based on their metastable heterogeneity characterized by co-expression of mesenchymal and ephitelial markers.

Harnessing the cell cycle to the advantage of regenerative medicine

M.G. Biferi, E.M.R. Puggioni, G. Falcone, D. Pajalunga, A. Mazzola, F. Michelini, M. Crescenzi
Dept of Cell Biology and Neurosciences, Istituto Superiore di Sanita', Roma, Italy

Despite spectacular progress in the understanding of molecular cell cycle control in the last quarter century, the new knowledge has rarely been translated into practical applications.We have demonstrated that terminally differentiated, senescent, and quiescent cells alike can be mitotically reactivated by KD of specific cell cycle inhibitors (CKIs), showing that all non-proliferating cells critically depend on CKIs to maintain their state.
Stem cell expansion, tissue engineering, and tissue repair are often hindered by poor cell proliferation. CKI ablation induces and hastens replication of quiescent cells, making this technique potentially applicable to a wide variety of regenerative medicine approaches, both in vitro and in vivo, whenever cell proliferation is a limiting factor.To demonstrate that short-term, reversible CKI ablation does not harm the cells, we induced proliferation of quiescent, primary human fibroblasts in the absence of serum by RNAi for the p21 CKI. The forcibly reactivated cells, compared with controls, showed no increases in H2AX phosphorylation, DNA breaks, cytogenetic abnormalities, or HPRT mutations. In addition, similarly treated skeletal muscle precursor (satellite) cells showed no impairment of the muscle differentiation program. These results suggest that short-term CKI suppression can be safely used in human therapy.
To demonstrate that CKI ablation is suitable to foster tissue repair in vivo, we infected intact, murine tibialis anterior muscles with AAV9 expressing an shRNA to p21. CKI suppression induced a wave of cell proliferation 5 to 20 days postinfection and greatly increased muscle cellularity. Satellite cells increased in number 2- to 10-fold and produced extensive muscle fiber neoformation, demonstrating the ability of CKI suppression to promote muscle growth even in the context of intact tissue. We are currently determining whether p21 RNAi accelerates tissue repair in damaged muscles.

CXCR6, a newly defined biomarker of tissue-specific stem cell asymmetric self-renewal, identifies more aggressive human melanoma cancer stem cells?

C.A.M. La Porta[1], R. Taghizadeh[2], M. Noh[2], Y.H. Huh[2], B. Arosio[1], J. Sherley[2]
[1]Dept of Biomecular Science, UniMI, Milan, Italy
[2]Boston Biomedical Research Institute, Watertown, MA, USA

A fundamental problem in cancer research is identifying the cell type that is capable of sustaining neoplastic growth and its origin from normal tissue cells. Recent investigations of a variety of tumor types have shown that phenotypically identifiable and isolable subfractions of cells possess the tumor-forming ability. In the present paper, using two lineage-related human melanoma cell lines, primary melanoma line IGR39 and its metastatic derivative line IGR37, two main contributes are reported. The first one is the first phenotypic evidence to support the origin of melanoma CSCs from mutated tissue-specific stem cells and the second one is the identification of a more aggressive subpopulation CXCR6+ in melanoma.
We defined CXCR6 as a new biomarker for tissue-specific stem cell asymmetric self-renewal. Thus, the relationship between melanoma formation and ABCG2 and CXCR6 expression was investigated Consistent with their non-metastatic character, unsorted IGR39 cells formed significantly smaller tumors than unsorted IGR37 cells. In addition, ABCG2+ tumors gave a 2-fold greater mass than tumors from unsorted cells or ABCG2- cells. Furthermore, CXCR6+ cells produced more aggressive tumors. These findings show that CXCR6 identifies a more discrete subpopulation of cultured human melanoma cells with a more aggressive melanoma cancer stem cell phenotype than cells selected on the basis of ABCG2, and CXCR6+/ ABCG2+ expression.
The association of more aggressive tumor phenotype with asymmetric self-renewal phenotype reveals a previously unrecognized aspect of tumor cell physiology. Namely, the retention of some tissue-specific stem cell attributes, like the ability to preserve self-phenotype, impacts the natural history of human tumor development. Knowledge of this new aspect of tumor progression may provide new targets for cancer prevention and treatment.

Pericytes resident in post-natal skeletal muscle differentiate into muscle fibers and enter the satellite cell pool

A. Dellavalle[1], G. Maroli[1,2], D. Covarello[1], E. Azzoni[1,3], A. Innocenzi[1], L. Perani[1], R. Sambasivan[4], S. Brunelli[1,3], S. Tajbakhsh[4], G. Cossu[1,2]
[1]Dept of Regenerative Medicine, San Raffaele Institute, Milan, Italy
[2]Dept of Biology, Univ. of Milan, Milan, Italy
[3]Dept of Experimental Medicine, Univ. of Milano-Bicocca, Milan, Italy
[4]Stem Cells & Development, Institute Pasteur, Paris, France

Adult skeletal muscle is a stable tissue that presents high regenerative potential after injury. A population of resident myogenic stem/progenitor cells, named satellite cells, is responsible for muscle post-natal growth and adult regeneration. In recent years, several studies have shown that non-myogenic cells, isolated from different tissues, can contribute to muscle regeneration in vivo after transplantation. However, there is currently no evidence that such a contribution represents a natural developmental option for non-myogenic cells, rather than cellular plasticity created by experimental manipulation. Here we show that skeletal muscle perivascular cells, genetically labelled with an inducible Cre-transgenic mouse (*TNAP-CreERT2*), contribute to post-natal muscle growth by fusing with developing myofibers and entering the satellite cell pool during unperturbed life of the animal. This is not observed when only endothelial cells are labelled in an inducible *VE-Cadherin-Cre* mouse. Pericytes contribution to myogenesis is increased after acute injury or in a model of muscular distrophy that leads to chronic degeneration/regeneration cycles. Pericytes-derived cells give rise to both smooth and skeletal myogenic clones, indicating the existence of a common progenitor within post-natal skeletal muscle. Finally, we show that skeletal myogenic differentiation of pericytes-derived cells is decreased in the absence of *Pax7*.

Inhibition of early B-cell factor 1 activity and modulation of the the B-lymphoid differentiation of primary hematopoietic progenitors by the transcription co-factor zinc finger protein 521 (EHZF/ ZNF521)

M. Lupia[1], T. Mega[1], S.J. Horton[2], N. Amodio[1], M. Mesuraca[1], D. Pelaggi[1], V. Agosti[1], E. Chiarella[1], S. Scicchitano[1], F. Bernaudo[1], R. Spina[1], M.A.S. Moore[3], M. Grieco[4], J. Schuringa[2], H.M. Bond[1], G. Morrone[1]
[1]Laboratory of Molecular haematopoiesis and cell biology, Univ. of Catanzaro Magna Græcia, Catanzaro, Italy
[2]Dept of Oncology, Groningen Univ. Medical Center, Groningen, The Netherlands
[3]Laboratory of Developmental hematopoiesis, Memorial Sloan-Kettering Cancer Center, New York, NY, USA
[4]Dept of Environmental Sciences, Second Univ. of Naples, Caserta, Italy

B lymphocyte development is a complex process, orchestrated by a network of transcription factors and epigenetic effectors, that leads to the progressive lineage restriction of pluripotent haematopoietic stem cells (HSCs) paralleled by the acquisition of specialized features. Early B-cell factor 1 (EBF1) is a central player in this process, and, in concert with E2A and/or Pax5, activates the transcription of a number of B-cell-specific genes such as CD79a, CD79b, Igll1, VpreB1 and CD19. Ebf1 knock-out abrogates B-lymphopoiesis and results in the accumulation of early "lymphoid" progenitors that lack signs of B-lineage priming but conserve T-lymphoid and myeloid potential. Enforced EBF1 expression in HSCs represses myeloid and T-lymphoid genes and restricts their differentiation to the B-cell lineage. Thus, modulation of EBF1 expression/activity may be essential to maintain the integrity of the immature haematopoietic cell compartment and to ensure a correct balance between T- and B-lymphopoiesis. Known inhibitors of EBF1 include the Notch1 receptor, the Polycomb protein Bmi1 and two transcription co-factors containing 30 fingers and an amino-terminal motif that binds to the NuRD complex: ZNF423 and ZNF521. ZNF521 was identified in our laboratory for its selective expression in human $CD34^+$ cells compared to mature leukocytes, and is believed to be a relevant player in the regulation of the homeostasis of the haematopoietic stem/progenitor cell compartment. We have dissected the ZNF521/EBF1 interaction and investigated the biological effects of ZNF521 in a B-lymphoid cellular context. Our data show that: *i)* ZNF521 binds to EBF1 via its carboxyl-terminal ZFs and this interaction is necessary for the repression of EBF1-dependent promoters; *ii)* the recruitment of NuRD complex components by ZNF521 is not essential for EBF1 inhibition; *iii)* ZNF521 represses EBF1 target genes in a human B-lymphoid molecular context; *iv)* silencing of ZNF521/Zfp521 in primary human and murine HSC/progenitors strongly augments the generation of B-lymphocytes in *in vitro* co-cultures with bone marrow stromal cells. Our data indicate that ZNF521 can antagonise B-cell development and strongly support the view that one important function of this factor may be to preserve the multipotency of primitive lympho-myeloid progenitors by preventing - or delaying - their EBF1-driven commitment toward the B-cell lineage.

Numb controls mode of division, proliferative rate and tumorigenic potential of mammary stem cells by regulating the Notch and the p53 pathways

D. Tosoni[1], S. Zecchini[1], M. Coazzoli[1], S. Pece[1,2,3], P.P. Di Fiore[1,2,3]
[1]Istituto Europeo di Oncologia, Milan, Italy
[2]Dipartimento di Medicina, Chirurgia ed Odontoiatria, Univ. di Milano, Milan, Italy
[3]IFOM, Fondazione Istituto FIRC di Oncologia Molecolare, Milan, Italy

Asymmetric self-renewal is the process through which a mother stem cell (SC) originates two daughter cells with an alternative stem or progenitor cell fate. Evidence from both invertebrate and mammalian models has established a causative link between faulty asymmetric self-renewal divisions and/or loss of polarity, increase in SC number and cancer initiation. The asymmetric segregation of the cell fate determinant Numb in mitotically dividing SC is an essential, highly evolutionary conserved, mechanism to maintain SC homeostasis, while promoting progenitor differentiation during asymmetric cell division (ACD). Here we characterize the role of Numb in the homeostasis of the normal mammary gland, where Numb is known to be asymmetrically distributed at mitosis of normal mammary SC (NMSC), and to exert a key role as a potent tumor suppressor by inhibiting the oncogenic potential of Notch and stabilizing the tumor suppressor function of p53. We provide direct evidence that, upon NMSC division, Numb is asymmetrically partitioned in the progeny that retains the SC identity. In compromising Numb function, we uncover profound defects in the behavior of NMSC, which shift from an asymmetric to a symmetric mode of self-renewal division and show a higher proliferative rate, with an ensuing increase in the SC number. Mechanistically, we implicate the Numb-p53 axis as a master regulator of ACD, whereas the Numb-Notch axis appears to be more relevant to the control of SC quiescence. We also unmask a link between loss of Numb, expansion of the SC compartment, aberrant mammary morphogenesis and increased frequency of mammary tumors. These tumors are characterized by the presence of cancer SC (CSC) with unlimited self-renewal potential. Pharmacological restoration of p53 and, albeit to a lesser extent, inhibition of Notch, dramatically affect tumor growth by decreasing the frequency of CSC symmetric divisions, and therefore ultimately inhibiting their unlimited replicative potential.

Special Interest Groups • Parallel Sessions
Membrane Trafficking and Organelle Biogenesis

COPII-dependent trafficking - implications for morphogenesis and development

A.K. Townley, K. Schmidt, L. Hodgson, D.J. Stephens
School of Biochemistry, Medical Sciences Building, Univ. of Bristol, UK

Epithelial morphogenesis is directed by interactions with the underlying extracellular matrix. Secretion of collagen and other matrix components requires efficient COPII vesicle formation at the endoplasmic reticulum. Here we show that suppression of the outer layer COPII component Sec13 in zebrafish embryos results in a disorganized gut epithelium. In human intestinal epithelial cells (Caco-2), Sec13 depletion causes defective epithelial differentiation on permeable supports. Defects are seen in the ability of cells to adhere to the substrate, form a monolayer, and make and maintain intercellular junctions. When embedded in a 3D matrix, Sec13-depleted Caco-2 cells form cysts but, unlike controls, are defective in lumen expansion. This correlates with a failure to assemble a type I collagen- and laminin-rich matrix. Incorporation of primary fibroblasts within the 3D culture substantially restores normal morphogenesis. We conclude that efficient COPII-dependent secretion, notably assembly of Sec13-Sec31, is required to drive epithelial morphogenesis in both 2D and 3D cultures in vitro, as well as in vivo. Our results provide insight into the role of COPII in epithelial morphogenesis and have implications for the interpretation of epithelial differentiation assays in cell culture.

Molecular mechanism of the dominant form of familial exudative vitreoretinopathy (FEVR)

M. D'Agostino, V. Lemma, R. Carmelo, M. Cannata Serio, G. Caporaso, M. Mallardo, S. Bonatti
Dept of Biochemistry and Medical Biotechnology, Univ. of Naples Federico II, Italy

Familial exudative vitreorethinopaty (FEVR) is an hereditary ocular disorder characterized by exudates and bleeding followed by scarring, retinal detachment and blindness. Mutations in Frizzled4 (Fz4), a member of the cell surface Wnt family receptors, were found in many FEVR patients. In a autosomal dominant form of FEVR (the most frequent and henceforth referred as Fz4-FEVR), the deletion of two nucleotides leads to the synthesis of a completely different and truncated cytosolic tail (L501fsX533). This receptor is retained in the ER and somehow traps Fz4 by oligomerization, thus performing its dominant effect.We have extensively mutagenized the cytosolic tail and intracellular loops of Fz4-FEVR. We found that the deletion of the last seventeen aminoacids rescues partially cell surface expression. This results suggested the presence of an ER retention motif in the distal portion of the cytosolic tail. Next we replaced the cytosolic tail of the G glycoprotein coded by the ts-045 mutant strain of VSV (VSVG) with the tail of wilde-type and mutant receptor: the chimeric VSVG-Fz4 FEVR tail was completely retained in the ER, whereas VSVG-Fz4 tail exited from the ER as well as VSVG protein. Therefore, we mutagenized by alanine substitution the cytosolic tail of the mutant receptor and found that the R519EERKWL525 sequence was necessary for Fz4-FEVR retention in the ER. Intriguingly, the mutagenesis suggests that also the third intracellular loop may be involved in ER retention. Moreover, we found with a proteomic approach a new interactor of both Fz4wt and Fz4-FEVR, the αB-Crystallin protein. Interestingly, this interaction was not tail dependent, suggesting that the three cytosolic loops may be involved. Current effort is focused to understand the role of αB-Crystallin and to identify the specific interactor(s) which decode the R519EERKWL525 motif responsible of the retention in the ER of Fz4-FEVR in order to identify possible therapeutic target(s).

Selective activation of ATF6 mediates endoplasmic reticulum proliferation triggered by a membrane protein

J. Maiuolo[1], S. Bulotta[1], C. Verderio[2], R. Benfante[2], N. Borgese[1,2]
[1]Dept of Pharmaco-Biological Science, Univ. of Catanzaro "Magna Graecia", Catamzaro, Italy
[2]CNR for Neuroscience and Dept of Pharmacology, Univ. degli Studi di Milano, Milano, Italy

It is well known that the Endoplasmic Reticulum (ER) is capable of expanding its surface area in response both to cargo load and to increased expression of resident membrane proteins. While the response to increased cargo load, known as the Unfolded Protein Response (UPR) is well characterized, the mechanism of the response to membrane protein load has been unclear. As model system to investigate this phenomenon, we have used a HeLa-TetOff cell line inducibly expressing a tail-anchored construct, consisting in an N-terminal, cytosolic, GFP moiety, anchored to the ER membrane by the tail of cytochrome b5 (GFP-b(5)tail). After removal of doxycycline, GFP-b(5) tail is expressed at moderate levels (1-2% of total ER protein), which nevertheless induce ER proliferation, assessed both by EM and by a 3-4 fold increase in phosphatidylcholine synthesis. We assessed a possible participation of each of the three arms of the UPR, and found that only the ATF6 arm was selectively activated after induction of GFP-b(5)tail expression; peak ATF6α activation preceded the increase in phosphtidylcholine synthesis. Surprisingly, upregulation of known ATF6 target genes was not observed under these conditions. Silencing of ATF6α abolished the ER proliferation response, whereas knockdown of Ire1 was without effect. Since GFP-b(5)tail lacks a lumenal domain, the response we observe is unlikely to originate from the ER lumen. Instead, we propose that a sensing mechanism operates within the lipid bilayer to trigger the selective activation of ATF6. Experiments aimed at elucidating this mechanism are currently in progress.

ERp44 acts as a pH-dependent chaperone to retrieve client proteins from the Golgi complex

S. Vavassori[1,2,4], M. Cortini[1,2,4], T. Anelli[1,2], I.R. Caserta[1], C. Fagioli[1], A. Fornili[1], S. Masui[3], M. Degano[1,2], K. Inaba[3], R. Sitia[1,2]
[1]Divisions of Genetics & Cell Biology, and Immunology, Transplantation & Infectious Diseases, San Raffaele Scientific Institute, Milan, IT
[2]Univ. Vita-Salute San Raffaele, Milan, IT
[3]Division of Protein Chemistry, Medical Institute of Bioregulation, Kyushu Univ., Fukuoka, JP
[4]Equally contributions

Newly synthesized secretory proteins undergo scrupulous quality control (QC) to avoid release of folding or assembly intermediates. High fidelity of secretion depends on retention in, or retrieval into the endoplasmic reticulum (ER), where immature cargoes are given another chance to complete folding and assembly . Exposure of unpaired cysteines is exploited in preventing secretion of intermediates that have undergone incomplete disulfide bond formation. Here we show that ERp44, a multifunctional chaperone of the PDI family, operates in a pH dependent manner, in synchrony with forward (ERGIC53)- and backward (KDEL-receptors) cargo transporters, to optimize secretion efficiency and fidelity. At ER-equivalent neutral pH, the ERp44 carboxy-terminal tail obscures the thiol-active cysteine and surrounding hydrophobic patches. At the cis-Golgi-equivalent, slightly acidic pH, however, the C-tail becomes flexible, unmasking both the active site to allow capture of client proteins and the RDEL motif to allow retrieval by KDEL receptors. Upon retrieval to the ER, the neutral pH ensures release of client proteins. Our results delineate a novel QC system, which works downstream the calnexin/calreticulin- and BiP-dependent cycles. The ERp44 cycle is paramount in the retrieval of orphan subunits of otherwise disulfide-linked oligomers such as IgM or adiponectin, whose recognition depends on free thiols.

Mitotic Golgi fragmentation: a G2/M transition checkpoint

R.I. Cervigni[1], M.L. Barretta[1], A. Persico[2], D. Corda[1], A. Colanzi[1,2]
[1]Institute of Protein Biochemistry, Naples, Italy
[2]Consorzio Mario Negri Sud, Santa Maria Imbaro (Chieti), Italy

The Golgi apparatus is composed of individual stacks that are laterally connected by tubules to form a ribbon structure. During G2/mitosis, the Golgi ribbon undergoes an extensive fragmentation that allows its correct partitioning by daughter cells. The first step of Golgi fragmentation occurs in G2 and involves the cleavage of the tubules interconnecting the stacks into a ribbon. Inhibition of this Golgi fragmentation results in cell-cycle arrest at the G2 stage, thus suggesting that correct inheritance of the Golgi complex is monitored by a 'Golgi mitotic checkpoint'.
Using a microinjection-based approach, we recently identified the first target of the Golgi checkpoint, whereby a block of this Golgi fragmentation impairs recruitment of the mitotic kinase Aurora-A to, and its activation at, the centrosomes. Overexpression of Aurora-A overrides this cell-cycle block, indicating that Aurora-A is a major effector of the Golgi checkpoint. Finally, we have found that the Golgi-dependent G2 block is not influenced by known G2 checkpoint mediators, and nor it is mediated by other kinases with essential roles in the activation of the cycB-Cdk1 complex, or by Golgi-localised cell-cycle regulators. Overall, our findings reveal the existence of novel mechanisms that upon a block of Golgi fragmentation, lead to inhibition of the recruitment of Aurora-A to the centrosome during early G2, by acting either directly on Aurora-A or indirectly on an Aurora-A activator.

Autophagic substrates clearance requires activity of the syntaxin-5 SNARE complex

M. Renna[1], C. Schaffner[2], A.R. Winslow[1], F.M. Menzies[1], A.A. Peden[3], R.A. Floto [2], D.C. Rubinsztein[1]
[1]Dept of Medical Genetics, Cambridge Institute for Medical Research, Univ. of Cambridge, Cambridge, UK
[2]Dept of Medicine, Cambridge Institute for Medical Research, Univ. of Cambridge, Cambridge, UK
[3]Dept of Clinical Biochemistry, Cambridge Institute for Medical Research, Univ. of Cambridge, Cambridge , UK

Autophagy is a lysosome-dependent cellular catabolic mechanism that mediates the turnover of intracellular organelles and long-lived proteins. Reduced autophagic activity has been shown to lead to the accumulation of misfolded proteins in neurons and might be involved in chronic neurodegenerative diseases. Here, we uncover an essential role for the syntaxin-5 SNARE complex in autophagy. Using genetic knock-down, we show that the syntaxin-5 SNARE complex regulates the later stages of autophagy after the initial formation of autophagosomes. This SNARE complex acts on autophagy by regulating ER-to-Golgi transport through the secretory pathway, which is essential for the activity of lysosomal proteases such as cathepsins. As a consequence of lysosomal disfunction, depletion of syntaxin-5 SNARE complex components results in the accumulation of autophagosomes, leading to decreased degradation of autophagic substrates. Our findings provide a novel link between a fundamental process such as intracellular transport and human diseases that might be affected by defective biogenesis and/or homeostasis of the autophagosome-lysosome degradation system.

Neurolign 3 misfolding mutations and activation of the unfolded protein response

D. Uccelletti, L. Ulbrich, F.L. Favaloro, E. Zanni, A. De Jaco
Dipartimento di Biologia e Biotecnologie "Charles Darwin". Univ. di Roma "La Sapienza"

Several studies have indicated that mutations in the neuroligin family of synaptic adhesion proteins play an important role in the etiology of autistic spectral disorders. Genetic alterations in the neuroligins genes, including point mutations, exon deletions, and premature truncations have been identified in a small percentage of autistic patients indicating that impairments in synaptic function leads to deficits in brain development. Mutations affecting function, amounts, folding and trafficking of these proteins may contribute to an imbalance in the excitatory/inhibitory network, leading to impaired neuronal signaling, abnormal brain circuits and neurotransmission. The R451C mutation in NLGN3 has been shown to induce a local misfolding in the extracellular domain of the protein that results mostly retained in the ER. The mutation slows down protein processing in HEK-293 and trafficking in neurons with a small fraction still reaching the cell surface. Stronger association with specific chaperone proteins has been shown for the R451C mutant compared to the wild type protein indicating the activation of the ER quality control system in blocking the protein from exiting the ER. We have investigated whether the ER retained protein is activating the UPR response in a yeast model system, K. Lactis and in a mammalian cell system, HEK-293 cells. From our results it emerges that the NLGN3 R451C mutant protein is activating the expression of KAR2, the yeast BiP homologue, that is not observed with the wild type protein. In mammals cells, we studied the activation of distinct UPR signaling pathways by monitoring the splicing of XBP-1 and the activation of ER stress-responsive promoter elements, using a UPR element (UPRE)-luciferase construct in HEK-293 overexpressing either wild type or mutant proteins. From our preliminary data we can conclude that the NLGN3 R451C mutation associated to autism is retained in the ER and is activating an ER stress response.

Agonists differently regulate endocytic trafficking of the "dual" receptor GPR17 in differentiating oligodendroglial cells

A. Fratangeli[1], E. Parmigiani[1], E. Giardino[1], S. Macchi[1], A. Buffo[2], D. Lecca[3], M. Fumagalli[3], M. P. Abbracchio[3], P. Rosa[1]
[1]CNR- Institute of Neuroscience, Department of Medical Pharmacology, Univ. of Milan, Italy
[2]Dept of Neuroscience, Section of Physiology, Univ. of Turin, Neuroscience Institute of Turin (NIT), Neuroscience Institute Cavalieri Ottolenghi (NICO), Regione Gonzole, Orbassano, Turin, Italy
[3]Laboratory of Molecular and Cellular Pharmacology of Purinergic Transmission, Dept of Pharmacological Sciences2, Univ. of Milan, Italy

The recent deorphanised G-protein coupled receptor GPR17 has been described as a new player in the myelination process. GPR17 is up regulated in in vivo injury models characterized by neuronal damage and demyelination, and a number of studies suggest that it is involved in the differentiation of oligodendrocyte precursor cells (OPCs). GPR17 is a "dual" receptor activated by uracil nucleotides and cysteinyl-leukotrienes. A number of studies indicated that receptor activation by both agonists causes inhibition of cAMP formation. Little information is available on the role of the agonists in regulating receptor availability at the cell surface and on GPR17 trafficking during cell-differentiation. We have investigated these aspects in immortalized mouse oligodendroglial cells (Oli-neu) and primary OPC cultures. Our data demonstrate that medium from neuronal cultures stimulates the time-regulated expression of GPR17 in Oli-neu with a kinetic similar to that detected in primary OPC. In differentiating Oli-neu, a consistent amount of GPR17 undergoes to constitutive internalization. The exposure to UDP-glucose increases the clathrin-dependent endocytosis of the receptor whereas LTD4 promotes a less efficient internalization, at least at early time points (15min). Recently, the fate of GPR17 has been analyzed using a homemade antibody directed against an extracellular epitope of the native receptor. Preliminary results suggest that GPR17 is also differently internalized in primary OPC cells. After 12 min-treatment with UDP-glucose, larger amounts of GPR17 compare to control and LTD4 enter the cells and colocalize with marker of early-recycling endosomes (TfR) and late-endosomes/lysosomes (lamp-1). These data suggest that the two different classes of physiological agonists differently regulate GPR17 endocytosis and responses. This may have important implications for myelination during development and after brain injury.

Special Interest Groups • Parallel Sessions
Cell Stress: Survival and Apoptosis

The Yin-Yang role of HSF1 in cancer cell survival

M.G. Santoro [1,2], A. Rossi[2], G. Belardo[1], M. Coccia[1], C. Brunelli[1]
[1]Dept of Biology, Univ. of Rome Tor Vergata, Rome, Italy
[2]Institute of Neurobiology and Molecular Medicine, CNR, Rome, Italy

The heat shock response represents a fundamental protective mechanism utilized by mammalian cells to preserve cellular function and homeostasis under stress conditions. Induction of the heat shock response requires the rapid activation of one or more heat shock factors (HSFs) that trigger the expression of genes encoding heat shock proteins (HSP). HSF type-1 (HSF1) is the main regulator of stress-induced HSP expression with pro-survival function, and contributes to establish a cytoprotective state in several pathological conditions. In cancer cells, however, activation of HSF1 has been associated with both anti- and pro-apoptotic responses. Antiapoptotic HSP expression generally results in cancer cell thermotolerance and chemoresistance; therefore, disruption of HSF1 signaling is considered therapeutic in several types of tumors. On the other hand, HSF1 activation may result in apoptosis induction in aggressive cancers presenting aberrations in survival signaling dependent on the nuclear factor NF-κB. We will describe the pro-apoptotic activity of a new class of potent HSF1 inducers, and will discuss how, in addition to heat shock proteins, HSF1 regulates the expression of proteins with non-chaperone function involved in survival and inflammatory signaling.

PTEN localizes at ER-mitochondria sites and regulates calcium signalling and apoptosis

A. Bononi, C. Agnoletto, M. Bonora, C. Giorgi, S. Marchi, G. Merli, S. Missiroli, S. Patergnani, F. Poletti, A. Rimessi, J. M. Suski, P. Pinton
Dept of Experimental and Diagnostic Medicine, Section of General Pathology, Interdisciplinary Center for the Study of Inflammation (ICSI) and LTTA center, Univ. of Ferrara, Ferrara, Italy

The tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10) has been identified as lost or mutated in almost all human tumour types and inherited PTEN mutation causes cancer-susceptibility conditions.
PTEN is a dual-specificity phosphatase that is capable of removing phosphates from protein and lipid substrates. At plasma-membrane, through its ability to dephosphorylate the phosphatidylinositol 3,4,5-trisphosphate (PIP3), PTEN antagonizes the activation of the highly oncogenic prosurvival phosphoinositide 3-kinase (PI3K)/Akt signalling pathway. Many PTEN effects on cell growth, proliferation and survival are believed to be mediated through its lipid phosphatase activity. However, PTEN exerts also multiple biological functions by its protein phosphatase activity or by protein–protein interaction, these include regulation of cell migration, cell cycle and genome stability. Recently, it has emerged that fine regulation of PTEN functions in tumor suppression is mediated through its different subcellular localization. Indeed several studies have demonstrated that PTEN is found in cell nuclei and in mitochondria where it acts as a mediator of mitochondrial-dependent apoptosis.
We show that PTEN localizes also at endoplasmic reticulum (ER) and Mitochondria-associated membranes (MAMs), signalling domains involved in ER-to-mitochondria calcium ions (Ca2+) transport and in induction of apoptosis.
We demonstrate that PTEN silencing impairs Ca2+ release from ER, thus lowering cytosolic and mitochondrial Ca2+ transient, evoked either by agonist stimulation or by apoptotic stimuli; consequently, cellular sensitivity to Ca2+ mediated proapoptotic stimulation is significantly reduced. Forcing PTEN to ER and MAMs, restores Ca2+ signals and thus re-establish sensitivity to apoptosis.
Our findings provide new insights into the mechanisms and the extent of PTEN involvement in cancer, highlighting new potential targets for therapeutic intervention.

Complex alterations of class IIa HDACs repressive influences observed in breast cancer cell lines: implications for cell survival

A. Clocchiatti, E. di Giorgio, C. Brancolini
Medical and Biological Sciences, Univ. of Udine, Udine, Italy

Class IIa HDACs subfamily includes HDAC4, 5, 7 and 9. Since HDAC4 is significantly mutated in human breast cancers, we investigated its function in breast cancer cell lines that mimic breast tumor heterogeneity. We observed that expression of HDAC4 is higher in ER- cell lines, with the exception of the HCC1937 cells, where we have found a point mutation in the ORF. To evaluate HDAC4 activity we investigated the mRNAs levels of a set of MEF2-target genes in MCF7 (estrogen positive) and MDA-MB-231 estrogen positive) cells. MEF2-target genes were validated in the breast cancer cell lines using different approaches: i) overexpression of HDAC4, ii) overexpression of a dominant negative form of MEF2 iii) treatment of cells with AMP-kinase activator AICAR, which promotes class IIa HDACs nuclear export. After validation we silenced HDAC4 expression. This silencing did not affected MEF2-target genes expression in both cell lines. By contrast, silencing of multiple class IIa HDACs (HDAC4, 5 and 9) promoted MEF2 dependent transcription in ER+ MCF7 cells but not in ER-, MDA-MB-231 cells. KLF2 was the only MEF2-target gene up-regulated after class IIa silencing. Although the siRNA experiments indicate a differential repressive competence of class IIa HDACs, binding between HDAC4 and MEF2 and the high-molecular weight HDAC4/MEF2 protein complexes (> 660 kDa) were similarly observed, in both cell lines. However, the reduced HDAC activity of the HDAC4-complex and the reduction of HDAC3 levels in the MDA-MB-231 cell line suggest an impairment of the repressive influence, in the estrogen negative cell line. KLF2 expression was subjected to additional de-regulation in MDA-MB-231 cells. Finally, silencing of HDAC4, 5 and 9 reduced proliferation by promoting apoptosis only in MCF7 cells. These results suggest an impairment of class IIa HDACs function in ER- cancer cells while they could represent a possible therapeutic target for ER+ breast tumours.

Ser46 phosphorylation and Pin1 mediated isomerization of p53 are key events in p53-dependent apoptosis induced by mutant Huntingtin

A. Grison[1,5], F. Mantovani[1,2], A. Comel[1,2], F. Persichetti[3,4], G. Del Sal[1,2]
[1]Laboratorio Nazionale CIB, Area Science Park, Padriciano, Trieste, Italy
[2]Dept of Life Sciences, Univ. of Trieste, Trieste, Italy
[3]Sector of Neurobiology, International School for Advanced Studies (SISSA), Trieste, Italy
[4]Dept of Environmental and Life Sciences, Univ. of Eastern Piedmont, Alessandria, Italy
[5]Present address: Sector of Neurobiology, International School for Advanced Studies (SISSA), Trieste, Italy

Huntington's disease (HD) is a neurodegenerative disorder caused by a CAG repeat expansion in the gene coding for Huntingtin protein. Several mechanisms have been proposed by which mutant Huntingtin (mHtt) may trigger striatal neurodegeneration, including mitochondrial dysfunction, oxidative stress and apoptosis. mHtt has been reported to induce DNA damage and to activate a stress response, and a critical role of p53 in mediating toxic effects of mHtt has been established. We have attempted to dissect the pathway of p53 activation by mHtt in human neuronal cells and in HD mice, with the aim of highlighting critical nodes that may be pharmacologically manipulated for therapeutic intervention. We demonstrated that expression of mHtt caused increased phosphorylation of p53 on Ser46 leading to its interaction with phosphorylation-dependent prolyl-isomerase Pin1 and consequent dissociation from the apoptosis inhibitor iASPP, thereby inducing the expression of apoptotic target genes. Knockdown of the HIPK2 kinase, as well as inhibition of either the upstream kinase ATM or the prolyl-isomerase Pin1 prevent mHtt-dependent apoptosis of neuronal cells. These results provide rationale for use of small molecule inhibitors of Pin1 and of stress-responsive protein kinases as a potential therapeutic strategy for treatment of HD.

Understanding the role of TRIM8, a new p53 target gene that modulates p53 activity, in the progression of glioma

L. Micale[1], M. F. Caratozzolo[2,3], C. Fusco[1], B. Augello[1], M. N. Loviglio[1], M. G. Turturo[1], G. Cotugno.[1], T. Lopardo[4], F. Galli[4], S. Cornacchia[2], F. Marzano[2], A. M. D'Erchia[3], L. Guerrini[4], G. Pesole[3, 5], E. Sbisà[2], A. Tullo[2], G. Merla[1]

[1]Medical Genetics Unit, IRCCS Casa Sollievo della Sofferenza, San Giovanni Rotondo (Foggia), Italy
[2]Institute for Biomedical Technologies - ITB, National Research Council (CNR), Bari, Italy
[3]Dept of Biochemistry and Molecular Biology "E.Quagliariello", Univ. of Bari "A. Moro", Bari, Italy
[4]Dept of Biomolecular Sciences and Biotechnologies, Univ. of Milano, Milano, Italy
[5]Institute of Biomembranes and Bioenergetics - IBBE, National Research Council (CNR), Bari, Italy

Impairment of p53 function has a crucial role in tumor evolution. p53 inactivation occurs through alteration of p53 function by mutations and deregulation of signaling pathways or p53 activators. The stabilization and activation of p53 are crucial in preventing cells from becoming cancerous. Thus, an important challenge for the development of cancer therapies is the discovery of p53-inactivating pathways or p53 modulators. We describe TRIM8 as a new p53 target gene that modulates the p53-mediated tumour suppression mechanism. We showed that, under stress conditions, p53 induces the expression of TRIM8, which in turn stabilizes p53. Furthermore, overexpression of TRIM8 regulates the p53 transcriptional activity by increasing p21 and GADD45 mRNA levels, that result in inhibition of cell cycle progression. Concurrently, TRIM8 overexpression decreases MDM2 levels suggesting that TRIM8-mediated p53 stabilization occurs through MDM2 degradation. TRIM8 is expressed in brain and it maps to 10q24.3, a region proposed to contain tumor suppressor genes involved in gliomas. By analyzing 100 human glioma cell lines we showed that TRIM8 is under expressed in these tumors, correlating with the tumor histological grade. Intriguingly, the restoring of TRIM8 expression level in U87 glioma cell line enhances the stabilization of endogenous p53 level and induces the suppression of cell proliferation. We assessed the molecular mechanisms responsible for TRIM8 downregulation, revealing TRIM8 LOH and hypermethylation of CpG islands. Finally we found that in glioma samples TRIM8 mRNA level anti-correlates with miR-17 expression, a microRNA involved in glioma progression. Consistently, target prediction tools and luciferase assay showed that miR-17 targets 3'UTR-TRIM8, resulting in TRIM8 downregulation. Our observations suggest the existence of a new p53-TRIM8 feedback mechanism and support the hypotheses that TRIM8 might participate to the development of gliomas through modulation of p53.

DNA ligase I deficient cells as model to study the cellular response to chronic replicational stress

V. Leva[1], C. Citrolo[1], S. Camerini[2], M. Crescenzi[2], G. Biamonti[1], A. Montecucco[1]

[1]Institute of Molecular Genetics, National Research Council, Pavia, Italy
[2]Dept of Cell Biology and Neuroscience, National Institute of Health, Rome, Italy

46BR.1G1 cells derive from a patient with a genetic syndrome characterized by drastically reduced replicative DNA ligase I (LigI) activity. BrdU-comet-assay and the analysis of subnuclear replication foci demonstrated that the maturation of replicating DNA is strongly delayed resulting in the accumulation of both single stranded and double stranded DNA breaks. This is accompanied by phosphorylation of the H2AX histone variant and the formation of γH2AX foci that mark damaged DNA. Single cell analysis demonstrates that the number of γH2AX foci in LigI defective cells fluctuates during the cell cycle: they form in S-phase, persist in mitosis and eventually diminish in G1-phase. Notably, replication-dependent DNA damage in 46BR1.G1 cells only moderately delays cell cycle progression and does not activate the S-phase specific ATR/Chk1 checkpoint pathway that also monitors the execution of mitosis. In contrast, the ATM/Chk2 pathway is activated. Proteomic and transcriptomic analyses indicated that the damage elicited by LigI-deficiency affects the posttranslational regulation of different splicing factors shifting the alternative splicing of target genes to control cell survival or cell death.
The phenotype of 46BR.1G1 cells is efficiently corrected by the wild type LigI but is worsened by a LigI mutant that mimics the hyper-phosphorylated enzyme in M-phase. Notably, the expression of the phospho-mimetic mutant drastically affects cell morphology and the organization of the cytoskeleton unveiling an unexpected link between endogenous DNA damage and the structural organization of the cell.

A role of the COP9 signalosome in modulating DNA-damage responses following DNA replicative stress

M. Panattoni[1], A. Lukacs[1], M.G. Savio[1], L. Maiorino[4], F. Sanvito[2], L.G. Guidotti[3], C. Doglioni[2], R. Pardi[1,4]
[1]Leukocyte Biology Unit, San Raffaele Institute, Milan, Italy
[2]Dept of Pathology, San Raffaele Institute, Milan, Italy
[3]Immunopathogenesis of Liver Infections Unit, San Raffaele Institute, Milano, Italy
[4]Vita-Salute San Raffaele Unive. Medical School, Milano, Italy

The COP9 signalosome (CSN) is a highly conserved protein complex, that was identified in plants on the basis of altered light response in loss of function mutants. CSN is critical for the proper development of all multicellular organisms. The CSN regulates proteasome-dependent degradation by controlling the activity of cullin-based E3 ubiquitin ligase complexes. CSN5/JAB1 mediates the function of the CSN via the regulation of neddylation levels of cullins resulting in the modulation of cullin-E3 ubiquitin ligase activity. The aim of our project is to understand the role of CSN, in vivo. For this reason we conditionally deleted CSN5/JAB1 in hepatocytes as the liver is a useful model to perform cell cycle and survival analysis during regeneration following liver damage. Mice carrying deletion of CSN5/JAB1 developed a severe liver disease that showed features of an abortive regenerative process, with atypical nuclei, necrotic foci and signs of progenitor cell expansion. Hepatic injury experiments also supported a role of the CSN in proper cell cycle progression and pro-survival mechanisms and showed that in the absence of CSN5/JAB1 there is an accumulation of proto-oncogene products. Gene expression analysis in regenerating livers show that CSN5/JAB1 depletion leads to a massive upregulation of DNA damage and repair related genes. Mutant phenotypes were rescued by crossing CSN5/JAB1 null mice with cdkn2a null mice, lacking both p19/ARF and p16/Ink4a. In our working hypothesis the CSN is a repressor of oncogene induced DNA damage response by controlling the protein levels of proto-oncogenes and effectors of the response upstream of p19/ARF. Collectively, our findings suggest that CSN5/JAB1 and in general the CSN, by controlling rapid post-translational turnover of critical substrates, exerts a pivotal role in the coordination of developmental programs entailing proliferation, differentiation, survival and DNA damage in response to environmental signals.

The mitochondrial chaperone TRAP1 regulates tumorigenesis in cancer cell models

M. Sciacovelli[1], G. Guzzo[1], F. Esposito[2], M. Landriscina[3], P. Bernardi[1], A. Rasola[1]
[1]Dept of Biomedical Sciences, Univ. of Padova, Padua, Italy
[2]Dept of Biochemistry and Medical Biotechnology, Univ. Federico II, Naples, Italy
[3]Dept of Medical Sciences, Univ. of Foggia, Foggia, Italy

I have investigated the role played by the mitochondrial chaperone TRAP1 in the process of tumorigenesis. TRAP1 is highly expressed in tumor cells, where it regulates oxygen consumption and the activity of respiratory chain complexes (see the abstract of Guzzo et al), and it is proposed to be involved in the regulation of the mitochondrial permeability transition pore (PTP). I have determined that TRAP1 interacts with the PTP regulator Cyclophilin-D, and I have characterized TRAP1 survival function against a wide spectrum of death stimuli inducing oxidative stress, including diamide, exposure to TNFα, and glucose deprivation. Moreover, I have found that knocking-down TRAP1 expression level through RNA interference in SAOS-2 osteosarcoma cells facilitates PTP opening, thus lowering the threshold for committing cells to death. TRAP1 forms multimeric complexes with respiratory chain complexes, inhibiting mitochondrial respiration and downmodulating ROS levels. Notably, cells were TRAP1expression had been knocked-down lose their tumorigenic potential both in vitro and in vivo; ROS scavenging restores the transforming capabilities of these cells. Altogether, these data indicate that the mitochondrial chaperone TRAP1 plays an important role in tumor progression, by prompting inhibition of oxidative phosphorylation and of intracellular ROS accumulation, thus ablating PTP and cell death and favouring tumor progression. Therefore, TRAP1 constitutes a possible target for anti-neoplastic intervention.

Poster Session A

(presenting authors are shown underlined)

Topic 1

Mechanisms of Signal Transduction

Topic 3

Membrane Trafficking and Organelle Biogenesis

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P1.1

The study of HCC-cell specific miRs reveals one novel human miR and miR-21, miR-24 and miR-27a differential expression in HCC

E. Abeni[1], A. Salvi[1], N. Portolani[2], S. Barlati[1], G. De Petro[1]
[1]Division of Biology and Genetics, Dept of Biomedical Sciences and Biotechnology, Univ. of Brescia
[2]Dept of Medical and Surgical Sciences, Univ. of Brescia

MicroRNAs (miRs) are a class of small non-coding RNA (18-25 nt) expressed in several organisms that negatively regulate gene expression in physio-pathological conditions.
We have developed a small RNA expression library in the human hepatocellular carcionoma (HCC) cell line HA22T/VGH to identify new miRs and to obtain a miRs panel. In total, 200 bacterial clones were sequenced, 118 clones corresponded to 31 known miRs cloned with different frequencies. A sequence of 36 nt that resulted homologous to the mus musculus pre-miR-xxxx allowed us to identify a human miR not yet present in the miRs databases. The results obtained by bioinformatic tools and Northern blot confirmed that miR-xxxx would be a new miR.
The most frequent miRs cloned were miR-21, miR-24 and miR-27a. We determined their expression levels by qPCR in HCC tissues (RQHCC) and in their PT counterparts (RQPT) from bioptic specimens of 33 HCC. The data were plotted considering the R (R=RQHCC/RQPT). The miR-21 resulted up-regulated in HCC respect to PT tissues (R=1.656; p=0.0316) and this became more evident in the group of HCC developed in non-cirrhotic liver (12/33) (R=2.824; p=0.0107). In the HCC raised in cirrhotic liver (21/33) there was unchanged miR-21 expression between PT and HCC (R=0.988). These results suggest that miR-21 up-regulation may be an early event during the hepatocarcinogenesis. miR-24 and miR-27a showed down-regulation in HCCs developed in cirrhotic liver tissues (R=0.529; $p<0.0001$ and R=0.404; $p<<0.0001$). This indicates that the down-modulation of these miRs could influence the hepatocyte transformation of the cirrhotic tissues. On the contrary, miR-27a resulted upregulated (R=2.129) in the non-cirrhotic group evidencing a different role of miR-27a in this HCC group. In summary, our results outline the miRs differential expression in cirrhotic/non-cirrhotic HCCs and this stimulates studies in the field of the molecular targeted therapy based on the miRs level restoration.

P1.2

RaLP expression favors the invasive potential of melanoma cells

E. Aladowicz[1], B. Fiore[1], G. Mazzarol[2], M. Capra[2], G. D'Ario[1], S. Confalonieri[2], M. Bianchi[2], A. Testori[3], P. Ferrucci[3], G. Viale[4], L. Lanfrancone[1]
[1]Dept of Experimental Oncology, IFOM - IEO Campus, Milan, Italy
[2]Molecular Pathology Unit, Cogentech, Milan, Italy
[3]Melanoma - Sarcoma Division, European Institute of Oncology, Milan, Italy
[4]Division of Pathology, European Institute of Oncology, Milan, Italy

Metastatic melanoma is one of the most aggressive cancers, whose response to therapy is poor. Understanding the molecular mechanisms of melanoma dissemination may lead to identifying new therapeutics to improve patient survival. RaLP, an adaptor protein of the SHC family, is a cytoplasmic protein selectively expressed in late stage melanomas and a positive regulator of melanoma migration *in vitro*. We have tested its role as melanoma prognostic marker in a large cohort of primary melanoma samples and found that high RaLP expression significantly correlates with the dominant melanoma prognostic factors. Patients carrying RaLP – expressing tumors show a reduced disease – free survival and overall survival. In addition, RaLP expression is predictive of lymph node metastasis, suggesting that RaLP is an important determinant in the acquisition of the metastatic phenotype by melanoma cells. *In vitro*, permanent RaLP silencing in metastatic melanoma cells does not interfere with cell proliferation, while it significantly decreases their migration. On the contrary, we show that ectopic RaLP expression increases migration and invasion of the cells toward extracellular matrices (ECM), by modulating collagen matrix digestion. Moreover, RaLP expression decreases the cell – cell adhesion properties of melanoma cells as well as their ability to adhere to ECM. We therefore hypothesize that RaLP expression facilitates dissociation of melanoma cells from the tumor mass by loosening cell – cell adhesion, increasing motility and invasion of surrounding tissues. RaLP is expressed in around 40% of melanoma metastases. Some studies have revealed several genes associated with melanoma metastases, even if a signature in clinical materials has not been defined yet. To better understand the molecular determinants involved in the metastatic process, we will correlate RaLP expression with the clinical features of the patients and with genes whose expression is modulated during progression.

P1.3

Heterogeneity in EGFR clathrin endocytosis: vesicles composition impact on receptor signaling and fate

V. Algisi[1], F. Aguet[4], G. Danuser[4], S. Polo[1,2], P.P. di Fiore[1,2,3], S. Sigismund[1]
[1]IFOM, the FIRC Institute for Molecular Oncology, Milan, Italy
[2]Dipartimento di Medicina, Chirurgia ed Odontoiatria, Universita' degli Studi di Milano, Milan, Italy
[3]European Institute of Oncology, Milan, Italy
[4]Harvard Medical School, Boston, MA, USA

Clathrin-mediated endocytosis (CME) is the best-characterized cellular endocytic route. This pathway involves the recruitment of PM-resident cargo into clathrin-coated pits, in a process in which adaptor molecules bridge the internalizing cargo with clathrin. AP2 (Assembly Protein 2) has long been considered to be the major adaptor for CME. More recent studies have, however, challenged the centrality of AP2 in the formation of the coat and other accessory proteins have been proposed to work as substitute adaptors for AP2, since they can bind both cargo and clathrin. Furthermore, the existence of such a wealth of proteins involved in CME – together with the large variety of sorting signals – has raised the possibility that they might be required for the formation of distinct types of clathrin pits, specialized in terms of cargo-selection and specific intracellular fate. Preliminary data collected in our lab on the EGFR (Epidermal Growth Factor Receptor) suggest the existence of 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 (followed by ongoing vesicle tracking in collaboration with Gaudenz Danuser and Francois Aguet, Harvard Medical School, Boston) were combined in the study in order to design a comprehensive picture.

P1.4

Toll like receptor-4 signaling and downstream transcription factor activated during nonalcoholic fatty liver disease

A. Alisi[1], N. Panera[1], A. Masotti[2], L. Da Sacco[2], M. Pezzullo[3], R. De Vito[4], S. Petrini[5], S. Ceccarelli[1], V. Nobili[1]
[1]Liver Research Unit, [2]Microarrays, [3]Core Facilities, [4]Pathology Unit, and [5]Microscopy Unit, "Bambino Gesù" Children's Hospital-IRCCS, Rome, Italy

Nonalcoholic fatty liver disease (NAFLD) is a burgeoning disease in developed countries. The multifactorial origin of NAFLD is now widely accepted, however, real pathogenetic mechanisms are still unclear. Genetic, epigenetic and environmental factors act in concert to induce a network of molecular interactions leading to NAFLD. Interestingly, recently the lypopolisaccharide(LPS)-mediated activation of toll like receptor(TLR)-4 signaling has been considered a starting point for pro-inflammatory signals that characterize NAFLD.
In this study we investigated which downstream molecule(s) can be activated by LPS/TLR-4 network during NAFLD development. To this aim we used a rodent model of NAFLD, a small cohort of children with biopsy-proven NAFLD, and different types of hepatic cells treated with LPS.
Rats were rendered NAFLD after 14 weeks high-fat and fructose diet (HFD-HF). Control rats were fed a standard diet (SD). We found that both TLR-4 mRNA and protein were enhanced in the liver of HFD-HF rats, concomitantly with an increased expression of tumor necrosis factor (TNF)-α mRNA, and augmented LPS and TNF-α serum levels. Noteworthy, we observed an increased expression, phosphorylation and nuclear translocation of two transcription factors: p65NF-kB and LPS-induced TNF-α factor (LITAF) in HFD-HF rats. Interestingly, LITAF over-expression was also found in liver tissues from 12 NAFLD children compared to healthy individuals. Moreover, we found that LPS treatment was able to enhance LITAF expression and its nuclear translocation in different liver cells types. Finally, preliminary data demonstrate that LPS induces nuclear translocation of LITAF and its binding to TNF-α promoter in hepatocyte like cells, and that this binding could be mediated by p38MAPK activity. In conclusion, here we suggest, for the first time, a possible role of LITAF as promoter of inflammatory liver response to LPS in NAFLD.

P1.5

The Aurora-A/TPX2 complex: novel regulatory mechanisms and potential role as an oncogenic holoenzyme

I.A. Asteriti, M. Giubettini, J. Scrofani, M. De Luca, P. Lavia, G. Guarguaglini
Institute of Molecular Biology and Pathology, CNR, Rome, Italy

Assembly of a bipolar spindle ensures balanced chromosome segregation at mitosis, preventing the generation of aneuploid daughter cells. The Aurora-A kinase is an important regulator of spindle assembly and function, frequently overexpressed in tumors. Small molecule inhibitors of its activity are currently undergoing clinical trials as anti-cancer agents. The microtubule (MT)-binding protein TPX2 contributes to activate Aurora-A and mediates its binding to spindle MTs. We are interested to understand the mitotic roles and regulation of the Aurora-A/TPX2 complex and its contribution to cell transformation.
We have identified a previously unrecognised role of TPX2 in control of Aurora-A protein stability. We found that Aurora-A abundance decreases in G2 and prometaphase cells lacking TPX2, in a proteasome- and Cdh1- dependent manner. This effect is counteracted by re-expressing, in a TPX2-silenced background, either full-length TPX2 or the Aurora-A binding region, but not a TPX2 mutant lacking the Aurora-A interaction domain. Furthermore, overexpression of the Aurora-A binding region of TPX2 prevents Aurora-A physiological degradation in telophase cells. This suggests that TPX2 downregulation at mitotic exit is a prerequisite to render Aurora-A available to the degradation machinery. Our observations indicate that TPX2, in addition to regulating Aurora-A localisation and activity, also modulates the abundance of the kinase. Given that Aurora-A levels are critical for cell transformation, they also suggest that increased levels of TPX2 contribute to elicit the full oncogenic potential of Aurora-A. Indeed, data mining analyses indicate that TPX2 is overexpressed in cancers and, moreover, that Aurora-A and TPX2 are frequently co-overexpressed in tumors. We therefore propose that the complex, when deregulated, acts as an oncogenic holoenzyme and contributes to tumor formation.

P1.6

Therapeutic targeting of CHK1 in NSCLC stem cells during chemotherapy

M. Bartucci[1], S. Svensson[2], P. Romania[1], R. Dattilo[1], M. Patrizii[1], M. Signore[1], S. Navarra[1], F. Lotti[1], M. Biffoni[1], E. Pilozzi[3], E. Duranti[3] , S. Martinelli[1], C. Rinaldo[4], A. Zeuner[1], A. Eramo[1], R. De Maria[1,2]
[1]Dept of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanità, Rome, Italy
[2]Mediterranean Institute of Oncology, Catania, Italy
[3]Dept of Experimental Medicine, Sant'Andrea Hospital, University 'La Sapienza', Rome, Italy
[4]Dept of Experimental Oncology, Molecular Oncogenesis Laboratory, Regina Elena Cancer Institute, Rome, Italy

Cancer stem cell (SC) chemoresistance may be responsible for the poor clinical outcome of non-small-cell lung cancer (NSCLC) patients. In order to identify the molecular events that contribute to NSCLC chemoresistance, we investigated the DNA damage response in stem cells derived from NSCLC patients. We found that after exposure to chemotherapeutic drugs NSCLC-SCs undergo cell cycle arrest, thus allowing DNA damage repair and subsequent cell survival. Activation of the DNA damage checkpoint protein Chk1 was the earliest and most significant event detected in NSCLC-SCs treated with chemotherapy, independently of their p53 status. In contrast, a weak Chk1 activation was found in differentiated NSCLC cells, corresponding to an increased sensitivity to chemotherapeutic drugs as compared to their undifferentiated counterparts. The use of Chk1 inhibitors in combination with chemotherapy dramatically reduced NSCLC-SC survival in vitro by inducing premature cell cycle progression and mitotic catastrophe. Consistently, the co-administration of the Chk1 inhibitor AZD7762 and chemotherapy abrogated tumor growth in vivo, whereas chemotherapy alone was scarcely effective. The combined use of Chk1 inhibitors and chemotherapy was associated to increased apoptosis and reduced the number of NSCLC-SCs in mouse xenografts. Furthermore, Chk1 inhibition effectively reduced NSCLC-SCs survival in freshly dissociated human tumor samples. Taken together, these observations argue in favor of a future clinical evaluation of Chk1 inhibitors in combination with chemotherapy for a more effective treatment of NSCLC.

P1.7

Involvement of bioactive sphingolipids in skeletal muscle atrophy

C. Battistini[1], A. Frati[1], F. Bini[1], F. Fontani[1], F. Penna[2], P. Costelli[2], E. Meacci[1]
[1]Dept of Biochemical Sciences, Univ. of Florence, Italy
[2]Dept of Experimental Medicine and Oncology, Univ. of Turin, Italy

Skeletal muscle atrophy is caused by several and heterogeneous conditions, such as cancer (cachexia), neuromuscular disorders, disuse and aging. In most types of skeletal muscle atrophy overall rates of protein synthesis are suppressed, protein degradation is consistently elevated and atrogenes, such as the ubiquitin ligase Atrogin-1/MAFbx, are up-regulated.
Sphingolipids represent a class of bioactive molecules capable of modulating the destiny of many cell types, including skeletal muscle cells. In particular, we have shown in previous studies that sphingosine 1-phosphate (S1P)-formed by sphingosine kinase (SphK), is able to act as morphogenic factor of C2C12 myoblasts [1,2].
Here, we report that the inhibition of SphK1 by specific gene silencing or pharmacological inhibition drastically reduced myotube size and the number of myonuclei and increased Atrogin-1/MAFbx expression. Notably, the atrophic phenotype of differentiated cells treated with dexamethasone (Dexa) and of muscle fibers obtained from cachectic mice inoculated with C26 adenocarcinoma cells, was characterized by increased expression of Atrogin-1/MAFbx and reduced levels of active SphK1. In addition, we found that treatment of myotubes with S1P was able to prevent Dexa-induced atrophy. Finally, by using specific agonists and antagonists of the S1P receptor subtypes, we extended the investigation on the role played by $S1P_2$ and $S1P_1$ receptor in the control of myotube atrophy.
Altogether, these findings provide the first evidence that S1P/SphK1/S1PR axis acts as a molecular regulator of skeletal muscle atrophy, thereby representing a new possible target for therapy in many physiological and pathological conditions.
[1] Sassoli et al.J.Cell Mol.Med. 2010
[2] Meacci et al.Cell Mol.Life Sci. 2010

P1.8

Characterization of the dynamics and functions of 4.1R and ICln interaction in cell morphology and osmotic swelling

L. Benedetti[1,2], C. Bazzini[3], D. Marchesi[2], M. Francolini[1,2], S. Rodighiero[2]
[1]CNR-Institute of Neuroscience, Dept of Medical Pharmacology, Univ. of Milan, Milan, Italy
[2]Fondazione Filarete, Milan, Italy
[3]Biomolecular Science and Biotecnology Dept, Univ. of Milan, Italy

The protein 4.1R is a cytoskeletal component that localizes to the membrane skeleton and stabilizes cell shape and membrane mechanical properties. Its role is not merely structural: it has been associated to cell polarity, tumor suppression, cell proliferation, architecture of the nucleus and the centrosome.
ICln is a highly conserved multifunctional protein that has been implicated in many cellular functions: from Cell Volume Regulation to RNA processing. We tried to elucidate the role of ICln-4.1R interaction in the context of Regulatory Volume Decrease, the process adopted by living cells to counteract osmotic swelling and of cell morphology regulation.
The dynamic interaction between the two proteins was investigated by the use of the FRET technique. Two different isoforms of 80kDa and 135kDa of 4.1R were used for the FRET experiments in hyper- and hypotonic conditions. The strongest FRET signal was measured between ICln and the 80kDa isoform of 4.1R and it increased after cell swelling. Both the 4.1R isoforms modified their subcellular localization in coexpression with ICln and colocalization experiments confirmed a significant reduction in membrane localization of 4.1R in hypotonic condition. We demonstrated that 4.1R of 135kDa interacts with cortical β-actin by FRET experiments, but in the presence of ICln this interaction decreased.
HEK cells transiently trasfected with the 135kDa isoform of 4.1R showed an increased area and a higher number of filopodia and the co-transfection of 4.1R with ICln reverted the phenotype. To better evaluate this phenomenon we established a protocol of Correlative Light-Scanning Electron Microscopy to precisely quantify at the ultrastructural level the surface area and the number and length of filopodia. We then hypothesized that 4.1R-ICln interaction is involved in volume regulation and might interfere with the activation of the pathways leading to filopodia emission probably destabilizing the β-actin-$4.1R^{135}$ complex.

P1.9

Akt phosphorylation at S404 regulates prelamin A through 14.3.3 binding and degradation

J. Bertacchini[1], Beretti[1], V.Cenni[2], M. Guida[1], O. Marin[3], G. Lattanzi[2], S. Marmiroli[1,2]
[1]Dept of Laboratory, CEIA, Univ. of Modena and Reggio Emilia, Modena
[2]I.G.M.-CNR, Unit of Bologna, c/o IOR, Bologna
[3]Dept of Biological Chemistry, Padova

In our previous work, we have identified A-type lamins as physiological substrates of Akt. Here we present the biological implications of lamins phosphorylation by Akt. Phosphorylation at Ser404 was examined during the cell cycle in C2C12 myoblasts synchronized by double thymidine block, then lysates were probed with a posphoSer404-lamin antibody, whose specificity we have demonstrated previously. Surprisingly, while S404-lamin A/C phosphorylation was stable throughout the cell cycle, S404-prelamin A phosphorylation was very weak during G1 and S phase, but peaked at G2 entry.
According to the "scansite" algorithm, phosphorylation at Ser 404 creates a putative binding site for 14.3.3. Our results demonstrate that S404-prelamin interacts in vitro with 14.3.3 in C2C12 myoblasts at G2/M and the association is largely dependent upon prelamin A phosphorylation by Akt. After 24 h of C2C12 myoblast transfection with caax-Akt, the content of prelamin clearly decreases, suggestive of increased degradation upon phosphorylation. Indeed, inhibitors of lysosomal enzymes abrogate the drop of prelamin A, while broad inhibitors of proteasome or caspase are uneffective. Further analysis show that pS404-prelamin relocates from the nucleus to the cytoplasm, where it co-localizes with the marker of phagosome degradation LC3B. Moreover, our data clearly show that non-farnesylated prelamin A is phosphorylated, and then degradated, to a much greater extent than the farnesylated or carboxymethylated forms. Deeper investigation with transgenic mice indicate that Akt controls not only prelamina A degradation but also its expression, though to lesser extent. These results have very important implication for treatment of diseases characterized by accumulation of prelamin, as Hutchinson-Gilford Progeria syndrome.

P1.10

Mitochondria dysfunction and differentiation of oligodendrocytes during proinflammatory conditions

M. Bonora, E. De Marchi, S. Patergnani, A. Bononi, J.M. Suski, F. Celsi, C. Agnoletto, S. Marchi, S. Missiroli, F. Poletti, A. Rimessi, C. Giorgi, P. Pinton
Dept of Experimental and Diagnostic Medicine, Section of General Pathology, Interdisciplinary Center for the Study of Inflammation (ICSI), Laboratory for Technologies of Advanced Therapies (LTTA), Univ. of Ferrara, Ferrara, Italy

Multiple sclerosis (MS) is a neurological disorder of the central nervous system characterized by demyelination and neurodegeneration. Recently mitochondrial dysfunction where found associated with the disease. Is also well established that mitochondria play a pivotal role in cell survival in large part because of their participation in the dynamic regulation of cellular Ca^{2+}. During normal conditions, the accumulation of Ca^{2+} in mitochondria stimulates oxidative metabolism, but also, overload of mitochondria with Ca^{2+}, results in dramatic alterations in mitochondrial function. Recent reports have documented impaired activity of several mitochondrial respiratory complexes in MS plaques.We investigated Ca^{2+} homeostasis in oligodendrocytes during conditions that mimic inflammation.
In particular, we evaluated the effects of TNFα and IFNγ on intracellular Ca^{2+} homeostasis in oligodendrocytes (OPCs), as these inflammatory cytokines are the major players in the autoimmune reaction against OPCs. Only TNFα alters intracellular Ca^{2+} homeostasis, whereas IFNγ has no effect. Moreover, the TNFα effects are restricted to the mitochondrial compartment, and accompanied by a reduction in mitochondrial membrane potential, suggesting specific effects on mitochondrial physiology. Finally, we found that OPCs forced to differentiate and pretreated with subtoxic doses of TNFα reveal a significant reduction of the percentage of cells that differentiate compared to untreated.Similar results were obtained by pharmacological impairment of mitochondrial membrane potential with FCCP. Interestingly succinate and Cyclosporin A where not able to recover the effects of TNFα on differentiation while CGP37157 (inhibitor of mitochondrial Na^{+}/ Ca^{2+} exchanger) almost abolish it. Taken together, these results suggest that TNFα impair oligodendrocyte differentiation by altering mitochondrial functions and specially impairing Ca^{2+} signaling.

P1.11

The RNA-binding protein Sam68 contributes to ALK-mediated signalling transduction

G. Borghetti, R. Bacchiocchi, L. Graciotti, F. Prattichizzo, F. Fazioli
Dept Clinical and Molecular Science, Univ. Polytechnic of Marche, Ancona, Italy

Many canonical steps of malignant transformation, in which the fusion protein NPM-ALK is directly involved in the pathogenesis of the Anaplastic Large Cell Lymphoma (ALCL), have been fairly well described and include increased mitogenity, protection from apoptosis and cell shape and motility modification. The molecular mechanisms underlying these processes are similar to those employed by other tyrosine kinases and rely on the activation of signalling molecules such as PI3K, PLC-?, p60src, alpha-DGK, Stat3 and MAPK. However, despite the progress obtained in the field, our present understanding of the pathogenic mechanisms leading to NPM/ALK–mediated lymphomagenesis is still limited and obviously further investigations are needed to characterize novel signalling pathways operative in NPM/ALK-positive ALCLs that could be relevant to identify new potential targets for drug therapy in these lymphomas.
A recent?protein-interaction proteomic study demonstrates that alpha-DGK associate in a complex with p60src, Vav1, Sam 68 as well as several proteins regulating the splicing of different mRNAs. Sam 68 is a molecule of particular interest, considering its role in various aspects of mRNA metabolism, including splicing, nuclear export and translation. Its ability to couple signal transduction to alternative pre-mRNA splicing, such as that regulating the expression of functionally different CD44, ciclinD1 or Bcl-x isoforms has been demonstrated.
Results obtained in our laboratory indicate Sam68 as a novel regulator of ALK-mediated biological properties. Indeed: 1) ALK kinase activity strictly correlate with Sam68 phosphorylation in different cell systems; 2) in vitro down-regulation of Sam68 by specific siRNA severely impairs cell migration and, to a lesser extent, cell growth in NPM/ALK positive cell lines; 3) Sam68 silencing reduced CD44v5 transcripts, whose expression has been correlated with enhanced malignancy and invasiveness of tumour cells.

P1.12

IL-1Ra a putative target gene through which mutant p53-R273H contributes to tumor microenvironment

G. Norelli[1], E. Cesareo[2], A. Facchiano[2], G. Bossi[1]
[1]Dept of Experimental Oncology, Regina Elena Cancer Institute, Rome, Italy
[2]Istituto Dermopatico dell'Immacolata IDI-IRCSS, Rome, Italy

p53 mutants exhibit gain-of-function (GOF) activities in vitro and in vivo. We Previously reported that conditional depletion of mutant p53 (mutp53) in HT29 xenograft tumors not only impacts on tumor growth but also reduces significantly tumor stroma invasion and tumor angiogenesis, suggesting that mutp53 along promoting cell survival and proliferation exerts GOF activities through modulation of tumor microenvironment. According to these outcomes, we explored whether mutp53 could play relevant role in modulating cytokines expression. Cytokines array performed on conditioned medium (CM) in a panel of human cancer cell lines showed that endogenous mutp53 is indeed involved in modulating the expression of specific cytokines. We found that mutp53-R273H negatively regulates IL-1 Receptor Antagonist (IL-1Ra) expression, as depletion of mutp53 determines a significant increase of IL-1Ra level in the CM in HT29 and MDA-MB468 cancer cells.
IL-1Ra is a naturally occurring cytokine and functions as a specific antagonist-receptor of pro-inflammatory cytokines (IL-1α, β). By interaction to the IL1 receptor, IL-1Ra blocks the IL-1α and IL-1β cascade of events. IL-1Ra is currently used as therapeutic in rheumatoid arthritis and, noteworthy, several data in literature suggest delivering of IL-1Ra as new therapeutic strategies for human cancer.
In a suggested scenario mutp53, by repressing the IL-1Ra expression, may contribute to maintaining a pro-inflammatory environment that promotes tumor malignancy. This study aims to explore the existence of molecular and functional link among mutp53 and IL-1Ra. Results that will be presented will contribute to highlight a novel target through which some p53 mutants might exert GOF activity.

P1.13

The absence of CCM3 results in an increase in β-catenin nuclear localization and signaling

L. Bravi[1], M.G. Lampugnani[1] , E. Dejana[1,2]
[1]IFOM, FIRC Institute of Molecular Oncology Foundation, Milan
[2]Dept of Biomolecular Sciences and Biotechnologies,School of Sciences, Univ. of Milan

The microvasculature of the brain, called blood-brain-barrier (BBB) is specialized to selectively control permeability between blood and the central nervous system. Recently, the canonical Wnt/β-catenin signaling has been shown to be a pivotal pathway implicated in BBB induction and differentiation.In addition to Wnt/β-catenin pathway, CCM3 (cerebral cavernous malformation 3), a protein expressed in endothelial cells (ECs), is emerging as crucial player in BBB maintenance. Mutation in CCM3 leads to a neurovascular pathology known as cerebral cavernous malformation characterized by dilated capillaries susceptible to ruptures and hemorrhages.
In order to clarify a possible crosstalk between CCM3 and β-catenin signaling during BBB formation and maintenance we established two in vitro models: brain- and lung-derived CCM3 positive and null ECs. We found that CCM3 null ECs show altered adherens and tight junctions (TJ), an aberrant actin organization, an increase in permeability and a higher amount of nuclear β-catenin. This last finding correlates with an upregulation of the β-catenin target gene axin 2 and a major downregulation of claudin 5, a component of TJ repressed by β-catenin, suggesting an increase in β-catenin transcriptional activity. Consistently in vitro treatment of CCM3 null ECs with Sulindac, an inhibitor of β-catenin, strongly reduces β-catenin nuclear amount and signaling and induces a partial rescue of permeability. Taken together these results suggest that the absence of CCM3 results in an altered b-catenin signaling, a possible mechanism responsible for the alteration of BBB in CCM.To further investigate this hypothesis an extensive analysis of β-catenin signaling is also under investigation in β-catenin reporter mice CCM3 wt and ko, in which a postnatal and endothelial specific CCM3 invalidation resulted in CCM-like vascular lesions in the cerebellum and in the retinas.

P1.14

Downregulating uPAR expression in human retinal endothelial cells by an antisense oligonucleotide inhibits angiogenesis in a mouse model of proliferative retinopathy

M. Lulli[1*], E. Witort[1], F.o Di Gesualdo[1], A. Lupia[1], R. Loffredo[1], I. Granucci[1], M. Dal Monte[2], S. Capaccioli[1]
[1]Dept of Experimental Pathology and Oncology, Univ. of Florence, Florence, Italy
[2]Dept of Biology, Univ. of Pisa, Pisa, Italy

Neoangiogenesis, that is new vessel formation by pre-existent endothelial cells, is the main pathogenetic event of a variety of severe retinal degenerations (the so-called proliferative retinopathies), included age related macular degeneration, diabetic retinopathy, neovascular glaucoma and retinopathy of prematurity. Here we demonstrate that the inhibition of uPAR expression by an antisense oligodeoxyribonucleotide (ASO) targeting uPAR mRNA significantly reduces neoangiogenesis in cultured human retinal endothelial cells (HRECs).
Furthermore, following its systemic delivering by intraperitoneal injection in a mouse model of prematurity retinopathy, the ASO-uPAR reaches the retina, where it inhibits retina neoangiogenesis, evaluated morphologically on the basis of the retinopathy score.
Finally, we demonstrated that the ASO-uPAR administration downregulated VEGF/VEGFR-1/VEGFR-2 expression in human cultured retinal cell lines, highlighting the therapeutic potential of the ASO-uPAR for the prevention and the treatment of the proliferative retinopathies.

P1.15

Signaling pathways that control ER-exit

A. Capalbo[1], J. Cancino[1], A. Luini[1,2]
[1]Telethon Institute of Genetics and Medicine, Napoli, Italy
[2]Institute of Protein Biochemistry, CNR, Napoli, Italy

Eukaryotic cells have evolved to compartmentalize enzymatic activities to specific organelles or plasma membrane domains. Compartmentalization requires that the cell transport macromolecules from their site of synthesis to the appropriate specialized compartment. In particular, secretory proteins are cotranslationally translocated into the Endoplasmic Reticulum (ER), where they undergo several post-translational modifications and are submitted to stringent quality control, ensuring that only molecules that have attained their correct structure are transported to the Golgi complex (GC) using the COPII machinery. New secretory proteins fold into their proper conformation in the ER by the action of several proteins acting as molecular chaperones that accelerate their folding. How cargo folding and export are coordinated is largely unknown. It has been reported that the presence of folded cargo modifies the formation and turnover of COPII vesicles. In addition the COPII export-machinery appears to be controlled by one or more (unidentified) kinases. Using controlled secretion pulses from the ER we have investigated phosphorylation changes during secretion. Our results by immunofluorescence (IF) using a pan-specific antibody against PKA-substrate indicate that 2 min (or less) after starting a pulse of traffic, a PKA-dependent phosphorylation signal appears over the ER, and in particular, on the ER exit sites. This finding correlates with the appearance of several PKA-phosphorylated bands by western-blotting (WB). This phosphorylation signal peaks about 2 min after cargo release, and then starts to decay to become low in 5 min. The inhibition of PKA with a specific blocker (PKI) inhibits the exit of cargo from the ER as well as the appearance of PKA-dependent phosphorylation. Strikingly, the PKA activity during ER-exit does not seem to be controlled by a Gαs-dependent mechanism since neither Gαs knock-down nor Gαs blocker peptides are able to block cargo exit from ER. We are trying to determine the mechanism and the role of PKA activation at the ER during secretion.

P1.16

The Golgi Apparatus: an heterogeneous intracellular Ca^{2+} store

P. Capitanio[1], V. Lissandron[1], M. Bortolozzi[2], T. Pozzan[1], P. Pizzo[1]
[1]Dept of Biomedical Sciences, Univ. of Padua, Padua, Italy
[2]Physics, Univ. of Padua, Padua, Italy

The Golgi apparatus (GA) represents, together with the endoplasmic reticulum (ER), the major IP_3-sensitive, rapidly mobilizable, intracellular Ca^{2+} store and its functionality is thus important for shaping cytosolic Ca^{2+} responses (1). Increasing evidence suggests that the GA is an heterogeneous Ca^{2+} handling organelle, equipped with a diverse molecular Ca^{2+} toolkit compared to the one expressed in the ER. For example, as Ca^{2+} uptake mechanisms, the GA expresses the classical sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) but also an additional Ca^{2+} pump, the secretory pathway Ca^{2+} ATPase1, SPCA1. The use of a specific Cameleon Ca^{2+} sensor targeted to the trans-Golgi, allowed us to directly demonstrate the functional GA heterogeneity by showing the distinct behavior of this sub-compartment: it takes up Ca^{2+} almost exclusively via SPCA1 (and not by SERCA); it does not release Ca^{2+} in response to IP_3 generation (but rather accumulates the cation as a consequence of the cytoplasmic Ca^{2+} rises) but is endowed (in some cells) with functional RyRs (2).
As regard to the other GA compartments, we are presently testing a new FRET-based Ca^{2+} indicator fused to the cis-Golgi targeting sequence of the enzyme β1,6 N-acetylglucosaminyltransferase (C2gnT) (3). The new probe very nicely co-localizes with the cis-Golgi marker GM130 and thus was used to study Ca^{2+} dynamics in this compartment at single cell level. The data collected were analyzed with a new "pixel-by-pixel" algorithm that discloses a strong Ca^{2+} handling heterogeneity in this Golgi compartment, both at cell resting conditions and after cell stimulation. Altogether these data suggest that the GA is unique in terms of Ca^{2+} homeostasis, with compartments that are separated by a few microns, and in very rapid equilibrium with each other, that still maintain quite substantial differences in terms of ion concentration and response to external stimuli.

1. Pizzo et al., 2011
2. Lissandron et al., 2010
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P1.17

Metastatic potential of BCSCs over their differentiated progeny

R. Dattilo[1], S. Navarra[1], G. Federici[1], M. Signore[1], M. Biffoni[1], E. Duranti[2], E. Pilozzi[2], M. Todaro[3], R. De Maria[1], Monica Bartucci [1]
[1]Dept of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanità, Rome, Italy
[2]Dept of Experimental Medicine, Sant'Andrea Hospital, Univ. 'La Sapienza', Rome, Italy
[3]Dept of Surgical & Oncological Sciences, Univ. of Palermo, Palermo, Italy

Metastasis is the major reason for breast cancer-related deaths. Understanding the mechanisms governing this property will greatly improve the survival of cancer patients. Many solid tumors, including breast, have been reported to contain stem cell-like cells termed cancer stem cells (CSCs) endowed with the ability to self-renew and produce a more differentiated tumor cell populations. In breast cancer, CSCs have also been proposed to be responsible for cancer metastasis, however, scarce evidence for their metastatic potential has been provided so far. In order to elucidate the molecular pathways that underpin cell motility and/or cell proliferation, we analyzed a set of six breast CSCs (BCSCs) lines, obtained from different patients, and compared them to their differentiated progeny. We determined through Reverse-Phase Protein MicroArray (RPMA) analysis the phosphorylation state of more than 100 key signaling proteins involved in proliferation, survival, migration and maintenance of the stem state. Quantitative analysis of the protein signaling states revealed differentially activated network architecture in BCSCs vs differentiated counterparts. In vitro, BCSCs over-express motility proteins, while, on the other hand, differentiated cells activate proliferation pathways to a greater extent. To verify the preferential ability of BCSCs to migrate, stem cells clones and differentiated cells were differentially transduced with lentiviral vectors coding for green and red reporter protein, mixed in defined ratios and orthotopically injected into mice mammary fat pad. We observed in vivo that BCSCs were the only population within the tumor mass capable to migrate and metastasize in distant organs. We believe that elucidation of the functional signaling architecture of BCSCs could help to uncover the molecular basis of tumor metastasis in the breast and identify more effective therapies.

P1.18

Cancer-associated E17K mutation of Akt1 is oncogenic in lung epithelial cells through the regulation of Oct-4 transcription factor

C. De Marco[1,2], M. Scrima[1], N. Rinaldo[1], S. Lovisa[3], L. Fabris[3], G. Baldassarre[3], R. Franco[4], D. Malanga[1,2], G. Viglietto[1,2]
[1]BIOGEM-Istituto di Ricerche Genetiche "G. Salvatore", Ariano Irpino (AV), Italy
[2]Dipartimento di Medicina Sperimentale e Clinica "G Salvatore", Univ. Magna Graecia, Catanzaro, Italy
[3]Centro di Riferimento Oncologico, Aviano, Italy
[4]Istituto dei Tumori, Fondazione G. Pascale, Napoli, Italy

The serine/threonine kinases of the Akt family represent the main downstream end-points of the phosphatidylinositol 3 kinase (PI3K) pathway. Signalling through the PI3K/Akt axis regulates multiple key cellular processes. Expectedly, aberrant PI3K/Akt signalling is frequently observed in human cancer. Recent studies have identified a gain-of-function mutation in the pleckstrin homology domain of Akt1 that results in a glutamic acid to lysine substitution at residue 17 (E17K) in multiple cancer types including lung carcinomas. So far, the contribution of somatic Akt1 mutations to the development of epithelial cancer has remained elusive. Herein, we examined the activity of the E17K mutant using immortalized human lung epithelial cells (BEAS-2B). Expression of E17K Akt1 mutant in BEAS-2B cells induced multiple phenotypic alterations characteristic of lung tumour cells, including growth factor-independent DNA synthesis and replication, anchorage-independent growth, increased capability to migrate and invade, resistance to anoikis and drug-induced apoptosis, capability to efficiently grow in nude mice. In particular, mutant Akt1 induced an expansion of a subset of putative cancer-initiating cells as determined by an increase in the efficiency of sphere formation as well as by enhanced expression of recognised stem cell markers. This leads to the emergency of a cell population endowed with the capability to form very aggressive, undifferentiated tumours in NOD/SCID mice at high efficiency. Finally, we demonstrate that the stem cell-related transcription factor Oct-4 is critical for the in vitro clonogenic potential and the in vivo tumorigenicity exerted by mutant Akt1. Together, these data support the notion that the cancer-associated E17K mutation in Akt1 may significantly contribute to lung cancer pathogenesis and represent an attractive target for therapeutic inhibition.

P1.19

Identification and characterization of the mitochondrial calcium uniporter

D. De Stefani[1,3], A. Raffaello[1,3], E. Teardo[2], I. Szabò[2], R. Rizzuto[1,3]
[1]Depts of Biomedical Sciences and [2]Biology and [3]CNR Institute of Neuroscience, Univ. of Padova, Italy

Mitochondrial Ca^{2+} homeostasis plays a key role in the regulation of aerobic metabolism and cell survival, but the molecular identity of the Ca^{2+} channel, the mitochondrial calcium uniporter, is still unknown. We have identified in silico a protein (renamed MCU) that shares the tissue distribution of MICU1, a recently characterized uniporter regulator, coexists with uniporter activity in phylogeny and includes two trasmembrane domains in the sequence. siRNA silencing of MCU drastically reduced mitochondrial Ca^{2+} uptake in intact and permeabilized HeLa cells, while leaving cytosolic Ca^{2+} transients and mitochondrial distribution and bioenergetic parameters unaffected. When overexpressed, MCU doubled the $[Ca^{2+}]_{mt}$ rise evoked by an IP_3-generating agonist or capacitative Ca^{2+} influx, and the increased mitochondrial uptake significantly buffered the cytosolic elevation. MCU overexpression increased mitochondrial Ca^{2+} uptake also in permeabilized cells, and the effect was abolished by Ruthenium Red (RuR). Experiments with a GFP-tagged MCU, retaining the functional properties of native MCU, demonstrated its localization in the inner mitochondrial membrane, with the N- and C-termini exposed to the intermembrane space. The purified MCU protein, produced in E. coli or in vitro and reconstituted in planar lipid bilayers, exhibited channel activity with electrophysiological properties and inhibitor sensitivity of the mitochondrial calcium uniporter. A mutant MCU, in which two negatively charged residues of the putative pore forming region were replaced, had no channel activity and reduced agonist-dependent $[Ca^{2+}]_{mt}$ transients when overexpressed in HeLa cells. Overall, these data demonstrate that the identified 40 kDa MCU protein is necessary and sufficient for rapid RuR-sensitive mitochondrial Ca^{2+} uptake, thus providing molecular basis for this process of utmost physiological and pathological relevance.

P1.20

The role of V-ATPase in Notch signaling

S. Duchi[1], K. Cortese[2], C. Tacchetti[2], D. Bilder[3], T. Vaccari[1]
[1]IFOM, Istituto FIRC di Oncologia Molecolare, Milano, Italy
[2]Centro di Ricerca MicroSCoBio/IFOM Fondazione Istituto FIRC di Oncologia Molecolare, Dipartimento di Medicina Sperimentale, Univ. di Genova, Italy
[3]Molecular and Cell Biology, Univ. of California, Berkeley, USA

Evidence indicates that activation of the signaling receptor Notch is finely regulated by endocytosis, but the mechanisms involved are not clear. Recently, we isolated mutants in Drosophila genes that encode subunits of the vacuolar ATPase (V-ATPase) proton pump, which control acidification of endocytic compartments. Drosophila epithelial cells lacking V-ATPase function display impaired acidification of the endosomal compartment and a correlated failure to degrade endocytic cargoes, including Notch. More importantly, they show a strong loss of Notch activation.
Consistent with a regulated role of V-ATPase in promoting Notch signaling, we, find that the V-ATPase subunit c is present in endosomes of Drosophila epithelial tissues.
We also show that the expression of the subunit c is upregulated at stages at which Notch activation is required. This suggests the existence of developmental regulation of VATPase expression.
To assess whether V-ATPase modulation of Notch signaling is conserved in mammals, we assayed Notch signaling activation in human cells in culture treated with drugs that impair V-ATPase function. We found that, similar to inhibitors of Notch cleavage, these strongly reduce Notch signaling activation in a number of normal and cancer cells, including leukemia cells with oncogenic Notch signaling.
Overall, our results shed light on the conserved role of V-ATPase in maintaining physiologic levels of Notch signaling. By virtue of its ability to sustain pathologic Notch signaling, V-ATPase could also represent a therapeutic target for cancers that display ectopic Notch signaling, such as some breast and lung carcinomas and some T-cell leukemias.

P1.21

Role of Mixed Lineage Kinase 3 (MLK3) in beta-arrestin 2 dependent cell migration

M. Fabbri, A. Giammarresi, R. Molteni, R. Pardi
Unit of Leukocyte Biology Vita-Salute University School of Medicine DIBIT-Scientific Institute San Raffaele, Milano, Italy

The multi-step leukocyte extravasation process is governed by adhesion molecules and chemotactic factors dynamically interplaying in the presence of shear forces. Responsiveness to chemotactic ligands is mediated by G protein-coupled receptors (GPCRs) which are finely regulated by a well characterized family of cytosolic proteins, beta-arrestin (b-arr). Recent evidence indicates that b-arrs may contribute to GPCR signalling by functioning as scaffolds for the recruitment of signaling proteins. In previous work by our lab, we demonstrated a role of b-arr2 in chemokine-driven activation of the Rap1 small GTPase, recently emerged as master regulator of integrin function.
Interestingly, b-arrs amino acid sequence and three-dimensional structure reveal a unique and evolutionary conserved proline-rich sequence in beta-arrestin 2, localized in a solvent exposed loop which may serve as a docking site for migration-associated transducers/adaptors. In order to find SH3 containing proteins that interact with b-arrs, we have performed an overlay screening assay of 153 different SH3 domains that revealed over 20 putative b-arrs putative interactors, some of which isoform specific. Among these, we focused our attention to Mixed Lineage Kinase 3 (MLK3). MLK3 is a ser/thr kinase that has an amino-terminal SH3 domain followed by the kinase domain and two leucine zippers, a cdc42/Rac1 binding (CRIB) domain and several other domains/motifs. Autophosphorylation of Thr277 and Ser281 is essential for MLK3 kinase activity. MLK3 functions in the SAPK/JNK and p38 stress pathways by directly phosphorylating MKK4 and MKK7. Here we demostrate that MLK3 becomes phosphorylated upon Keratinocyte-derived Chemokine treatment of mCXCR2-RBL2H3 cells. MLK3 phosphorylation is dependent on b-arr2. Stimulation with KC causes phosphorylation of both p38 and c-jun, but only the latter is partially b-arr2 dependent. This results suggest a direct recruitment of MLK3 by b-arr2 upon chemotactic stimulus.

P1.22

A role for NAADP in Ca^{2+}-dependent pathways activated by VEGF in human endothelial cells

A. Favia, B. Esposito, A. D'Alessio, A. Filippini
Dept of Histology and Medical Embryology, SAPIENZA University of Rome, Italy

The release of Ca^{2+} from intracellular stores is a key process involved in a variety of signaling pathways. The second messenger nicotinic acid adenine dinucleotide phosphate (NAADP) mobilizes Ca^{2+} from lysosome-like acidic stores but not from IP3-sensitive or ryanodine-sensitive Ca^{2+} stores in different cell types. To date, two receptors for NAADP have been discovered, namely TPC1 and TPC2. Interestingly, intracellular calcium dynamics plays a central role in angiogenesis of endothelial cells (EC) which is primarily triggered in response to growth factor such as vascular endothelial growth factor (VEGF). In addition to its well known role in the formation of new blood vessels, VEGF is also involved in a number of different EC functions such as proliferation, migration, regulation of vascular permeability and survival through the binding to two receptors (VEGFR1 and VEGFR2) both stimulating intracellular calcium mobilization. The aim of our study is to characterize the role of Ca^{2+} in VEGF-dependent signal transduction and to correlate its intracellular signaling to specific biological functions. Our data propose the involvement of NAADP in VEGFR2- but not VEGFR1-induced calcium mobilization. We show that both pharmacological inhibition of NAADP signaling with Ned-19 (a reported specific antagonist of NAADP) and the use of small interfering RNA (siRNA) targeting TPC1 and TPC2, significantly reduces VEGF-stimulated calcium release in Human Umbilical Vein Endothelial Cells (HUVEC). Our data suggest that both Ned-19 and silencing of TPC receptors by RNAi down regulates VEGF-stimulated ERK1/2 phosphorilation. Moreover, we observed that Ned-19 modulates Akt activation and inhibits VEGF-induced migration of EC. All together our data suggest that NAADP plays an important role in VEGF-induced Ca^{2+} signaling via tyrosine kinase receptor and open new strategies for studying EC functions.

P1.23

Experimental Morphogenetic Field (EMF) promotes cell adhesion, cell spreading and modifies F-actin distribution pattern of TCam-2 seminoma cells

F. Ferranti[1], G. Ricci[2], M. Caruso[1], M. Bizzarri[3], M. Stefanini[1], F. D'Anselmi[4,5], A. Catizone[1]
[1]Dept of Anatomy, Histology, Forensic Medicine and Orthopedics, "Sapienza" Univ. of Rome
[2]Dept of Experimental Medicine, Second Univ. of Naples
[3]Dept of Experimental Medicine, "Sapienza" Univ. of Rome
[4]ASI Italian Space Agency
[5]Dept of Surgery Pietro Valdoni, "Sapienza" Univ. of Rome

Seminomas are the most frequent solid malignant tumours among young men and occur as a consequence of germ cell differentiation arrest followed by malignant transformation. According to the current model a disturbance of the original niche of Primordial Germ Cells (PGC) or gonocytes may lead to imbalance between proliferation and differentiation resulting in neoplastic transformation. It is well known that germ cell development is regulated by adhesion to specific substrates, such as laminin and fibronectin, and to somatic cells of the testis and that the adhesion of germ cells is modified due to cancer progression. A growing body of evidence has suggested that re-establishment of appropriate interactions between cancer cells and the surrounding microenvironment can reverse the neoplastic phenotype. Therefore in this study we tested the hypothesis that a set of regulatory morphogens extracted from chicken egg albumen, called Experimental Morphogenetic Field (EMF), could affect seminoma cells malignant features such as adhesion capability. Today only few cell lines are available for seminoma and among them the most accredited is the TCam-2 seminoma cell line. We performed adhesion assay on this cell line and we found, after forty minutes of culture with EMF, an higher percentage of TCam-2 cells adhering to the culture dish respect to the control samples. Time lapse analyses showed that EMF induced a more rapid spreading of TCam-2 cells respect to control cells with a significant difference in cell spreading even after the first ten minutes. Actin cytoskeleton is also modified by EMF: after 48 and 72 hours of culture F-actin appears distributed in dots compatible with focal contacts in control cells while in EMF-treated cells it is distributed in the cortical cytoplasm. These data demonstrate that microenvironment can modulate TCam-2 adhesion capability suggesting a role in the tumour progression/reversion.

P1.24

Blocking ErbB-3/p85 interaction by a phosphotyrosyl-peptide improves Trastuzumab responsiveness of metastatic ErbB-2 positive breast cancers

V. Folgiero[1], S.E. Di Carlo[1], G. Bossi[1], E. Spugnini [2], G. Bon[1], A. Di Benedetto[3], S. Germoni[2], A. Accardo[5], M. Milella[4], G. Morelli[5], M. Mottolese[3], R. Falcioni[1]
[1]Regina Elena Cancer Institute, Dept of Experimental Oncology
[2]SAFU, Via delle Messi d'Oro, Rome, Italy
[3]Dept of Pathology and 4Department of Oncology, Rome, Italy
[5]Dept of Biological Sciences, CIRPeB, University of Naples "Federico II" & IBB CNR, Naples, Italy

Amplification of the ErbB-2 oncogene occurs in almost 25% of breast cancers and is associated with poor patient outcome. Trastuzumab (Herceptin), a humanized monoclonal antibody directed to the ectodomain of ErbB-2, induces positive clinical responses in 95% of cases of ErbB-2-overexpressing primary tumors. In contrast, most metastatic breast cancers escape Trastuzumab treatment despite expression of ErbB-2. It has been suggested that in these tumors activation of PI3K/Akt pathway by the ErbB-2/ErbB-3 heterodimer could counteract the effects of Trastuzumab. Here we demonstrate that inhibition of the interaction between ErbB-3 and p85, the regulatory subunit of PI3K, by siRNA targeting of ErbB-3 or by a phosphopeptide interfering with complex formation, induces apoptosis and augments responsiveness to Trastuzumab in metastatic ErbB-2-positive breast cancer cells. The in vivo uptake of MCF7 tumors is inhibited by 80% by the phosphopeptide alone and is abolished in combination with Trastuzumab therapy. The administration of phophopeptide in vivo, by electroporation into tumors, induces apoptosis and necrosis, and inhibits by 50% and 58% tumor growth compared to scramble peptide or untreated tumors, respectively. Combined therapy with Trastuzumab inhibits tumor growth up to 72%. Treatment of artificial metastases, by liposomes containing the phosphopeptide, strongly inhibits metastases formation and almost abolishes it when administered in combination with Trastuzumab. These results indicate that the ErbB-3/p85 complex is a limiting and clinically relevant factor in predicting the responsiveness to Trastuzumab therapy in metastatic ErbB-2-expressing breast cancers. The therapy we propose with this phosphopeptide may represent a new efficient strategy to overcome Trastuzumab resistance in breast cancers.

P1.25

hERG1 ion channels are involved in cell proliferation and pharmacoresistence in colorectal cancer models

A. Fortunato, S. Falsini, M. D'Amico, S. Pillozzi, E. Morelli, M. Masselli, O. Crociani, A. Arcangeli
Dep. of Experimental Pathology and Oncology, Florence, Italy

Ion channels are involved in cancer progression of several different type of cancers and there are evidences of their role in the acquisition of a pharmacoresistant phenotype.
We have investigated the function of hERG1 potassium channel in colorectal adenocarcinoma. Both cell lines and mice have been used to study the function of hERG1.
We have found a wide variation in the expression of hERG1 in the cell lines analysed. HCT116 cell line has one of the highest expression of hERG1. In order to study the function of hERG1 in HCT116 cells, we have generated a stable silenced cell lines derived from HCT116 cells by interference of RNA mediated by lentivirus transduction. Then, we have characterized these cell lines by gene expression, cell biology and electrophysiological analysis.
We have found that the knock-down of the hERG1 gene in the HCT116 cell line has changed the phenotype and reduced the proliferative capacity of the silenced cells. In vivo experiments have confirmed that the proliferative capacity of the HCT116-hERG1 silenced cells inoculated in mice is reduced.
Microarray analysis of silenced cells compared with control cells has been used to identify genes that genetically interact with hERG1. Gene expression analysis has suggested an involvement of hERG1 in the signalling pathways.
In addition we have found that the over-expression of hERG1 correlate with cisplatin resistance suggesting a role of this ion channel in pharmacoresistence. HCT116 cells, silenced for hERG1 are more sensitive to cisplatin compared to control cells and also the treatment with a pharmacological inhibitor of hERG1 channels has had the same effect, confirming the role in pharmacoresistence of hERG1 gene.
Thus, the hERG1 expression in cancer cells has a function both in cell proliferation and pharmacoresistence. These finds suggest an important role of hERG1 ion channels in the biology of cancer cells and consequently their importance as drug targets.

P1.26

Identification of a MSCs induced potassium elk-like current in a B- chronic leukemia cell line, MEC1

L. Gasparoli[1], M. D'Amico[1], M. Masselli[1], A. Becchetti[2], A. Arcangeli[1]
[1]Dept of Experimental Pathology and Oncology, Univ. of Florence, Italy
[2]Dept of Biotechnology and Biosciences, Univ. of Milano Bicocca, Milano, Italy

Since many years there is a growing interest regarding the relationship between ion channels, cancer cells and the tumor microenvironment (TM). For example, recent evidence showed that both β1 integrin activation, through binding to extracellular matrix (ECM) proteins, and SDF-1 lead to an increase in hERG1 potassium channels activity, in neuroblastoma and leukemia cells (1). In this context it is becoming relevant to assess the effect of the TM on ion channels expression and activity. We focused our attention on the expression of eag-like potassium channels (eag, elk and herg) in MEC1 cells, a line of B-chronic leukemia, cultured for different time (0-6 days) with or without MSCs (bone marrow mesenchymal cells), and on their electrophysiological profile through patch clamp technique.
At day 0, in both culture conditions, none of the analyzed cells showed eag-like currents. After 3 days, only in co-culture conditions, a small percentage of cells (12,5%) began to show an inward inactivating potassium current with a density of 28,62±12,5 pA/pF. This current was partially E4031 insensitive (hERG specific blocker, 2μM) but totally inhibited by CsCl (10mM). After 6 days 34,6% of the cells expressed this current with an increase in current density up to 71,8±17,8 pA/pF. In conclusion we proved that the MSCs induce two inward inactivating potassium currents with similar electrophysiological features (inward rectification) but different pharmacological properties (E4031 sensibility). In particular the insensitivity of the larger component of this current to this compound led us to hypothesize that this current was due to hELK2 channel (2), whose expression is detected for the first time in leukemia cells.
The onset of this current in MEC1 cells could be probably related to the β1 integrin or CXCR4 activation through binding to MSCs.

1. Pillozzi et al, Blood, 117: 902-914, 2011
2. Becchetti A. et al, European Journal of Neuroscience, Vol. 16, 415-428, 2002

P1.27

Bimodal pattern of chemokine induced Rap1 activation: dissecting the molecular mechanisms

A. Giammarresi, M. Fabbri, R. Molteni, R. Pardi
Unit of Leukocyte Biology Vita-Salute Univ. School of Medicine DIBIT-Scientific Institute San Raffaele, Milano, Italy

The multi-step leukocyte extravasation process is governed by adhesion molecules and chemotactic factors dynamically interplaying in the presence of shear forces. Responsiveness to chemotactic ligands is mediated by G protein-coupled receptors (GPCRs) which are regulated by beta-arrestin. Recent evidence indicates that, in addition to playing a regulatory role in GPCR desensitization and internalization, beta-arrestins may contribute to GPCR signaling by functioning as scaffolds for the recruitment of signaling proteins into complexes with agonist-occupied receptors. On this basis, we investigated the physiological role of ARRB2 in chemokine-driven dynamics associated with leukocyte extravasation, with special interest to the activation of the Rap1 small GTPase, recently emerged as master regulator of integrin function. The analysis of KC (Keratinocyte-derived Chemokine) induced Rap1 activation profile in RBL (Rat Basophilic Leukemia) cells expressing mCXCR2 shows a bimodal kinetic, with the first peak at 30" and the second at 5'. RNA interference-mediated depletion of ARRB2 specifically inhibits Rap1 activation. Rap1-GTP formation is catalyzed by a series of guanine nucleotide exchange factors (GEFs). Among these, C3G, which is consitutively bound to the adaptor molecule CrkII, is a good candidate for KC dependent Rap1 activations,, which is activated through Crk adaptor protein. We showed that, in these cells, KC stimulates the rapid tyrosine phosphorilation of endogenous C3G and that knockdown of C3G or CrkII by siRNA inhibits the KC-dependent Rap1-GTP formation. KC induces rapid CXCR2 internalization through G protein-coupled receptor kinases 2 and 6. We have demostrated that these GRKs mediate KC-dependent activation of Rap1, suggesting that receptor internalization is necessary for Rap1 activation. Together, these results provide a model whereby ARRB2, switching from G-protein dependent to independent signaling regulates Rap1 activity.

P1.28

Importin beta beyond intracellular transport: a novel regulator of kinetochore function in mitotic cells

M. Giubettini[1], E. Roscioli[1], A. Bolognesi[1], L. Di Francesco[2], A. Ruggiero[1], A. Harel[3], M.E. Schininà[2], P. Lavia[1]
[1]Institute of Molecular Biology and Pathology, CNR National Research Council, c/o Sapienza Univ. of Rome, Rome, Italy
[2]Dept of Biochemical Sciences, Sapienza University of Rome, Rome, Italy
[3]Dept of Biology, Technion-Israel Institute of Technology, Haifa, Israel

Mitosis is highly dynamic process in which the mitotic spindle is rapidly assembled. Central to mitosis is the ability of the spindle microtubules (MTs) to contact the kinetochore (KT) of each chromosome so as to ensure even chromosome segregation between daughter cells. KTs promote the nucleation of MTs that contribute to spindle assembly in a process requiring the GTPase RAN. They also recruit mitotic checkpoint proteins that monitor the state of MT/KT attachments before chromosome segregation begins.
We previously demonstrated that importin beta, a RAN effector and the main vector for protein import in interphase nuclei, acts as a regulator of spindle organization when cells reach mitosis.
In this work we show a novel role of importin beta in temporal control of mitotic KT function. We show that importin beta regulates spindle pole organization via its C-terminal region (binding cargo proteins via importin alpha in nuclear import), and also regulated MT dynamic activity and interaction with KTs via its central region, which interacts with nuclear pore complexes in interphase.
Underlying the role of importin beta at the MT/KT level is the formation of a complex including importin beta itself, RANBP2/NUP358, a nucleoporin with SUMO ligase activity, and SUMO-conjugated RANGAP1 (the GTP hydrolysis-activating factor for RAN). We demonstrate that importin beta regulates the timely delivery of RANGAP1 to MT-attached KTs, hence modulating the functional status of RAN at KTs before and after MT attachment.
In conclusion, importin beta, via the RANBP2/SUMO-RANGAP1 complex, modulates a critical switch between early mitosis, when RANGTP-dependent MT nucleation is predominant, and the activation, after MT/KT interaction, of correction of improper attachments by the chromosome passenger complex (Aurora-B and its partners). We propose that importin beta regulates RANGTP hydrolysis at the KT level to arrest MT nucleation and enable the correction system to operate.

P1.29

Deregulation of the RNA binding protein KSRP-mediated post-transcriptional control is involved in melanoma metastatic phenotype

I. Granucci, M. Lulli, E. Witort, R. Loffredo, A. Lupia, F. Di Gesualdo, S. Capaccioli
Dept of Experimental Pathology and Oncology, Univ. of Florence and Phoenix Onlus Stem Cell Foundation for Human Life

K homology splicing regulatory protein (KHSRP) is involved in different biological processes, including mRNA stability control. We focus our interest on its role in post-transcriptional control. Indeed, KSRP is known to recognize and bind to the 3'UTR of some mRNAs and to promote their decay recruiting the exosome and the degradation machinery. To date, only few KSRP mRNA targets have been identified, including iNOS, the let7 family, IL-8 and beta-catenin already reported to be deregulated in invasive melanoma. On the basis of these considerations, we have hypothesized that KHSRP could play a key role in melanoma progression.
The aim of this work is to validate our hypothesis by demonstrating that expression of KHSRP and/or its function are deregulated in melanoma, which could leads to alterations of its targeted protein pattern thereby promoting the metastatic phenotype.
In keeping with our hypothesis, our preliminary results indicate that KHSRP levels undergo a progressive reduction, concomitantly with alterations of melanoma cell proliferation and motility.
Experiments are now in progress to demonstrate that the exogenous up-regulation of KHSRP leads to a reduction of the metastatic phenotype and to a decrease in the levels of its already known targets or of some additional mRNAs we have hypothesized to be KSRP targets.

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P1.30

How to modulate Tie2 activity. From a phage display-based approach to a new interacting protein: Neurexin

S. Graziano, B. Assenzio, L. Mascia, M. Arese*, F.B ussolino*
Dept of Oncological Sciences and Institute for Cancer Research and Treatment, Univ. of Torino School of Medicine, Candiolo (TO), Italy
*These authors equally contribute to the project

Several studies suggest a functional association between tyrosine-kinase receptors and adhesion molecules. A screening of a peptide library to identify binding partners for the endothelial cell- specific tyrosine kinase receptor Tie2 has been carried out. Homology analysis of the interacting peptides led to the identification of the synaptic transmembrane adhesion protein Neurexin. While Neurexins have been previously studied exclusively in the nervous system, we have recently demonstrated that they are expressed by cells of the blood vessels wall (1). Interestingly, Neurexins are physically associated with Tie2 in exogenously expressing cells and also in chick embryo arteries. To begin the analysis of the molecular/cellular mechanisms and the functions of this interaction in the vascular system we decided to use the Neurexin peptides isolated by phage display. These putative peptides, seven amino acids long, were fluoresceinated and tested by Elisa and immunofluorescence assays for their binding affinity on cells expressing Tie2. The ability of the peptides to modulate Tie2 function was also assessed in endothelial cell migration, tubulogenesis and sprouting. Since the Ang/Tie2 signaling is typically involved in the angiogenic remodeling process during development, we also evaluated the activity of these peptides in vivo, in the chick chorioallantoic membrane (CAM) angiogenesis assay.
Finally, the tight link between angiogenesis and vessel tone/hemodynamics, is leading us to study the role of Neurexin/Tie2 in other vascular activities. We have previously shown that an antibody against Neurexin inhibits vessel contraction[1]. On the other hand the Tie2 agonist Ang1 restores the LPS induced vessel hypotension. In this framework we are now analyzing the reciprocal influences of Ang1, LPS, anti-Neurexin antibody, and Neurexin peptides on the contractile properties of blood vessels.
1. Bottos et al. Proc Natl Acad Sci U S A. 2009 Dec 8;106(49)

P1.31

The PAR/aPKC Complex controls the vectorial migration of medaka macrophages *in vivo*

C. L. Crespo[1], B. Clissi[1], P.Keller[2], B. Wittbrodt[5], P.Wadhwani[3], A.Ulrich[3], M. Mione[4], J. Wittbrodt[5] and R. Pardi[1]
[1]Leukocyte Biology Unit, S. Raffaele Univ. School of Medicine, Milan, Italy
[2]Janelia Farm Research Campus, Howard Hughes Medical Institute, Helix Drive, USA
[3]Karlsruhe Institute of Technology, Institute of Organic Chemistry and CFN, Karlsruhe, Germany
[4]IFOM, the FIRC Institute for Molecular Oncology Foundation, Milan, Italy
[5]Centre for Organismal Studies, Univ. of Heidelberg, Heidelberg, Germany

The establishment and maintenance of cell polarity is a requirement for leukocyte migratory response to inflammatory cues. A conserved polarity complex consisting of Par3, Par6 and atypical Protein kinase C (aPKC) in conjunction with small GTPases of the Rho Family temporally and spatially controls polarization in several cell types. To explore the functional role of this signaling complex *in vivo*, we have been using mYFP-labeled Medaka (*Oryzias latipes*) macrophages exposed to the fish tailfin wounding. We combine a cell-specific transient transgenesis strategy to target the complex in a mosaic fashion with 4D *in vivo* live imaging to access overall parameters of stimulus-induced leukocyte migration. When compared with their wild-type counterpart, macrophages overexpressing the constitutively active myristoylated PKCξ showed a significant defect on global motility towards wounding. Accordingly, blocking the kinase activity of the complex with the kinase dead version PKCξKW decreased macrophage directionality and wound-directed speed. The genetic displacement of Par6 or Par3/aPKC interactions did impaired as well cell straightness and directional speed towards the injury.
We are currently exploring the *in vivo* impact of the PAR polarity complex on the highly dynamic cytoskeleton of directionally moving leukocytes. Medaka double transgenic line with GFP-labeled microtubule tips (EB3-EGFP) and RFP-labeled F-actin (RFP-Lifeact) on macrophages was generated to transiently coexpress the PAR mutants together with a CFP-labeled nuclear marker (H2B-CFP). Preliminary evidences suggest altered speed-dependent F-actin oscillations and nuclear/MTOC reorientation in wound-activated macrophages interfered for the PAR complex activity or assembly. Our data support a functional role of the PAR/aPKC complex in regulating the directional migration of myeloid cells responding to inflammation *in vivo*.

P1.32

Effects of $A\beta_{42}$ oligomers on Ca^{2+} homeostasis in primary cortical neurons

M. Kipanyula[1], C. Lazzari[1], E. Zampese[1], T. Pozzan[1], A. Piccini[2], M. Tabaton[2], C. Fasolato[1]
[1]Dept of Biomedical Sciences, Univ. of Padua, Padua, Italy
[2]Dept of Neuroscience, Ophthalmology and Genetics, Univ. of Genova, Genova, Italy

Alzhimer's disease is the majority cause of dementia and cognitive decline that affects humans. The most important hallmark of disease is the extracellular deposition of senile plaques mainly formed by aggregation of β-42 amyloid peptide ($A\beta_{42}$) that triggers neuronal dysfunction and death in the brain. Recently is become apparent that soluble $A\beta_{42}$ oligomers, rather than deposited amyloid plaques are associated with the onset of dementia. These oligomers, produced within neurons or entering from outside, can specifically cause perturbation on Ca^{2+} homeostasis that is at the base of neuronal toxicity.
A mechanistic explanation for this effect is still lacking. $A\beta_{42}$ oligomers can elevate the intracellular Ca^{2+} concentration by either functioning as artificial channels or by activating Ca^{2+} channels (1). Using the Fura-2/AM Ca^{2+} imaging technique we focused our study on the effect of $A\beta_{42}$ oligomers on intracellular Ca^{2+} stores of primary cortical neurons obtained from newborn C57B6J mice. Cells were pre-incubated with $A\beta_{42}$ oligomers (0.5 μM) (2), during the Fura-2 loading procedure. Imaged cells were depolarized for six minutes with KCl (30 mM) to allow neuronal identification; then intracellular Ca^{2+} stores were quickly assayed by stimulation with charbachol (0.5 mM), DHPG (10 μM) or caffeine (20 mM) in a Ca^{2+} free-EGTA containing medium. $A\beta_{42}$ oligomers, but not monomers, reduced Ca^{2+} release induced by IP3 generating agonists albeit increased that induced by caffeine. Notably all these effects occurred without reduction of the total store Ca^{2+} content, as assayed by the Ca^{2+} released by ionomycin. Furthermore, no effect was observed on the resting Ca^{2+} levels as well as on the long lasting Ca^{2+} influx caused by KCl-induced depolarization.

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P1.33

Modulation of blood brain barrier permeability for a better access of anti-cancer drugs to brain tumors: the CCM/β-catenin crosstalk

L. Maddaluno[1], N. Rudini[1], M.G. Lampugnani[1], E. Dejana[1,2]
[1]IFOM, The FIRC Institute of Molecular Oncology Foundation , Milan, Italy
[2]Dept of Biomolecular Sciences and Biotechnologies, School of Sciences, Univ. of Milan, Milan, Italy

Primary brain malignancies and brain metastasis are more resistant to chemotherapies compared to other types of tumors. This is due to the very specialized phenotype of the brain vessels that constitute an extremely selective barrier, the so-called blood brain barrier (BBB). In these vessels endothelial cells (ECs) present a highly developed system of tight junctions (TJs), absence of fenestration and low pinocytotic activity. Furthermore, astrocytes contribute to the BBB-coverage with their foot processes. As a consequence, circulating solutes do not readily enter the brain parenchyma unless through specific endothelial "transporters". Thus, BBB also limits the passage of anti-cancer drugs from the blood to the brain. Therefore, it would be therapeutically useful to develop systems to modulate BBB permeability. Data from our laboratories, suggest a key role of the Wnt/β-catenin signaling pathways in the induction, regulation and maintenance of the BBB characteristics. Besides β-catenin, other three proteins, CCM1, CCM2 and CCM3, expressed by brain ECs, are emerging as key modulators of the organization and function of the BBB. Indeed, mutations occurring in any of the genes encoding these proteins, leads to Cerebral Cavernous Malformation (CCM), a pathology characterized by brain vascular malformations. Preliminary data produced in our laboratory point to a possible link between the β-catenin pathway and the functions of CCM proteins in the regulation of BBB stability. Thus, we are clarifying the mechanisms through which β-catenin and CCM proteins collaborate in the regulation of BBB permeability. The knowledge generated from our work will contribute to the development of new molecular tools for the modulation of vessel permeability during anti-cancer therapy.

P1.34

Estrogen Receptor β modulates mitochondrial functions in human Malignant Pleural Mesothelioma cells

A.G. Manente[1], D. Valenti[2], G. Pinton[1], L. Rossi[3], L. Mutti[4], D. Fennell[5], A.R. Vacca[2], L. Moro[1]
[1]DiSCAFF, Univ. of Piemonte Orientale "A. Avogadro", Novara, Italy
[2]CNR-IBBE, Bari, Italy
[3]Dept of Human Morphology and Applied Biology, Univ. of Pisa, Pisa, Italy
[4]GIME, Italy
[5]Centre for Cancer Research and Cell Biology, Queen's University of Belfast, UK

Our group was the first to show that Estrogen receptor beta (ERβ) is the predominant ER isoform expressed in human Malignant Pleural Mesothelioma (MMe), functioning as a tumour suppressor. However the underlying mechanisms to exploit this anti-cancer activity of ERβ have not yet been explored. Based on a bioinformatics approach, we identified genes differentially expressed between High ERβ and Low ERβ expressor MMe patients. Among gene down-regulated in High ERβ group we identified the SDHB gene that code for the B subunit of mitochondrial respiratory chain complex II. On the basis of this observation, the aim of this work was to assess whether ERβ acts as a tumor repressor also by interfering with mitochondrial functions.
We firstly confirmed that in vitro highly expressed ERβ negatively modulates the SDHB protein levels in, epithelioid MMe derived, REN cells and causes a remarkable reduction in MRC complexes II and IV activities. Moreover, we observed a significant reduction in the mitochondrial ATP production even in the presence of the complex II substrate, succinate, confirming that alterations in complex II activity negatively affected mitochondrial functions. These mitochondrial dysfunctions lead to mitochondrial fragmentation that we demonstrated by electron microscopy analysis evidencing an increase in the number and a reduction in the size of mitochondria and by measure of the mitochondrial DNA content. Probably due to mitochondrial dysfunctions, the ERβ over-expressing cells increase glucose-dependence; indeed we observed an increase in L-Lactate production and loss of capability to survive in a glucose-deprived medium.
On the basis of these data, we can hypotize that in part ERβ exerts its role of tumor repressor in Malignant Pleural Mesothelioma, trough interference with mitochondrial functions.

P1.35

A deubiquitinating enzyme for receptor-smads

M. Inui[1], A. Manfrin[1], A. Mamidi[1], G. Martello[1], L. Morsut[1], E. Enzo[1], S. Soligo[1], S. Moro[2], S. Polo[3], S. Dupont[1], M. Cordenonsi[1], S. Piccolo[1]
[1]Dept of Medical Biotechnologies, Section of Histology and Embryology, Univ. of Padua, Italy
[2]Dept of Pharmaceutical Sciences, Molecular Modeling Section, Univ. of Padua, Italy
[3]IFOM, Fondazione Istituto FIRC di Oncologia Molecolare, Istituto Europeo di Oncologia, Milan, Italy

The TGFβ pathway is critical for embryonic development and adult tissue homeostasis. Upon ligand stimulation, the TGFβ/BMP receptors phosphorylate the Receptor-activated Smads (R-Smads), which then associate with Smad4 to form a transcriptional complex that regulates gene expression through site-specific DNA recognition. Several ubiquitin ligases serve as inhibitors of R-Smads, yet no deubiquitinating enzyme (DUB) for these molecules has so far been identified. This contributed to leave unexplored the possibility that ubiquitination of R-Smads is reversible and engaged in regulating Smad function, in addition to degradation. Here we identify USP15 as a DUB for R-Smads. USP15 is required for TGFβ and BMP gene responses and biological effects in mammalian cells and Xenopus embryos. At the biochemical level, USP15 primarily opposes regulative ubiquitination of R-Smads, that hits their DNA binding domain and is incompatible with promoter recognition. As such, USP15 is critical for the occupancy of endogenous target promoters by the Smad complex. These data identify a new layer of control by which the ubiquitin system regulates TGFβ biology.

P1.36

Thyroid hormone T3 counteracts stz induced diabetes in mouse

C. Mangialardo[1], C. Verga Falzacappa[2], L. Madaro[3], M. Bouchè [3], S. Misiti[2]
[1]Dept Medicina Molecolare, Sapienza,Univ. di Roma,Roma, Italy
[2]Dept Medicina Sperimentale, Sapienza,Univ di Roma,Roma, Italy
[3]Dept Istologia ed Embriologia Medica, Sapienza,Univ. di Roma,Roma, Italy

This study intended to demonstrate that the thyroid hormone T3 counteracts the onset of a Streptozotocin (STZ) induced diabetes in wild type mice. To test our hypothesis diabetes has been induced in Balb/c male mice by multiple low dose Streptozotocin injection; and a group of mice was contemporary injected with T3. After 48h mice were tested for glucose tolerance test, insulin serum levels and then sacrified. Whole pancreata were utilized for morphological and biochemical analyses, while protein extracts and RNA were utilized for expression analyses of specific molecules. The results showed that islets from T3 treated mice were comparable to age- and sex-matched control, untreated mice in number, shape, dimension, consistency, ultrastructure, Insulin and Glucagon levels, Tunel positivity and caspases activation, while all the cited parameters and molecules were altered by STZ alone. The T3-induced pro survival effect was associated with a strong increase in phosphorylated Akt. Moreover, T3 administration prevented the STZ-dependent alterations in glucose blood level, both during fasting and after glucose challenge, as well as in insulin serum level.
In conclusion we demonstrated that T3 could act as a protective factor against STZ induced diabetes.

P1.37

Cellular and molecular mechanisms of honey-mediated wound healing

E. Ranzato, S. Martinotti, B. Burlando
Dept of Environment and Life Sciences, DiSAV, Univ. of Piemonte Orientale "Amedeo Avogadro", Alessandria, Italy

Skin lesions generally heal rapidly and efficiently, but some pathological or severe wounds do not heal completely. Hence, great interest has been attracted by therapies providing healing acceleration and reducing wound-related complications. Honey has a number of properties that are believed to facilitate the healing process. Its acidic pH, H2O2 production, high sugar content, and specific plant-derived chemicals serve to inhibit microbial growth. Empirical evidence established honey as a treatment for wounds and sores in ancient times, and an extensive body of scientific literature on the wound healing capabilities of honey has confirmed its value. However, little is known about the cellular and molecular mechanisms involved in honey-promoted wound healing. Given the overlapping phases of the wound healing process, this kind of remedy should affect at least two different processes before it can be said to have some scientific support for wound healing use. For this reason, we included in our experiments keratinocytes and fibroblasts, which play main roles in wounded skin repair. Fibroblasts are attracted into the site of wound to initiate the proliferative phase of repair and matrix deposition, while keratinocytes are involved in the re-epithelialisation phase. By using in vitro scratch wound models we have shown an increase of wound closure induced by selected monofloral honey types (acacia, manuka, buckwheat) on both keratinocytes and fibroblasts. In addition, cell migration assays have revealed honey chemoattractant effects on both cell types. ELISA assay has shown honey modulation of syndecan 4 expression in keratinocytes. Antibody and PCR array analyses have indicated cytoskeletal rearrangement and traits of epithelial-mesenchymal transition in honey-exposed keratinocytes. In conclusion, data show that different monofloral honeys are able to induce skin wound healing responses at both the epithelial and dermal levels.

P1.38

Analysis of the bioenergetic properties of ρ^0 cells

I. Masgras, L. Azzolin, V. Giorgio, M. Sciacovelli, A. Rasola, V. Petronilli, P. Bernardi
Dept of Biomedical Sciences and CNR Institute of Neurosciences, Univ. of Padova, Italy

Mammalian mitochondrial DNA (mtDNA) contains 37 genes, which encode 13 core components of the respiratory chain complexes and of the F_0F_1-ATP synthase, and 24 RNAs required for mtDNA gene expression. Mutations in some of these genes lead to mitochondrial diseases, a group of disorders endowed with respiratory dysfunction caused by inefficient proton pumping and/or compromised ATP production. The study of mitochondrial diseases is made extremely complex by their heterogeneous phenotype and by the lack of suitable cell and disease models. Cells completely devoid of mtDNA (termed ρ^0 cells) can be repopulated with mutated mtDNA, thus mimicking specific diseases. Moreover, ρ^0 cells, which still maintain a mitochondrial membrane potential, constitute an extreme and fascinating model of mutations impairing oxidative phosphorylation. Here we analyze the bioenergetic processes that contribute to the maintenance of the inner mitochondrial membrane potential in the absence of a working respiratory chain, with a particular focus on the electrogenic exchange of ATP^{4-} for ADP^{3-} by the adenine nucleotide translocator, coupled in the matrix to ATP hydrolysis by an incomplete F_0F_1-ATP synthase working in reverse. Moreover, we investigate the properties of the permeability transition pore (PTP) of ρ^0 cells. This is of pivotal importance, as prolonged PTP opening leads to mitochondrial membrane depolarization and cell death. We observe that some PTP inducers maintain their effectiveness in ρ^0 cells, and that cyclosporin A, a drug that inhibits the pore following binding to the mitochondrial chaperone cyclophilin D, is still active in ρ^0 cells. These observations pave the way to investigating the interplay between the mechanism responsible for the maintenance of the mitochondrial membrane potential and PTP regulation. These results can be extended to biological settings where a partial impairment of respiration occurs, such as mitochondrial diseases or cancer.

P1.39

Identifying the signalling mechanism that regulates Nedd4 activity

E. Maspero[1], S. Mari[1], E. Valentini[1], A. Musacchio[2], S. Pasqualato[2], S. Polo[1,3]
[1]IFOM, Fondazione Istituto FIRC di Oncologia Molecolare, Milan, Italy
[2]Dipartimento di Oncologia Sperimentale, Istituto Europeo di Oncologia, Milan, Italy
[3]Dipartimento di Medicina, Chirurgia ed Odontoiatria, Univ. degli Studi di Milano, Milan, Italy

The key signature of the Nedd4 family E3 ligase is the HECT domain that consists of a larger N-terminal lobe containing the E2-binding site and a smaller C-terminal lobe including the active Cys residue. Nedd4 family members directly catalyze protein ubiquitination by a sequential mechanism process and have been found to interact non-covalently with ubiquitin. We report here crystal structures of the Nedd4 HECT domain, alone and in complex with Ub, that reveal a novel binding mode involving two surfaces on Ub and both subdomains of the HECT N-lobe. Our structures suggest a model for HECT-to-substrate Ub transfer in which the growing chain on the substrate is kept close to the catalytic cysteine to promote processivity. An intrinsic level of regulation of Nedd4 activity is provided by the fact that an intramolecular inhibitory interaction between the HECT and the C2 domain of the ligase has been described. One intriguing possibility is that upstream signalling events may regulate the release of this auto-inhibition, leading to ligase activation and its membrane relocalization via the displaced C2 domain. EGF-induced phosphorylation and/or ubiquitination at critical sites might help opening the conformation and thus creating a Nedd4 molecule in the "activated state".
To gain insight on a possible regulative role exerted by post-translation modifications on the ligase activity we set up a mass spectrometry approach to identify phosphorylation and ubiquitination sites present in Nedd4 protein upon EGF stimulation.

P1.40

Phosphodiesterase 4 specific inhibitors control cell growth of human hepatoma HepG2 cells

M. Massimi, V. Castelli, M.F. Giardi, F. Galli, M. Giorgi
Dept Basic and Applied Biology, Univ. of L'Aquila, L'Aquila, Italy

Cyclic nucleotides, as part of the signal transduction system, play a key role in coordinating many cellular functions. Their involvement in the control of cell proliferation is widely recognized. Cyclic nucleotide levels are regulated by the action of cyclases as biosynthetic enzymes, and of phosphodiesterases (PDEs) as degradative enzymes. It is well known that in different cell types the increase of the intracellular concentration of cyclic nucleotides leads to many physiological changes, including blockade of cell proliferation and induction of cell differentiation.
In this study, we investigated the effects of PDE inhibitors on the growth of human hepatoma HepG2 cells. Previous experiments showed a significant control of cell proliferation with the use of PDE4 inhibitors, such as rolipram and DCTA-46. Treatment of HepG2 cells with 100 μM rolipram or 1 μM DC-TA-46 caused a marked increase of cAMP, 6 and 2.7 fold respectively. The effects of a permanent increase of cyclic nucleotides were also investigated in these cells using dbcAMP as a membrane permeant-unhydrolysed cAMP analogue. Dose-response experiments showed dose- and time-dependent effects of PDE-inhibitors on cell growth. The concentrations of inhibitors that halved cell numbers after 96 h of treatment were used for subsequent experiments and were: 1 μM for DC-TA-46, 100 μM for rolipram, and 1mM for dbcAMP. Treatment with rolipram and especially with dbcAMP induced apoptosis as demonstrated by DNA fragmentation following DAPI staining and cytofluorimetric assays. In addition, all the inhibitors produced a decrease in cyclin D1 that was highly significant in dbcAMP-treated cells; dbcAMP also caused a slight decline of cyclin A.
The results demonstrate an involvement of PDE inhibitors in cell cycle control and suggest that they might be useful as potential chemotherapeutic and/or chemopreventive agents in hepatocellular carcinoma.

P1.41

Numb activates the E3 ligase Itch to control Gli1 function through a novel degradation signal

L. Di Marcotullio[1,5], A. Greco[1,5], D. Mazzà[1], G. Canettieri[1], L. Pietrosanti[1], P. Infante[1], S. Coni[1], M. Moretti[2], E. De Smaele[2], E. Ferretti[2], I. Screpanti[1,3] , A. Gulino[1,3,4]
[1]Dept of Molecular Medicine, Sapienza Univ., Rome, Italy
[2]Dept of Experimental Medicine, Sapienza Univ., Rome, Italy
[3]Pasteur Institute, Cenci Bolognetti Foundation, Sapienza Univ., Rome, Italy
[4]Neuromed Institute, Pozzilli, Italy

Hedgehog pathway regulates tissue patterning and cell proliferation. Gli1 transcription factor is the major effector of Hedgehog signaling and its deregulation is often associated to medulloblastoma formation. Proteolytic processes represent a critical mechanism by which this pathway is turned off. Here, we characterize the regulation of an ubiquitin-mediated mechanism of Gli1 degradation, promoted by the coordinated action of the E3-ligase Itch and the adaptor protein Numb. We show that Numb activates the catalytic activity of Itch, releasing it from an inhibitory intramolecular interaction between its HECT and WW domains. The consequent activation of Itch together with the recruitment of Gli1 through direct binding with Numb, allow Gli1 to enter into the complex, resulting in Gli1 ubiquitination and degradation. This process is mediated by a novel Itch-dependent degron, composed of a combination of two PPXYs and a phospho-Serine/Proline (pSP) motifs, localized in Gli1 C-terminal region, indicating the role of two different WW-docking sites in Gli1 ubiquitination. Remarkably, Gli1 protein mutated in these modules is no longer regulated by Itch and Numb, and determines enhanced Gli1-dependent medulloblastoma growth, migration and invasion abilities as well as in vitro transforming activity. Our data reveal a novel mechanism of regulation of Gli1 stability and function, that influences Hedgehog/Gli1 oncogenic potential.

P1.42

Ceramide kinase/C1P axis: a new signalling pathway involved in the antiproliferative action of Vitamin D_3 in human neuroblastoma cells

F. Bini[1], M. Garcia-Gil[2], M. Granado[3], C. Battistini[1], I. Perrotta[1], A. Frati[1], F. Fontani[1], M. Caleo[4], A. Gomez-Muñoz[3], E. Meacci[1]
[1]Dept of Biochemical Sciences, Univ. of Florence, Italy
[2]Dept of Biology, Univ. of Pisa, Italy
[3]Dept of Biochemistry and Molecular Biology , Univ. of the Basque Country, Spain
[4]Istituto di Neuroscienze CNR, PISA, Italy

1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), a crucial regulator of calcium/phosphorus homeostasis, has important physiological effects on growth and differentiation in a variety of malignant and non-malignant cells. Synthetic structural hormone analogues, with lower hypercalcemic side effects, are currently under clinical investigation. Sphingolipids appear to be crucial bioactive factors in the control of cell fate: the phosphorylated forms, sphingosine-1-phosphate (S1P) and ceramide-1-phosphate (C1P), are mitogenic factors, whereas sphingosine and ceramide (Cer) usually act as pro-apoptotic agents. Although many studies correlate S1P function to impaired cell growth, the relevance of C1P/Cer system and its involvement in neuroblastoma cells remain to be clarified.
Here, we demonstrated the anti-proliferative effect of $1,25(OH)_2D_3$ as well as of its structural analogues, ZK156979 and ZK191784, in human SH-SY5Y cells, as judged by [3H]thymidine incorporation, cell growth and evaluation of active ERK1/2 levels. $1,25(OH)_2D_3$ and ZK191784 treatment induced a significant decrease in CerK expression. Notably, the treatment of SH-SY5Y cells with ZK159222, antagonist of $1,25(OH)_2D_3$ receptor, trichostatin A, inhibitor of histone deacetylases, and COUP-TFI-siRNA prevented the decrease of CerK expression elicited by $1,25(OH)_2D_3$ supporting the involvement of VDR/COUP-TFI/istone deacetylase complex in CerK regulation.
Altogether, these findings provide the first evidence that CerK/C1P axis act as molecular effector of the anti-proliferative action of $1,25(OH)_2D_3$ and its analogues, thereby representing a new possible target for anti-cancer therapy of human neuroblastoma.

P1.43

Characterization of the SNARE protein SNAP29 in trafficking, signaling and tumor suppression

E. Morelli, P.p. Ginefra, T. Vaccari
IFOM, Istituto FIRC di Oncologia Molecolare, Milan, Italy

In cancer cells, signaling pathways activated by growth factors are often deregulated due to altered trafficking. Intracellular trafficking requires that membranes fuse with each other in a tightly regulated and highly specific manner. The fusion machinery includes Syntaxins, Synaptobrevins and SNAPs that, as part of the SNARE complex, bring membranes together. At present, it is not clear whether deregulation of membrane fusion is involved in tumorigenesis.
Recently, in a screen to isolate putative tumor suppressor genes in Drosophila we have identified B6, a mutation in the gene encoding the SNARE protein SNAP29. We have found that snap29B6 carries a nonsense mutation leading to production of a truncated protein that lacks the Cterminal SNARE domain. mRNA expression and lethal phase analysis suggest that the B6 mutant is likely a genetic null.
Consistent with a role of SNAP29 in membrane fusion, we have found that snap29B6 mutant cells present defects in vesicle trafficking. An ex-vivo cargo uptake assay performed on developing Drosophila epithelial organs - the larval imaginal discs - mutant for snap29 reveals cargo accumulation in early endosomes and in exocytic vesicles.
Interestingly, snap29B6 mutant imaginal discs over-proliferate, are unable to polarize and to terminally differentiate. Since these traits are frequently associated to tumorigenesis, snap29 might act as a tumor suppressor. Defects in snap29B6 mutant tissue can be rescued by ectopic overexpression of SNAP29, indicating that they are caused by impairment of snap29 function.
Taken together, our evidence indicates that SNAP29 exerts a role in regulating vesicle fusion and that compromised function leads to tumorigenesis. Future experiments will clarify SNAP29 localization, will define in which vesicle trafficking process SNAP29 acts, and will assess whether and how SNAP29 might control signaling. By shedding light on the fundamental functions of SNAP29 in epithelial tissues, I hope to gain a deeper understanding of how intracellular trafficking might control tumor suppression.

P1.44

A Pin1/mutant p53 axis promotes aggressiveness in breast cancer

M. Napoli[1,2,#], J. E. Girardini[1,2,#], S. Piazza[1], A. Rustighi[1,2], C. Marotta[1,2], V. Capaci[1,2], A. Thompson[3], A. Rosato[4], T. Crook[3], A. R. Means[5], G. Lozano[6] and G. Del Sal[1,2,*]
[1]Laboratorio Nazionale CIB (LNCIB), Area Science Park, Trieste, Italy
[2]Dipartimento di Scienze della Vita, Univ. degli Studi di Trieste, Trieste, Italy
[3]Dundee Cancer Centre, Univ. of Dundee, UK
[4]Dipartimento di Scienze Oncologiche e Chirurgiche, Univ. degli Studi di Padova, e Istituto Oncologico Veneto IRCCS, Padova, Italy
[5]Dept of Pharmacology and Cancer Biology, Duke Univ., Durham, NC, USA
[6]Dept of Cancer Genetics, M.D. Anderson Cancer Center, Houston, Texas, USA
[#]These authors contributed equally to the work

TP53 missense mutations dramatically influence tumor progression, however their mechanism of action is still poorly understood. Here we demonstrate the fundamental role of the prolyl isomerase Pin1 in mutant p53 oncogenic functions. Pin1 enhances tumorigenesis in a Li-Fraumeni mouse model and cooperates with mutant p53 in Ras-dependent transformation. In breast cancer cells, Pin1 promotes mutant p53 dependent inhibition of the anti-metastatic factor p63 and induction of a mutant p53 transcriptional program to increase aggressiveness. Furthermore, we identified a transcriptional signature associated with poor prognosis in breast cancer and, in a cohort of patients, Pin1 overexpression influenced the prognostic value of p53 mutation. These results define a Pin1/mutant p53 axis that conveys oncogenic signals to promote aggressiveness in human cancers.

P1.45

Quantitative proteomics to identify molecular determinants of EGFR clathrin-independent endocytosis

G. Nappo[1], A. Cuomo[2], T. Bonaldi[2], S. Polo[1,3], S. Sigismund[1], P. P. Di Fiore [1,2,3]
[1]IFOM , the FIRC Institute for Molecular Oncology Foundation, Milan, Italy
[2]European Institute of Oncology, Milan, Italy
[3]Dipartimento di Medicina, Chirurgia ed Odontoiatria, Univ. degli Studi di Milano, Milan, Italy

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) pathway, as the receptor becomes ubiquitinated. 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.
The molecular mechanisms involved in the NCE pathway of the EGFR are mainly unknown. Essentially, NCE is defined by its insensitivity to functional ablation (KD) of clathrin and by its sensitivity to cholesterol-interfering drugs, hence its definition as a "raft-dependent pathway".
In order to identify the molecular components of NCE, we employed 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 SILAC quantitative proteomics, allowed us to select a list of candidate players, particularly enriched for specific raft-mediated endocytic proteins, which have been validated by RNA interference in different functional assays. In this way, we were able to restrict our list to those proteins that exert a specific role in EGFR NCE and do not function in CME. In addition, in two cases we also unveil an impact of these players on EGFR degradation and signalling. Morphological and localization studies are still on going. Given the degradative function of NCE, this approach might be useful to identify negative regulators of EGFR, which might represent novel potential targets for cancer therapies.

P1.46

Characterization of KCASH2 and KCASH3, two novel BTB/POZ proteins involved in cerebellar development and medulloblastoma tumorigenesis

M.A. Occhione[1], E. De Smaele[1], L. Di Marcotullio[2], M. Moretti[1], M. Pelloni[1], P. Infante[2], D. Cucchi[1], A. Greco[2], L. Pietrosanti[2], J. Todorovic[1], S. Coni[2], G. Canettieri[2], E. Ferretti[1], R. Bei[3], M. Maroder[4], I. Screpanti[2], A. Gulino[2,5]
[1]Dept of Exp. Medicine, [2]Dept of Mol. Medicine, [4]Dept of Medical Surg. Sciences and Biotechnol., Sapienza Univ. of Rome, Italy
[3]Dept of Exp. Medicine and Bioch. Sciences, "Tor Vergata" Univ. of Rome, Italy

Medulloblastoma (MB) is the most common pediatric malignant brain tumor, and arises from aberrant cerebellar precursors development, a process mainly controlled by Hedgehog (Hh) signaling pathway. Histone deacetylase HDAC1 has been recently shown to modulate Hh, deacetylating its effectors Gli1/2 and enhancing their transcriptional activity. HDAC represents therefore a potential therapeutic target for Hh-dependent tumours, but still little information is available on the physiological mechanisms of HDAC regulation. We describe here a new family of proteins named KCASH, as "KCTD containing, Cullin3 Adaptor, Suppressor of Hedgehog". This family is composed by three members: KCASH2^{KCTD21}, KCASH3^{KCTD6} and the previously characterized KCASH1/REN^{KCTD11}. KCASHs share a number of features, such as a BTB domain (required for the formation of a Cullin3 ubiquitin ligase complex and HDAC1 ubiquitination and degradation capability) and the ability of suppressing the acetylation-dependent Hh/Gli signaling. Expression of KCASH2 and -3 is observed in cerebellum while epigenetic silencing and allelic deletion is observed in human medulloblastoma. Rescuing KCASHs expression reduces the Hedgehog-dependent medulloblastoma growth, suggesting that loss of members of this novel family of native HDAC inhibitors is crucial in sustaining Hh pathway mediated tumorigenesis. The ongoing search for additional interactors or targets of the KCASH proteins will shed light on further roles played by these proteins in the complex processes of neural development and cancerogenesis.

P1.47

Vaccination to kitL: a new approach to inhibit tumor angiogenesis

C. Olgasi[1], P. Dentelli [1], A. Trombetta [1], A. Rosso [1], G. Togliatto [1], A. Iavello [1], A. Castelli [1], M. Gili [1], A. Orsello [1], S. Prestigio [2], G. Forni [2], F. Cavallo [2], MF. Brizzi [1]

[1] Dept. of Internal Medicine, University of Turin, Italy
[2] Dept. of Clinical and Biological Science, University of Turin, Italy

Endogenous angiogenesis inhibitors have shown promise in preclinical studies, but in clinical trials they have not been as effective as expected. Here, we describe a strategy that targets the membrane-bound c-kit ligand (mbKitL) by DNA vaccination. The vaccination procedure generated antibodies that detected the mbKitL on tumor endothelial cells (TECs). Purified Ig inhibited adhesion of c-Kit expressing cells to TECs. In vivo, DNA vaccination interfered with angiogenesis in the matrigel plug assay and reduced tumor growth. Inhibition of tumor cell proliferation and vessel formation and stabilization were crucial for the in vivo effect of DNA vaccination. We also found that DNA vaccination was associated with a reduction of foxp3 expressing cells into tumor microenvironment. No bone-marrow toxicity or impairment of normal blood vessels could be detected. These data provide evidences that DNA vaccination targeting the mbKitL may represent an valuable approach to interfere with tumor angiogenesis.

P1.48

Developing sensors for the visualisation of adaptive and ER stress-driven Unfolded Protein Response

A. Orsi, E van Anken

San Raffaele Scientific Institute, Milan, Italy

Upon synthesis, secretory proteins enter the endoplasmic reticulum (ER) where they fold and oligomerize with assistance of resident chaperones and foldases. If the folding machinery can no longer cope - eg due to increased folding load, the imbalance is detected by ER stress sensors that invoke the unfolded protein response (UPR). In mammals, the UPR is orchestrated via at least three transmembrane sensors: Ire1, Perk1 and ATF6. The integration of UPR signalling via these parallel pathways determines how homeostasis is restored or whether cells succumb to the stress imposed.

During chronic ER stress Ire1 is recruited to distinct foci on the ER membrane, from which the UPR signaling is initiated. We are now studying if Perk and ATF6 are also recruited these UPR broadcasting centres, as they might represent hubs for UPR signal integration. Interestingly, Ire1, Perk1 and ATF6 are involved in the regulation of other physiological and developmental progresses, such as B cell differentiation and pancreatic beta cell homeostasis. In these programmes, UPR sensors can be activated independently from each other, suggesting that adaptive UPR differs from the one elicited pharmacologically.
To investigate these aspects, we are using a B cell line that we can trigger to differentiate into highly secreting plasma cells. This process is accompanied by a massive expansion and remodeling of the ER, and depends on the UPR machinery. Using lentiviral vectors we are setting up the expression of fluorescently-tagged UPR sensors in these cells, in order to visualise the ER stress sensing by live cell imaging. This visual approach and the use of a differentiable cell line will allow us to compare physiological and pharmacological UPR in the same cellular system.
The comparison of how different branches of the UPR are specifically employed or muted in different

P1.49

RNA polymerase III drives alternative splicing of the Potassium Channel Interacting Protein contributing to brain complexity and neurodegeneration

S. Massone[1*], I. Vassallo[1*], M. Castelnuovo[1*], G. Fiorino[2], E. Gatta[3], M. Robello[3], R. Borghi[4], M. Tabaton[4], C. Russo[5], G. Dieci[2], R. Cancedda[1], A. Pagano[1]
[1]Oncology Biology and Genetics Department (DOBiG), Univ. of Genoa, Genoa-Italy and National Institute for Cancer Research (IST), Genoa-Italy
[2]Dept of Biochemistry and Molecular Biology, Univ. of Parma, Parma-Italy
[3]Dept of Physics (DIFI), Univ. of Genoa, Genoa-Italy
[4]Dept of Experimental Medicine (DiMeS), Univ. of Genoa, Genoa-Italy
[5]Dept of Health Sciences, Univ. of Molise, Campobasso-Italy
* These authors contributed equally to this work

Alternative splicing generates protein isoforms that are conditionally or differentially expressed in specific tissues. Discovery of factors that control alternative splicing might clarify the molecular basis of biological and pathological processes. We found that IL1-α? dependent upregulation of 38A, a small RNA polymerase III-transcribed RNA, drives the synthesis of an alternatively spliced form of the Potassium Channel Interacting Protein (KCNIP4). The alternative KCNIP4 isoform cannot interact with the γ-secretase complex, resulting in modification of γ-secretase activity and Amyloid Precursor Protein processing and increased secretion of beta-amyloid enriched in the more toxic Aβ x-42 species. Notably, synthesis of the variant KCNIP4 isoform is also detrimental to brain physiology, as it results in the concomitant blockade of the fast kinetics of potassium channels. This alternative splicing shift is observed at high frequency in tissue samples from Alzheimer's disease (AD) patients, suggesting that RNA polymerase III co-genes may be upstream determinants of alternative splicing that significantly contribute to homeostasis and pathogenesis in the brain.

P1.50

Rab5 couples migratory protrusions and pericellular proteolysis during tumor cell invasion

A. Palamidessi[1], E. Frittoli[1], C. Malinverno[1], P. P. Di Fiore[1,2,3], G. Scita[1, 2]
[1]IFOM, Fondazione Istituto FIRC di Oncologia Molecolare, Milan, Italy
[2]Dipartimento di Medicina, Chirurgia e Odontoiatria, Univ. degli Studi di Milano, Milan, Italy
[3]Istituto Europeo di Oncologia, Milan, Italy

Cells, and in particular tumor cells, can adopt different modes of cell motility. The ability to switch between diverse modes of migration enables tumors to adapt to micro-environmental conditions and to metastasize. The critical pathways and cellular processes underlying the plasticity of tumor cell motility have only begun to be identified. An appealing hypothesis, supported by recent evidence, is that endocytosis, originally thought of as a device to internalize nutrients and membrane-boundmolecules, is a connectivity infrastructure (which we call "the EndocyticMatrix") of different cellular networks necessary bound molecules ion of various cellular programs. A primary role of the Endocytic Matrix is the delivery of space- and time-resolved signals to the cell, and is thus essential for the execution of polarized functions during 3D cell migration and invasion. Here, we will focus on the endocytic and signaling functions of Rab5, a smallGTPases essential for endosome biogenesis. We will discuss experimental evidence that support the general paradigm that intracellular trafficking, controlled by Rab5, is needed to re-direct molecules to restricted regions of the plasma membrane and to couple the formation of Rac-dependent migratory protrusions with proteolytically active adhesive site, ultimately mediating tumor cell invasion in 3D matrices.

P1.51

β1integrin/hERG1 complex as a novel molecular target for antineoplastic therapy

F. Zanieri[1], A. Sette[1], S. Crescioli[1], R. Mercatelli[2], S. Pillozzi[1], L. Carraresi[1], M.F. Brizzi[3], P. De Filippi[4], A. Arcangeli[1]
[1]Dept Experimental Pathology and Oncology, Firenze, Italy
[2]Ist. Sistemi Complessi, CNR di Firenze, Italia
[3]Dept of Internal Medicine, Torino, Italy
[4]Molecular Biotechnology Center, Torino, Italy

hERG1 channels are aberrantly expressed in several human cancers where they control different aspects of the neoplastic cell biology, since they form on the plasma membrane of tumor cells macromolecular complexes with integrin receptors, mainly β1 subunit. Therefore, the β1/hERG1 complex may represent a novel molecular target in cancer therapy and the blocking of such complex may represent an alternative strategy to inhibit hERG1 channel activity in cancer cells. Immunoprecipitations (IP) experiments evidenced that that the formation of this complex occurs selectively in cancer cells but not in the corresponding normal cells. Basing on this premise its molecular characterization can represent a very useful task to design novel antineoplastic strategies. Fundamental to design such strategy is our demonstration that β1 subunit and hERG1 protein directly interact on the plasma membrane of cancer cells. Such demonstration was obtained performing FRET and IP experiments, and clearly indicated that the two proteins directly interact to form a plasma membrane complex characterized by an intermolecular distance between the two proteins lower than 4 nm. Pull down experiments are ongoing to better define the protein domains implicated in hERG1 binding with its partners. Preliminary experiments have identified in the cytosolic C-terminus of beta1 and the cytosolic C terminus of hERG1 the regions of the proteins involved in complex formation. As regards cancer therapy our aim is to develop inhibiting peptides which may unlock the complex, and a bifunctional diabody targeting β1 and hERG1 extracellular domains in order to prevent the complex formation. The first step was the isolation of the variable domains of heavy chain (VH) and light chain (VL) of the monoclonal antibody against hERG1 produced in our laboratory, and the isolation of the same domains of the antibody against β1. VH and VL sequences will be cloned in an opportune vector to assemble the bispecific diabody.

P1.52

PARP1-1 inhibition as a novel therapeutical approach for malignant pleural mesothelioma

G. Pinton[1], A.G. Manente[1], D. Valente[2], L. Rossi[3], B. Murer[4], A.R. Vacca[2], L. Mutti[5], L. Moro[1]
[1]DiSCAFF and Drug and Food Biotechnology Center, University of Piemonte Orientale A. Avogadro, Novara, Italy
[2]IBBE-CNR, Bari, Italy
[3]Dept oh Human Morphology and Applied Biology, Univ. of Pisa, Pisa, Italy
[4]Dept of Anatomic Pathology, Mestre Hospital, Italy
[5]Italian Group for the Study and Therapy for Mesothelioma (G.I.Me), Italy

Malignant Pleural Mesothelioma (MMe) is an asbestos-related, highly aggressive neoplasm. Asbestos is cytotoxic for human mesothelial cells; it's able to trigger an inflammatory process that results in release of cytokines and reactive oxygen species (ROS). Human mesothelial cells exposed to asbestos activate the nuclear enzyme PARP-1 to repair DNA single-strand breaks (SSBs). PARP-1, known as a molecular nick-sensor, binds SSBs specifically and utilizes β-NAD+ as a substrates to catalyze the synthesis of (ADP-ribose) polymers (poly-ADP ribosylation) on nuclear proteins, including PARP itself with the increase of PARP-1 activity. Activation of PARP depletes ATP content; therefore, a loss of energy supply also contributes to cell death. Immunohistochemical analysis revealed low PARP-1 staining in peritumoral mesothelia tissues and a progressive increase in expression in the more aggressive MMe phenotypes, inversely correlated with Estrogen receptor beta (ERβ) expression, that we firstly demonstrated to be the predominant ER isoform expressed in MMe with function of tumour suppressor. PARP-1 protein represents an important novel target in cancer therapy, so we tested a newly synthesized PARP-1 inhibitor, AGO14699 compound, and demonstrated that it significantly reduced MMe cells viability, interfering with the activity of the enzyme. Moreover, we observed a direct correlation between ERβ expression and mitochondrial ATP production. In ERβ over-expressing cells, alterations in MRC complex II and IV activities negatively affected mitochondrial functions and increased glucose-dependency.

P1.53

Phagocytosis of hemozoin alters MMP-9/TIMP-1 balance in human monocytes: role of cytokines, lipids and NF-κB

G. Giribaldi[1,2], E. Valente[1,2], M. Polimeni[1], D. Ulliers[1,2], A. Khadjavi[1], G. Mandili[1], P.E. Van den Steen[3], G. Opdenakker[3], M. Prato[1,2]
[1]Dipartimento di Genetica, Biologia e Biochimica, Univ. di Torino, Torino, Italy
[2]Italian Malaria Network - Centro Interuniversitario di Ricerche sulla Malaria (CIRM), Torino, Italy
[3]Laboratory of Immunobiology, Rega Institute, Univ. of Leuven, Leuven, Belgium

INTRODUCTION. Phagocytosis of malarial pigment hemozoin (HZ) impairs several monocyte functions and promotes production of inflammatory molecules. In the present study, the effects of HZ on MMP-9/TIMP-1 balance in human monocytes were studied, along with dependence on production of pro-inflammatory molecules, role of lipid moiety of HZ, involvement of NF-κB activation and following biological effects.
RESULTS. HZ enhanced MMP-9 and TIMP-1 mRNA expression and protein secretion, as well as net total gelatinolytic activity and cell invasiveness. HZ-dependent MMP-9/TIMP-1 enhancement was abrogated by anti-hTNFα, anti-hIL-1β and anti-hMIP-1α blocking antibodies and was mimicked by recombinant cytokines. Lipid-free HZ did not reproduce HZ effects, whereas 15-HETE did. Quercetin, artemisinin and parthenolide, three NF-κB inhibitors showing anti-malaria properties, abrogated HZ effects.
CONCLUSIONS. Phagocytosis of HZ by human monocytes promotes an inflammation-mediated and NF-κB-dependent increase of expression and secretion of both MMP-9 and TIMP-1; the lipid moiety of HZ appears to be relevant, and a role for 15-HETE, a potent lipoperoxidation derivative generated by HZ from arachidonic acid via heme-catalysis, is likely. Nevertheless, HZ enhances net gelatinolytic activity and cell invasion ability, suggesting that TIMP-1 enhancement is not sufficient to counterbalance MMP-9 increase, which might be detrimentally instrumental for monocyte extravasation during CM. In conclusion, a role for MMP-9 and TIMP-1 as potential markers of malaria severity, as well as the potential use of synthetic MMP inhibitors as adjuvant therapy for CM, should be carefully considered in future studies.

P1.54

Human keratinocyte early differentiation: involvement of KGFR/FGFR2b expression and signalling

V. Purpura, F. Belleudi, M.R. Torrisi
Dipartimento di Medicina Clinica e Molecolare, "Sapienza" Univ. di Roma, Rome, Italy

The keratinocyte growth factor receptor (KGFR/FGFR2b) is a fibroblast growth factor receptor 2 (FGFR2) splicing variant expressed exclusively on epithelial cells and playing a key role in skin homeostasis.
To analyze the role of KGFR in the early differentiation of human keratinocytes, we modulated the receptor expression by cDNA transient transfection or siRNA microinjection and we induced a rapid synchronous wave of differentiation in pre-confluent HaCaT cells by treatment with Thapsigargin, an inhibitor of endoplasmic reticulum Ca-ATPase pump family. Quantitative RT-PCR, western-blot and immunofluorescence analysis showed that KGFR overexpression increases the early differentiation marker keratin 1 (K1), while receptor depletion reduces it and that the receptor ligand-dependent activation and signaling are required for the differentiative effect. Overexpression of kinase negative KGFR mutant or Tyr769 KGFR signaling mutant, which is not able to recruit and activate PLC-γ? showed that the receptor kinase activity, but not its PLCγ-?mediated signaling, is required for KGFR-mediated differentiation. The reduction of K1 expression obtained by AKT inhibition indicated that the PI3K/Akt signaling pathway is involved in this process. Our in vitro experimental model indicates that KGFR/FGFR2b expression represents a key event regulating keratinocyte early differentiation during the switch from undifferentiated to differentiating cells.

P1.55

Filamin A-mediated interaction of Prostate Specific Membrane Antigen (PSMA) with beta1 integrin regulates survival of advanced prostate cancer cells

S. Grasso[1], P. Shukla[1], M.E. Perico[1], V. Antonini[3], G. Fracasso[1], D. Ramarli[2]
[1]Immunology, Dept of Pathology and Diagnostics, Univ. Verona, Italy
[2]Immunology Unit, Hospital-Univ., Verona, Italy
[3]Institute of Biophysics, CNR, Trento, Italy

Prostate cancer (Pc) is the second cause of cancer-related death in males of Western countries, due to the poor prognosis of the advanced, androgen-independent, metastatic disease. Advanced Pc cells over-express PSMA, in a drug-resistant, apoptosis-insensitive cell microenvironment where PI3K/AKT/mTOR and RAF/MEK/ERK pathways are constitutively activated, STAT3/NF-kB transactivators increase their function, IL-6 expression is maximized and betat1/beta3 integrins are aberrantly or overexpressed.
Whether and how PSMA acts in the generation/maintenance of the advanced Pc phenotype was the topic of our study. PSMA is a trans-membrane folate-hydrolase/carboxypeptidase bearing a binding site for Filamin A (FLNa) in its cytodomain. Previous and herein detailed results by our group demonstrate that PSMA cross-linking signals to PI3K/AKT/mTOR and RAF/MEK/ERK1/2 activation in LNCaP cells (a cell model of advance Pc) and rescues LNCaP from apoptotic stimuli. Both pathways regulate survival and PSMA-mediated rescue. The relevance of FLNa-mediated PSMA/beta1 interaction in these phenomena was shown by the finding that:i) PSMA and beta1 co-localized at the surface of LNCaP cells ii) PSMA cross-linking induced the exposure of HUT-21 activation epitopes on beta1 and the assembly of a complex including PSMA itself, FLNa, beta1 integrin, pp130CAS and pSrc iii) PSMA- mediated beta1 activation, AKT or ERK1/2 phosphorylation were all hampered by FLNa silencing or Src inhibition with PP1.
Collectively, these results first highlights a key role for PSMA/beta1 integrin cooperation in regulating the activation state of advanced Pc cells. Moreover they show that the bridging activity of FLNa can extend the cross-talk of beta1 cytodomain to molecules other than growth factors or cytokine receptors.

P1.56

Epigallocatechin-3-gallate inhibits the growth of malignant mesothelioma cells

E. Ranzato[1], S. Martinotti[1], V. Magnelli[1], S. Biffo[1,2], B. Burlando[1]
[1]Dept of Environment and Life Sciences, DiSAV, Univ. of Piemonte Orientale "Amedeo Avogadro", Alessandria, Italy
[2]Molecular Histology and Cell Growth Laboratory, San Raffaele Science Institute, Milan, Italy

Malignant mesothelioma (MMe) is a lethal tumor arising from the mesothelium of serous cavities. This cancer shows a close relationship with asbestos exposure and its incidence has been increasing as a result of widespread use of asbestos. An effective therapy for MMe has not been established yet: surgery is very invasive, radiotherapy is not a radical treatment, while chemotherapy has reached encouraging but not definitive results. So, there is an urgent need for new approaches. Epigallocatechin-3-gallate (EGCG), the most abundant polyphenol of green tea, inhibits the growth of various tumor cells and has been proposed as a potential chemopreventive agent. We have demonstrated a selective cytotoxicity of EGCG to MMe cells, compared to nonneoplastic mesothelial cells, and have studied its mechanism of action. EGCG produces H_2O_2 outside the cells, and its cytotoxic effects have been almost abolished by exogenous catalase (CAT). Moreover, EGCG produces an intracellular increase of ROS that is reduced by exogenous CAT. EGCG induces intracellular Ca^{2+} rise that is abolished by extracellularly-applied CAT or by specific Ca^{2+} channel blockers. MMe cells have also been found to hyperexpress a T-type Ca^{2+} channel subunit with respect to normal mesothelium, and siRNA of this subunit almost abolishes intracellular Ca^{2+} rise, ROS production and noxious effects on cells. Taken together, these data allow to hypothesize that extracellular H_2O_2 produced by EGCG triggers Ca^{2+} influx into the cells, thereby leading to intracellular ROS production and apoptosis or necrosis. The Ca^{2+} influx seems to involve T-type channels and could explain the selective toxicity of EGCG to MMe cells. These findings suggest the possible use of EGCG for MMe therapy, and indicate T channels as a novel pharmacological target.

P1.57

Oncogenic RAS-induced neoplastic phenotype is sustained through alterations of calcium homeostasis

A. Rimessi, S. Patergnani, S. Marchi, C. Agnoletto, A. Bononi, M. Bonora, E. De Marchi, C. Giorgi, S. Missiroli, F. Poletti, J. Suski, P. Pinton
Dept of Experimental and Diagnostic Medicine, Section of General Pathology, Interdisciplinary Center for the Study of Inflammation (ICSI), Laboratory for Technologies of Advanced Therapies (LTTA), Univ. of Ferrara, Ferrara, Italy

The RAS oncogene is mutated in 33% of all human cancers, with highest frequency in cancers where there are currently poor therapeutic options. For this reason, there is an acute need for innovative and novel research to identify new avenues for the development of pharmacologic inhibitors of RAS activity. This project outlines studies to address roles for the caveolin-1 (cav-1) and Ca^{2+} regulation in RAS function. The identification of a "plasma membrane/endoplasmic reticulum/mitochondrial functional module" acting during oncogenic-RAS expression, is the focus of the present study. We hypothesize that at the base of tumorigenesis and tumor maintenance there is compartmentalized oncogenic RAS able to maintain an altered intracellular Ca^{2+} homeostasis, in part compromising Caveolin-1 functionality. Mechanistically, we think that RAS is associated with a dynamic protein complex consisting of pivotal calcium modulator in both Plasma membrane-Associated Membranes (PAM are the putative plasma membrane/endoplasmic reticulum contact sites) and Mitochondria-Associated Membranes (MAM are the endoplasmic reticulum/mitochondria contact sites) sites. The redistribution of RAS during transformation impairs the stability/functionality of the molecular complex promoting neoplastic phenotype.
The focus on Ca^{2+} signalling by RAS is interesting and it is an avenue unexplored, in fact represents an important and emerging area of cancer research.
The overall goal of this study is to broaden our knowledge about role of RAS in tumor pathogenesis, mediating its effect on Ca^{2+} signalling and mitochondrial physiology and its molecular understanding will be instrumental in designing innovative therapeutic strategies.

P1.58

Identification and characterization of a myopathic phenotype affecting skeletal muscles of NG2 null mice

E. Rizzo[1], P. Sabatelli[2], A. Angelin[3], B. Blaauw[4], C. Reggiani[4], P. Bernardi[3], N.M. Maraldi[2], W.B. Stallcup[5], P. Bonaldo[1]
Depts of [1]Histology, Microbiology & Medical Biotechnologies, [3]Biomedical Sciences and [4]Human Anatomy and Physiology, Univ. of Padova, Padova, Italy
[2]National Research Council, IGM at IOR, Bologna, Italy
[5]Burnham Institute for Medical Research, Cancer Research Center, La Jolla, CA, USA

Collagen VI (ColVI) is a large extracellular matrix protein with a widespread distribution in several tissues. Mutations of human genes encoding ColVI cause various forms of muscular dystrophy including Bethlem Myopathy and Ullrich Congenital Muscular Dystrophy. Previous studies demonstrated that ColVI null mice display an early onset myopathic phenotype affecting skeletal muscles, characterized by mitochondrial dysfunction and spontaneous apoptosis. Molecular pathway transducing colVI signals is largely unknown. NG2 is a transmembrane proteoglycan acting as cell surface receptor for ColVI. It is expressed by developing tissues and tumors. Notably, NG2 is present on postnatal skeletal muscles and it is selectively downregulated in myofibers from Ullrich patients and ColVI null mice. The aim of this study is to investigate the role of NG2 and its interplay with ColVI in skeletal muscles. Although NG2 null (Cspg4–/–) mice appear phenotypically normal, careful investigation of muscles revealed a myopathic syndrome. In particular, Cspg4–/– myofibers show increased incidence of apoptosis and mitochondrial dysfunction. When Cspg4–/– animals were subjected to exercise, they displayed muscle weakness and decreased resistance to fatigue. Electron microscopy of Cspg4–/– mice revealed increased thickness of basement membrane and proliferation of pericytes in the capillary vessels of skeletal muscles. These 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. These findings suggest that NG2 may influence pericyte homeostasis and contribute to muscle fiber degeneration in ColVI related disorders.
In conclusion, the phenotype of Cspg4–/– mice is partially similar to that of ColVI null mice, strongly pointing at an interaction between these two molecules in muscle. Future studies will allow elucidating further the role of NG2 in normal and diseased muscle.

P1.59

The mutations that cooperate with PML/RAR in leukemogenesis form a unique network of functionally homogenous transcriptional deregulations

C. Ronchini[1], A. Brozzi[1], A. Gruszka[1], L. Luzi[1], D. di Bernardo[2], P.G. Pelicci[1]
[1]Dept Experimental Oncology, European Institute of Oncology, IFOM-IEO Campus, Milan, Italy
[2]Telethon Institute of Genetics and Medicine, Naples, Italy

The oncogenic fusion protein PML/RAR requires secondary cooperating alterations to induce fully developed myeloid leukemias. In order to identify these cooperators, we performed an insertional mutagenesis screen, infecting the PML/RAR KI mice with the Murine Leukemia Virus (MLV). MLV infection significantly accelerated the onset and increased the penetrance of the PML/RAR-dependent leukemias. From 48 accelerated tumors, by IPCR and sequencing, we identified 215 common viral integration sites (CIS) and 271 putative target genes. Each animal developed multiclonal leukemias, indicating that a very high number of independent cooperators of PML/RAR exist. Analyzing the mechanisms of gene deregulation by viral insertion, we predicted a loss of function mutation for 35% and a gain of function mutation for 65% of the CIS-targets. Performing an expression profile analysis both on murine and human leukemias, we were able to validate around the 70% of the targets tested. However, very surprisingly, we observed a general deregulation of the gene targets with the majority of the leukemias showing concomitant deregulated expression of many cooperating-genes. These data led us to hypothesize the existence of a network of regulatory interactions among the targets of the genetic lesions, such as the mutation of one gene could modify the expression of one or several other genes. Indeed, using Netview, we showed that the majority of the CIS-targets (the 80% in mouse and the 70% in human) form a single complex network, indicating that they are indeed co-expressed. We, therefore, asked if they could be functionally related. More than 70% of targets we identified could be assigned to cellular pathways that control cellular proliferation. Our data suggest that the high genetic-heterogeneity of cooperating mutations of PML-RAR is converted into a unique network of deregulation of functionally homogenous cellular pathways that would lead to common leukemogenic mechanisms.

P1.60

ESCRT-0 controls Jak/Stat signaling and tumor suppression in ***Drosophila***

N. Wöβner[1], X. Wang[2], D.J. Montell[2], T. Vaccari[1]
[1]IFOM, Istituto FIRC di Oncologia Molecolare, Milano, Italy
[2]Johns Hopkins School of Medicine, Baltimore, MD, USA

Sorting of ubiquitylated proteins to be degraded in lysosomes occurs in forming Multi-Vesicular Endosomes (MVEs) and depends on four Endosomal Sorting Complex Required for Transport (ESCRT-0, -I, -II, -III) complexes. ESCRT-I, -II, -III proteins have been implicated in tumor suppression in Drosophila due to their ability to sort, among other cargoes, signaling receptors. Despite this, the tumor suppressive role of ESCRT-0 - the entry point of MVE sorting - has not been studied, and the mechanisms by which ESCRTs govern signal transduction are far from clear.
We have found that developing Drosophila epithelia lacking activity of both hrs and stam, the two known components of ESCRT-0 complex, accumulate ubiquitylated proteins in endosomes, consistent with loss of MVE sorting activity. More surprisingly, hrs and stam double mutant tissue fails to polarize apico-basally, to terminally differentiate and loose proliferation control. Since these traits are acquired during tumorigenesis, our evidence suggests that the activity of the ESCRT-0 complex is normally tumor suppressive.
To elucidate the mechanisms by which ESCRT-0 might contribute to tumor suppression, we profiled the signaling ability of Drosophila epithelial tissues mutant for both hrs and stam. Distinctly from cells that lack ESCRT-I, -II, -III activity, hrs stam double mutant cells do not display alteration of Notch signaling activation. In contrast, they show high levels of Jak/Stat signaling. Reduction of Jak/Stat signaling in hrs stam double mutant cells rescues epithelial integrity, suggesting that Jak/Stat signaling plays a major role in ESCRT-0-mediated tumorigenesis in developing Drosophila epithelia.
Overall, our data indicate that the initial steps of cargo sorting at MVEs are tumor suppressive, as previously reported for the subsequent ones controlled by ESCRT-I, -II, -III complexes. ESCRT-0 are required to downregulate Jak/Stat signaling, but otherwise dispensable for Notch activation, reveling unexpected diversity among ESCRT complexes in control of signaling.

P1.61

mRNA targeting to endoplasmic reticulum stress signaling sites

E. van Anken[1,2,3], T. Aragón[1,2,4], D. Pincus[1,2], S. Coyle[1], A.V. Korennykh[1,2], P. Walter[1,2]
[1]Univ. of California, San Francisco
[2]Howard Hughes Medical Institute
[3]current address: San Raffaele Scientific Institute, Milan
[4]current address: University of Navarra, Pamplona

Eukaryotic proteins destined for the cell surface or secretion fold and assemble in the endoplasmic reticulum (ER). The unfolded protein response (UPR) detects when the burden on the ER protein folding machinery exceeds its capacity, and readjusts the abundance of the organelle according to need. Conserved from yeast to human is the UPR pathway that is transduced via Ire1, a transmembrane kinase/endoribonuclease. Ire1 initiates the non-conventional splicing of the mRNA encoding a key transcription activator, Hac1 in yeast or XBP1 in metazoans. In the absence of ER stress, ribosomes are stalled on unspliced *HAC1* mRNA. The translational control is imposed by a base-pairing interaction between the *HAC1* intron and the *HAC1* 5' untranslated region (UTR). After excision of the intron, transfer RNA ligase joins the severed exons, lifting the translational block and allowing synthesis of Hac1 from the spliced *HAC1* mRNA to ensue. Hac1 in turn drives the UPR gene expression program comprising 7–8% of the yeast genome to counteract ER stress. We show that, on activation, Ire1 molecules cluster in the ER membrane into discrete foci of higher-order oligomers, to which unspliced *HAC1* mRNA is recruited by means of a conserved bipartite targeting element contained in the 3' UTR. Disruption of either Ire1 clustering or *HAC1* mRNA recruitment impairs UPR signaling. The *HAC1* 3' UTR element is sufficient to target other mRNAs to Ire1 foci, as long as their translation is on hold. Translational repression afforded by the intron fulfils this requirement for *HAC1* mRNA. Surprisingly, the kinase/endonuclease domain of Ire1 is dispensable for recruitment. Instead, a conserved positively charged motif in the juxtamembrane cytosolic linker domain of Ire1 facilitates the docking of *HAC1* mRNA onto Ire1 foci. Recruitment of mRNA to ER stress signaling centers provides a new paradigm for the control of eukaryotic gene expression.

P1.62

Multiple effects of presenilin-2 mutations linked to familial Alzheimer's disease on Ca2+ homeostasis: a single organelle FRET-based analysis

A.K.C. Wong, R. Filadi, P. Capitanio, E. Zampese, C. Fasolato, T. Pozzan, P. Pizzo
Dept of Biomedical Sciences, Univ. of Padua, Padua, Italy

Calcium (Ca2+) is a key intracellular messenger and Ca2+ dyshomeostasis has been demonstrated in Alzheimer's Disease (AD) (1), in particular in the rare genetically inherited AD cases (Familial AD, FAD), mostly due to mutations in presenilin (PS) 1 and 2 genes. We have recently shown that PS2 FAD mutants reduce the Ca2+ content of the major intracellular Ca2+ store, the endoplasmic reticulum (ER), mostly by inhibiting SERCA activity, as demonstrated at the cell population level by using ER-targeted aequorin Ca2+ sensor (2,3).
The aim of our work is to study in details, by employing specifically targeted "Cameleon" probes, the effect of FAD PS2 mutants on Ca2+ homeostasis, at the single cell level, considering different intracellular Ca2+-handling compartments (ER, Golgi apparatus and mitochondria). We created a novel ER-targeted Cameleon probe with optimized Ca2+ affinity, in order to investigate the effect of the expression of the PS2-T122R FAD mutation on the ER Ca2+ homeostasis in single cell FRET-experiments. In addition, since the Golgi apparatus is very heterogeneous in terms of Ca2+ handling and substantial differences have been found between the cis- and the trans-side of the organelle (4), we employed both the already available trans- and the novel cis-Golgi targeted "Cameleon" probes to dissect the possible differential effects of PS2-T122R mutation on this peculiar Ca2+ handling organelle. Mitochondria are also key players in Ca2+ homeostasis and neurodegeneration; thus we applied specific "Cameleon" probes also to study PS2 mutations' effects on these organelles. Additionally, we investigated whether and how PS2 FAD mutations affect ER-mitochondria interactions (5), a critical aspect in determining cell fate. Our data provide novel insights into the complex functions of PSs (wt and FAD–linked mutants) on Ca2+ homeostasis and AD pathogenesis.

1. Zampese et al., 2009
2. Zatti et al., 2006
3. Brunello et al. 2009
4. Lissandron et al. 2010
5. Zampese et al., 2011

P1.63

Unacylated ghrelin and ghrelin counteract skeletal muscle atrophy

N. Filigheddu[1], S. Reano[1], M. Ferrara[1], P. E. Porporato[1], E. Angelino[1], G. Ronchi[2], S. Geuna[2], Y. Sun[3], A. Graziani[1]

[1]Dept of Clinical and Experimental Medicine and Biotechnolog Center for Applied Medical Research, Univ. of Piemonte Orientale "Amedeo Avogadro", Novara, Italy

[2]Dept of Clinical and Biological Sciences, Univ. of Torino, Orbassano (TO)

[3]USDA ARS Children's Nutrition Research Center, DepTs of Pediatrics & Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX, USA

Acylated and unacylated ghrelin (AG and UnAG, respectively) are the two forms of the circulating peptide encoded by the ghrelin gene. AG, but not UnAG, stimulates growth hormone release and positive energy balance through binding to the receptor GHSR1a. However AG and UnAG share several biological activities, including PI3K/Akt-mediated inhibition of apoptosis and induction of skeletal myoblasts differentiation through activation of p38. Skeletal muscle atrophy is defined as the loss of muscle mass triggered by a specific genetic program carried out by atrogenes expression and induced by either fasting or denervation. Here we show in vivo that up-regulation of circulating UnAG obtained either pharmacologically or in transgenic mice inhibits skeletal muscle atrophy induced by either fasting or denervation without inducing muscle hypertrophy. Moreover we provide both biochemical and genetic evidence indicating that UnAG anti-atrophic protection does not involve GHSR1a-mediated activation of the GH/IGF-1 axis. Skeletal muscle atrophy can be opposed by activation of the protein kinase mTOR, which, by forming two distinct protein complexes, mTORC1 and mTORC2, triggers distinct pathways leading, respectively, to increased protein synthesis and to inhibition of protein degradation. In order to identify the biochemical mechanisms underlying AG/UnAG anti-atrophic activity, skeletal muscle atrophy was induced in vitro upon dexamethasone (DEXA) treatment of C2C12 myotubes. Both AG and UnAG inhibit DEXA-induced atrophy of myotubes, measured as myotubes diameter and atrogenes expression, through stimulation of mTORC2 and p38 pathway, while not activating mTORC1 pathway, protein synthesis and hypertrophy. These findings unveil a novel mechanism by which AG/UnAG, by acting directly in the skeletaol muscle through a novel yet unidentified common receptor, contribute selectively to prevent muscle protein degradation without inducing protein synthesis and muscle hypertrophy.

P3.1

Role of ErbB1 in Trastuzumab-induced ErbB2 signaling and internalization

P. Bagnato[*1,2,3], A. Rabellino[*1,2,3], A. Castagnino[1,2,3], M. Di Benedetto[1,3], P. Bianchini[1] , M. Bono[1,2,3], K. Cortese[1,2,3], C. Tacchetti[1,2,3]
[1]MicroScoBiO Research Center, Univ. of Genoa, Genova, Italy
[2]IFOM Center of Cell Oncology and Ultrastructure, Genova, Italy
[3]Dept of Experimental Medicine, Univ. of Genoa, Genova, Italy
*these authors contributed equally to this work

The ErbB family of receptors comprises four closely related members: EGFR (ErbB1 or HER1), ErbB2 (HER2), ErbB3 (HER3), and ErbB4 (HER4). These receptors play important roles in cell proliferation, survival, migration and differentiation, and also in development and progression of cancer. In particular, ErbB2 is over-expressed in approximately 30% of invasive breast cancers, which often display a highly invasive and metastatic phenotype, resistance to conventional chemotherapy and hormone-therapy. The anti-proliferative effect of the ErbB2 specific antibody Trastuzumab in cells over-expressing ErbB2 has lead to its effective use in current therapeutic protocols. However, notwithstanding the extensive use, the mechanism of action of this antibody has not been defined yet and some controversial data have been reported. We suggest that some of these controversial results can be reconciled by showing that Trastuzumab treatment induces two levels of activity, depending on the length of the treatment.
In short term treatment, Trastuzumab induces activation and phosphorylation of ErbB2, and its heterodimerization with ErbB1. This activation of ErbB2 generates a signaling cascade that leads first to phosphorilation of Erk1/2 and subsequently to the de-phosphorylation of AKT. Moreover, at early time points, Trastuzumab promotes the endocytosis and recycling of the receptor back to the plasma membrane. Silencing of ErbB1, by RNA interfering, leads to early degradation of the activated ErbB2, probably rerouting it from recycling endosomes to a degradative compartment.
In this work we provide a model of the Trastuzumab mechanism of action in ErbB2 over-expressing cells: low levels of ErbB1 lead to a more efficient down-regulation of ErbB2, determining a consistent reduction of the receptor from the PM and attenuation of its specific proliferation signals.

P3.2

[WITHDRAWN]

P3.3

Alix is a substrate of the Ozz-E3 ligase and modulates actin remodeling and plasma membrane stability in skeletal muscle

D.P. Romancino[1,*], Y. Campos[2,*], G. Paterniti[1], X. Qiu[2], S. Moshiach[2], V. Di Felice[3], A. d'Azzo[2], A. Bongiovanni[1]
[1]Institute of Biomedicine and Molecular Immunology (IBIM), National Research Council (CNR), Palermo, Italy
[2]Dept of Genetics and Tumor Cell Biology, St. Jude Children's Research Hospital, Memphis, TN, USA
[3]Dept of Experimental Biomedicine and Clinical Neurosciences, Univ. of Palermo, Italy
*These authors contributed equally to this work

Alix/AIP1 is a multifunctional adaptor protein that participates in basic cellular processes, such as membrane trafficking and actin cytoskeleton assembly, by binding selectively to a variety of partner proteins. However, the mechanisms regulating Alix turnover, subcellular distribution and mode of action in muscle cells are unknown. We now report that Alix is expressed in skeletal muscle throughout myogenic differentiation. In the differentiated myotubes, specific pool of Alix colocalizes in discrete subcellular regions with Ozz, the substrate-binding component of the muscle-specific ubiquitin ligase complex, Ozz-E3. We have shown previously that Ozz-E3 plays an active role in skeletal muscle differentiation and regeneration, by ubiquitinating the sarcolemmal-associated pool of β-catenin and the sarcomeric embryonic myosin heavy chain (MyHCemb) (Nastasi et al., 2004; Campos et al., 2010). Here, we demonstrate that Ozz interacts with and promotes the ubiquitination of Alix. This modification appears to regulate the levels of Alix in specific sub-compartments, such as the multivesicular bodies (MVBs) and the compartment containing the actin polymerisation factor cortactin. These findings suggest that the Ozz-E3 ligase regulates Alix at sites in which the endocytic machinery connects to the actin cytoskeleton. In line with this observation, we show that knockdown of Alix' expression affects the level and distribution of filamentous (F)-actin and leads to plasma membrane instability, in myotubes. These alterations result in aberrant cell morphology and exosome secretion, impaired formation of sarcolemmal protrusions, and defective cell motility. Our results point to a crucial role of Alix in the process of muscle cell migration and exosome biogenesis, by regulating the dynamics of the plasma membrane and the actin cytoskeleton.

P3.4

Vimentin assembly is regulated by the small GTPase Rab7

F. Piro, L. Cogli, C. Bucci
Dept of Biological and Environmental Sciences and Technologies (DiSTeBA), Univ. of Salento, Lecce, Italy

Intermediate filaments are a dynamic component of the cytoskeleton characterized by rapid movement and dynamic exchange of subunits. Indeed, they can be quickly reorganized by phosphorylation or glycosylation. Several recent data demonstrate that intermediate filaments are closely linked to other cellular machineries as cytoskeleton or membrane traffic machineries. Indeed, intermediate filaments are clearly well integrated with the actin and tubulin cytoskeletons and their motors, and contribute to the regulation of membrane traffic.
We have identified vimentin, the major intermediate filament protein of mesenchymal cells, as a Rab7 interacting protein using the two-hybrid system. Vimentin is a regulator of cell adhesion and migration, and it is a modulator of protein kinases. Rab7 is a small GTPase involved in the regulation of transport to late endosomes and lysosomes in the endocytic pathway. We confirmed the interaction using co-immunoprecipitation and pull-down; we also established that the interaction is direct using bacterially expressed recombinant proteins. Our data demonstrate that Rab7 is involved in vimentin phosphorylation and thus in the regulation of the vimentin network. Indeed, depletion of Rab7 causes a decrease of phosphorylated vimentin, while overexpression of Rab7 causes a strong increase of vimentin phosphorylation at different sites. Increased phosphorylation is associated with an increased presence of vimentin in the soluble fraction demonstrating vimentin disassembly. Thus, Rab7 regulates vimentin assembly possibly by, directly or indirectly, acting on specific kinases.
Recent work has established that the endo-lysosomal sorting machinery interacts with members of the intermediate filaments and, in particular, with vimentin. The relationship between Rab7 and vimentin points out to a strong connection between intermediate filaments and molecules controlling the endo-lysosomal vesicular transport.

P3.5

Intracellular trafficking of tail-anchored proteins

P. Cassella[1], S. F. Colombo[1], Nica Borgese[1,2]
[1]Consiglio Nazionale delle Ricerche Institute of Neuroscience and Dept of Pharmacology Univ. degli Studi di Milano, Milano, Italy
[2]Dept of Pharmacobiological Science, Univ. di Catanzaro "Magna Graecia"

Membrane proteins must be targeted from their site of synthesis on cytosolic ribosomes to the proper target membrane, into which they are then integrated via specific translocation machinery. A group of membrane proteins called tail-anchored (TA) have a transmembrane domain near the C terminus and an N-terminal cytosolic moiety; because of this topology they do not have access to the SRP-mediated co-translational pathway for insertion into the ER, and can be inserted into their target membranes only posttranslationally. It is not clear how these tail-anchored (TA) proteins select their target, but C-terminal charged residues play an important role. To investigate how discrimination between the mitochondrial outer membrane (MOM) and ER occurs, we used mammalian cytochrome b5, a TA protein existing in two, MOM or ER localized, versions (b5 ER and b5 MOM). Substitution of the seven C-terminal residues of the ER isoform with two arginines results in a MOM-targeted protein (b5 RR) in vivo. However, the in vivo targeting specificity is not reproduced in vitro, inasmuch as both proteins are able to insert into protein-free liposomes, and neither of them shows a preference for ER or MOM-derived vesicles even when presented with both these membranes together. We have therefore set up an in vivo targeting system to investigate the mechanism underlying specific targeting within cells. The method consists in microinjecting cultured cells (CV1) with the b5 ER and b5 RR isoform recombinant proteins, produced in bacteria, as GST fusion proteins. We find that 15 minutes after microinjection each isoform is correctly localized to the appropriate target compartment. Thus, no ribosome-associated factors are required to capture the newly synthesized polypeptide immediately after chain termination. We are presently investigating the involvement of known chaperones in this targeting process both by pharmacological and by RNAi approaches.

P3.6

Oxytocin receceptor trafficking: role of different G-proteins

E. Donà[1], B. Silva[1], M. Busnelli [1,2], R. Longhi[3], B. Chini[1]
[1]CNR, Institute of Neuroscience, Milan, Italy
[2]Dept. Pharmacology, Univ. of Milan, Milan, Italy
[3] CNR, Institute of Chemistry of Molecular Recognition, Milan, Italy

The oxytocin receptor (OTR) is a promiscuous G-protein coupled receptor that couples to both Galphaq and Galphai and whose stimulation leads to the activation of different intracellular signaling pathways. As in the case of most G-protein coupled receptors (GPCRs), agonist stimulation of the OTR leads to desensitization and internalization but the role played by the promiscuous receptor coupling in this process has not been investigated. Oxytocin (OT) derived peptides that activate selectively either the Galphai or Galphaq pathways were characterized in our laboratory. In order to assay the capability of these analogs to promote G-protein selective internalization, we developed two fluorescent agonists: dLVT-Alexa568 and atosiban-Alexa568. dLVT-Alexa568, that promotes both OTR/Galphaq and OTR/Galphai coupling, led to a very efficient receptor internalization. On the contrary, atosiban-Alexa568, a selective OTR/Galphai, did not lead to any change in receptor localization at the plasma membrane even after one hour of stimulation. Consistently, only dLVT-Alexa568 induced beta-arrestin recruitment. These data suggest that OTR internalization is depending on Galphaq activation. To further explore receptor/G-protein internalization mechanisms, we analyzed the possibility of a redistribution of G-proteins after receptor activation. As no change in the Galphai or Galphaq localization was observed after ligand-induced internalization, our data indicate that both G-proteins are actively excluded from the vesicles containing the internalized receptor. Finally, when atosiban pretreated cells were stimulated with OT, OTR endocytosis was still observed and was accompanied by atosiban co-internalization, indicating that atosiban did not block OTR internalization and suggesting a dimeric/oligomeric supramolecular organization of the receptor.

P3.7

Characterization and identification of new proteins involved into the TRC-40 mediated insertion of tail anchored proteins into the ER membrane

S.F. Colombo[1], N. Borgese[1,2]
[1]Consiglio Nazionale delle Ricerche Institute for Neuroscience and Dept of Pharmacology, Univ. of Milan, Milano, Italy
[2]Dept of Pharmacobiological Science, Univ. of Catanzaro "Magna Graecia", Roccelletta di Borgia (CZ), Italy

Tail anchored (TA) proteins are membrane proteins localised on many different intracellular membranes. Most TA proteins must first translocate into the ER membrane and then travel through the secretory pathway to reach their final destination. Thus, insertion into the ER represent a crucial step in TA protein biogenesis. TA proteins contain an N-terminal domain exposed to the cytosol and a single transmembrane segment near the C-terminus followed by few or no polar residues. Due to their peculiar topology they can't be inserted into the ER by the SRP dependent co-translational mechanism but, once released in the cytosol, they must be inserted into the ER post-translationally.

In the last few years many efforts have been dedicated to identify the machinery involved in TA proteins insertion. A cytosolic chaperone (GET3 in yeast/ TRC40 in mammals) has been demonstrated to play a fundamental role in ER insertion. In yeast GET3 delivers the TA proteins at the ER membrane interacting with two receptors (GET1 and GET2) whose counterparts in mammals have not still been identified. The role as a TRC40 receptor of WRB, the homologue of the yeast GET1 in mammals, is still debated.

To identify the receptor/s involved in TRC40 mediated insertion of TA proteins into the mammalian ER, we have taken advantage of the observation that rat liver microsomes contain abundant TRC40, presumably bound to its receptor. We have set up a functional assay, based on the reconstitution of proteoliposomes active in TA protein insertion, from microsomal extracts. If these extracts are depleted of TRC40-associated proteins by immunoaffinity isolation with anti-TRC40 antibodies, the resulting reconstituted proteoliposomes are inactive. Activity can, however, be reconstituted by addition of the fraction eluted from the immunoaffinity resin. We are presently analysing this eluate by mass spectrometry with the aim of identifying the putative receptor.

P3.8

RILP and V1G1: functional interaction in mammalian cells

M. De Luca, L. Cogli, C. Progida, R. Bramato, S. Bramato, A. Carucci, C. Bucci
Dept of Biological and Environmental Sciences and Technologies (DiSTeBA), Univ. of Salento, Italy

The pH of intracellular compartments and extracellular environment is a carefully controlled parameter. Proton transport is mediated by vacuolar (V)-ATPases, large multisubunit complexes composed of 14 different subunits, that are organized into an ATP-hydrolytic domain (V1) and a proton-translocation domain (V0) working together as a rotary machine.

The interaction with specific partners plays a crucial role in controlling V-ATPase activity. Using different approaches, we have identified the G1 subunit of the V-ATPase as a RILP (Rab-Interacting Lysosomal Protein) interactor. RILP is required for biogenesis of MVBs (multivesicular bodies) and, together with Rab7, acts in the regulation of late endocytic traffic. Acidification, triggered by V-ATPase in early endosomes, is required for the formation of MVBs. Thus, RILP through V-ATPase, may trigger a downstream pathway, giving rise to MVBs formation.

Here we show that V1G1 directly interacts and co-localizes with RILP on late endosomal and lysosomal membranes.

Our study demonstrates that RILP is a negative regulator of V1G1 and it controls V1G1 protein stability through a proteasome-dependent mechanism. The regulatory role of RILP is specific for the V1G1 subunit, as other subunits are not affected by RILP.

In addition, our data show that Rab7, a functional RILP interactor, doesn't affect V1G1 abundance suggesting that the effect of RILP on V1G1 is specific. Interestingly, the concomitant over-expression of RILP and Rab7 restores normal V1G1 levels suggesting that the interaction between Rab7/RILP and RILP/V1G1 is mutually exclusive and fundamental for V1G1 stability.

Deficient functions of V-ATPases and defects of vesicular acidification are recognized as important mechanisms in a variety of human diseases, thus elucidating a new regulatory mechanism of V-ATPase activity will surely help to develop new therapeutic approaches for different pathologies.

P3.9

Mechanisms of transmembrane domain-dependent partitioning of membrane proteins whithin the endoplasmic reticulum

M. Fossati[1,2], E. Fasana[1], JB. Manneville[3], B. Goud[3], N. Borgese[1,2,4]
[1]Consiglio Nazionale delle Ricerche Institute of Neuroscience, Milan, Italy
[2]Dept of Pharmacology, Univ. degli Studi di Milano, Milan, Italy
[3]Laboratoire Mécanismes moléculaires du transport intracellulaire, Institut Curie, CNRS UMR 144, Paris, France
[4]Dept of Pharmacobiological Science, Univ. of Catanzaro, Catanzaro, Italy

Our group recently characterized a novel sorting mechanism within the ER that depends only on transmembrane domain (TMD) length/hydrophobicity. Two fluorescent tail-anchored (TA) proteins differing only in TMD length (FP-22 and FP-17, with TMDs of 22 and 17 residues) are segregated from each other within the ER: FP-22 partitions into ER tubules and ER exit sites (ERES), while FP-17 is excluded from ERES and has access to both ER tubules and sheets. Since FP-22's TMD is predicted to be longer than the thin ER bilayer, we hypothesized that this hydrophobic mismatch could be reduced by FP-22's segregation into curved and/or thicker ER subdomains.
To investigate the role of membrane curvature in TMD-dependent sorting, purified FP-17 and FP-22 were reconstituted in Giant Unilamellar Vesicles (GUVs) of uniform lipid composition. By inducing tubulation of the GUVs with molecular motors, we investigated whether FP-22 preferentially partitions into the pulled tubules. By analyzing the protein distribution, no preferential segregation of FP-22 in tubes was observed. Rather, the two proteins seem to be homogenously distributed in both flat and curved domains, suggesting that membrane curvature is not sufficient to induce TMD-dependent segregation.
Concerning the involvement of lipid microdomains in protein segregation, we carried out photo-crosslinking analysis to investigate whether FP-17 and FP-22 reside in different lipid environments within the ER. Cells pulsed with radiolabeled photolabile phosphatidylcholine (PC) were semi-permeabilized with digitonin. After the post-translational insertion of FP-17 and FP-22 into the ER and the exposure of cells to UV light, lipid-protein complexes were isolated by immunoprecipitation. By comparing the radioactive

P3.10

[WITHDRAWN]

P3.11

TRIM50 forms a complex with HDAC6 and p62/SQSTM1 that localizes to aggresomes

C. Fusco[1], L. Micale[1], M. Egorov[2], M. Monti[3], E.V. D'Addetta[1], B. Augello[1], G. Cotugno[1], F. Cozzolino[3], A. Calcagnì[1], M.N. Loviglio[1], R.S. Polishchuk[2], P. Pucci[3], G. Merla[1]
[1]Laboratory of Medical Genetics, IRCCS Casa Sollievo Della Sofferenza Hospital, San Giovanni Rotondo, Italy
[2]Telethon Institute of Genetics and Medicine, Naples, Italy
[3]CEINGE Advanced Biotechnology and Dept of Organic Chemistry and Biochemistry, Federico II Univ., Naples, Italy

TRIM50 is hemizygous in patients affected by Williams Beuren syndrome, a neurodevelopmental genomic disorder, caused by a 1.5-1.8 Mb deletion at 7q11.23 including 28 genes. We recently demonstrated that TRIM50 encodes a cytoplasmic E3-ubiquitin ligase that catalyzes the binding of specific substrates to the ubiquitin leading them to the degradation by activating the Proteasome or autophagy system.
Further characterization of this protein was able to show that TRIM50 forms highly labile and dynamic cytoplasmic bodies that are aggresome precursors. Indeed in response to proteasome inhibition, TRIM50 localizes to the aggresome in a microtubule dependent manner. In addition, we identified HDAC6 as a new binding partner of TRIM50, an association that is strengthen under conditions of protein impairment. Using HDCA6-deficient fibroblasts we demonstrated that HDAC6 is required for the proper localization of TRIM50 within the aggresome.
Furthermore, we provide experimental compelling evidences that TRIM50 localizes and interacts with p62, a multifunctional adaptor protein implicated in various cellular processes including autophagic clearance of aggregation-prone polyubiquitinated proteins. Of note TRIM50 modulates the protein level of HDAC6 and p62, mainly increasing their insoluble fractions.
Our work showed that TRIM50 bodies, by its E3-ubiquitin ligase are aggresome precursors. When TRIM50 fails, by its E3-ubiquitin ligase, to drive its proteins target to proteasomal degradation, an alternative route is taken to ensure their sequestration transporting them to the aggresome via the association with HDAC6 and possibly their subsequent removal by p62-mediated autophagy.

P3.12

Imaging calcium-regulated exocytosis in RBL mastocytes with fluorescent polystyrene and mesoporous nanoparticles

A. Giovia, M. Ekkapongpisit, M. D'Aquino, G. Nicotra, M. Ozzano, C. Isidoro
Laboratorio di Patologia Molecolare and NanoBioimaging, Centro di Biotecnologie per la Ricerca Medica Applicata, Univ. del Piemonte Orientale "A. Avogadro", Dipartimento di Scienze Mediche, Novara, Italy

Mast cells contain a peculiar class of inflammatory granules that belong to the family of secretory lysosomes. Mastocytes possess two different subpopulation of inflammatory granules. Upon antigen-mediated crosslinking of IgE-membrane receptors these granules are exocytosed in a calcium-dependent fashion. It is not know whether the two subpopulations of mast cell granules follow identical or different pathways for calcium-regulated exocytosis. In this study, we employed two different types of fluorescent nanoparticles (NP) to trace the calcium-regulated exocytosis of inflammatory secretory granules in rat basophilic/mast cells (RBL). We used cyanine-doped silica mesoporous MCM-41 IRIS-3 NPs of 10 nm (produced by Cyanine Technologies, Turin – Italy; Gianotti et al., ACS Appl Mater Interfaces. 2009) and the FITC-conjugated polystyrene Latex beads of 30 nm (purchased from Sigma-Aldrich). MCM-41 IRIS-3 NPs were rapidly internalized by RBL and, at least temporarily, they co-localized with Lysotracker (which labels acidic compartments). Endosomes and lysosomes were traced by transgenic expression of Green Fluorescent Protein (GFP)-linked chimeric cathepsin D. At steady-state, MCM-41 IRIS-3 NPs did not co-localize with CD-GFP. Polystyrene NPs also were internalized, though less efficiently, by RBL cells. MCM-41 IRIS-3 NPs and Polystyrene NPs showed only temporarily co-localization. The intracellular compartments of final destination were clearly distinct for the two types of NPs. Granule exocytosis was stimulated by means of A23187, a calcium ionophore, and by specific IgE-receptor stimulation with appropriate antigen (DNP-BSA). Kinetic study of induced exocytosis revealed that Polystyrene NPs were promptly extruded by the cells, whereas MCM-41 IRIS-3 NPs were exocytosed at a much later time. This study demonstrate the usefulness of fluorescent NPs for monitoring the secretory compartments in living cells. Research supported by "Poli di Innovazione BANP", Regione Piemonte.

P3.13

Study of the role of EFA6A and EFA6As in the axon initial segment of rat hippocampal neurons

R. Guarascio, V. Matafora, A. Bachi, D.a Talarico
Division of Genetics and Cell Biology, San Raffaele Scientific Institute, Milan

The processes of neurite extension and remodeling require a close coordination between the cytoskeleton and the cell membranes. The small GTPase ARF6 plays a central role in regulating membrane traffic and actin dynamics, and its activity has been demonstrated to be involved in neurite elaboration.
EFA6A is a guanine nucleotide exchange factor (GEF) for ARF6. We have shown that two distinct isoforms of the EFA6A gene are expressed in murine neural tissue: a long isoform (EFA6A), and a short isoform (EFA6As). The two isoforms have distinct functional activities when overexpressed in primary cortical neurons: EFA6A promotes neurite extension, whereas EFA6As (that lacks the Sec7 domain, endowed with GEF activity on ARF6) induces dendrite branching. Our results suggest that EFA6A could play a role in neuronal morphogenesis through the regulated expression of two functionally distinct isoforms.
We have shown that both EFA6A and the EFA6As are phosphorylated in neuronal cells; we are currently analyzing the role of phosphorylation in regulating the biological activity of the EFA6A isoforms in primary neuronal cells.
Using an antibody that recognizes endogenous EFA6A and EFA6As, we have detected a distinct labeling in the Axon Initial Segment (AIS) of rat hippocampal neurons. The AIS is a specialized compartment that contains a high density of Na+ and K+ channels, and it is involved in the initiation of action potentials, and in the establishment and maintenance of neuronal polarity. Alterations of AIS properties contribute to nervous system diseases, as epilepsy.
We are currently analyzing rat hippocampal neurons infected at different stages of differentiation with recombinant lentiviruses encoding isoform-specific shRNAs, to evaluate the effect of downregulation of EFA6A/EFA6As on AIS formation/maintenance and on neuronal polarity.

P3.14

Both *in vitro* and *in vivo* evidences of CD63 tetraspanin impacts on malignant melanoma-associated phenotype: role of TIMP-1, uPAR and MMP

A. Lupia[1], I. Granucci[1], M. Lulli[1], F. Di Gesualdo[1], R. Loffredo[1], E. Witort[1], V. Carloni[2], L. Borgognoni[3], N. Pimpinelli[4], D. Massi[4], F. Bianchini[1], L. Calorini[1,4], S. Capaccioli[1]
[1]Dept of Experimental Pathology and Oncology, University of Florence (Florence)
[2]Dept of Internal Medicine, Univ. of Florence (Florence)
[3]Plastic Surgery Unit, Regional Melanoma Referral Center, Istituto Toscano Tumori (ITT), Santa Maria Annunziata Hospital, Florence
[4]Dept of Critical Care Medicine and Surgery, Univ. of Florence (Florence)

Premises. CD63 protein, belonging to the Tetraspanins superfamily, is highly expressed in several normal tissues as well as in the early stage of melanoma cell progression. We have previously observed in a high number of tissue samples of human melanocytic nevi, dysplastic nevi, thin melanomas, thick melanomas and metastasizing melanomas, a progressive decrease of CD63 levels with tumour progression and its disappearance in metastasis.
Working hypothesis. We have hypothesized that CD63 could play a functional role in controlling cell motility and invasion, probably by means of functional interactions with other proteins playing key role in invasiveness and metastasis. Based on data reported in the literature, the tissue inhibitor of metalloproteinase-1 (TIMP-1) and the pro-metastatic proteins uPAR and MMP seemed the best candidates.
Results. a) A375 human melanoma cells, endowed with high levels of CD63 tetraspanin, show a significant reduction in cell motility and invasiveness due to the abrogation of uPAR and MMP and promotion of TIMP expression, compared to control tumour cell line expressing CD63 tetraspanin levels.
b) In B16 murine melanoma cells stimulated by inflammatory cytokines, reduction of CD63 tetraspanin expression is associated with the promotion of a metastatic phenotype, characterized by a high capacity to colonize host lungs and expressing a high level of uPAR and MMP-9.
Conclusions. Altogether, our results suggest that CD63 tetraspanin, most probably interacting with TIMP-1, uPAR and MMP, is a suppressor of the melanoma metastatic potential.
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P3.15

Role of EGFR in regulation of solute carriers in cell homeostasis

W. Maruwge[1], S. Polo[1,2]
[1]Fondazione IFOM, Istituto FIRC di Oncologia Molecolare, Milano, Italia
[2]Dipartimento di Medicina, Chirurgia ed Odontoiatria, Univ. degli Studi di Milano, Milano, Italia

Recent results from our laboratory couple the regulation of solute carriers (SLCs) to EGF through ubiquitination, a post translational modification that is appearing to have a regulatory role for the internalization and/or degradation for these proteins1, 2. SLCs have a regulatory role as gatekeepers for cells and organelles, controlling the uptake and efflux of metabolites and maintaining cellular homeostasis3.In this study we aim at investigating how the EGF receptor (EGFR) can bring about the ubiquitination of SLCs and how this in turn is translated into functional cell context, that is, whether and how EGFR regulates the physiology (activity and transport system) of SLCs, and if SLCs are crucial for EGF mediated signaling. One of the identified solute carriers is SLC3A2, a cell surface, transmembrane protein that is implicated in integrin signaling and tumorigenesis, and is involved in transport of L-type amino acids, acting as bidirectional amino acid exchanger4-6. SLC3A2 is not constitutively associated with the plasma membrane but internalizes upon amino acid deprivation7. In our initial experiments we wanted to assess if this process is dependent on or regulated by EGF. Depriving cells of methionine before following the internalization of SLC3A2, we found that it does in fact internalize upon methionine deprivation, and that simultaneous stimulation with EGF leads to accelerated internalization and partial colocalization.

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P3.16

Molecular mechanisms of human diseases caused by serpin polymerisation

E. Miranda[1], V. Timpano[1], U. Ekeowa[3], J. Perez[2], D. A. Lomas[3]
[1]Dept Biologia e Biotecnologie 'Charles Darwin', University of Rome 'La Sapienza', Rome
[2]Dept Biologia Cellular, Genetica y Fisiologia, University of Malaga, Spain
[3]Dept of Medicine, Cambridge Institute for Medical Research, Cambridge, UK

Serpins are an ample family of evolutionary conserved proteins that inhibit serin proteases, mainly in the extracellular space. The inhibitory mechanism involves extensive conformational change in the serpin molecule, which requires high flexibility. This renders serpins prone to destabilisation of the tertiary structure as a consequence of point mutations. Several aminoacidic changes have been reported in different serpins in connection with human disease. In most cases, the mutant proteins form homopolymers that cannot exit the endoplasmic reticulum as efficiently as the wild type proteins. This causes cell toxicity and eventually disease, with a phenotype that depends on the specific serpin affected and the rate of polymerisation of the mutant protein. The exact mechanism of polymerisation and the pathways of cell toxicity are still under active research. We use mammalian cell models and monoclonal antibodies to better understand the mechanisms underlying alpha-1 antitrypsin deficiency, which is due to mutations on the prototypic serpin alpha-1 antitrypsin, and the dementia FENIB (familial encephalopathy with neuroserpin inclusion bodies), which is caused by point mutations in neuroserpin.

P3.17

Plasma membrane organization of GPI-anchored proteins in polarized epithelial cells

S. Paladino[1,2], S. Lebreton[3], S. Tivodar[1] F. Formiggini[2], G. Ossato[4], E. Gratton[4], M. Tramier[5], M. Coppey-Moisan[5], C. Zurzolo[1,3]
[1]Dipartimento di Biologia e Patologia Cellulare e Molecolare, Univ. Federico II, Napoli, Italy
[2]CEINGE Biotecnologie Avanzate, Napoli, Italy
[3]Unité de Trafic Membranaire et Pathogénèse, Institut Pasteur, Paris, France
[4]Laboratory for Fluorescence Dynamics, Univ. of California, Irvine, California
[5]Complexes macromoléculaires en cellules vivantes, Institut Jacques Monod, Univ. Paris-Diderot, France

Cholesterol has been shown to be an active player in the organization of GPI-anchored proteins (GPI-APs) in nanoclusters at the plasma membrane of fibroblast. On the other hand, in polarized epithelial cells GPI-APs travel to the apical surface in oligomeric complexes, which form in the Golgi apparatus and are essential for their apical sorting. Thus, in polarized epithelial cells GPI-AP clustered organization appears to be important both for their sorting in the Golgi and for their function at the plasma membrane. However, what is the driving force for this clustered organization and whether these structures represent similar entities is unclear. We have used different imaging and biochemical approaches to investigate the plasma membrane organization of two apical model GPI-APs, GFP-FR and PLAP. Differently from fibroblasts, in polarized MDCK cells each GPI-AP is organized in cholesterol-independent homo-clusters, which can in turn coalesce into cholesterol-dependent hetero-clusters. Importantly homo-clusters are required for subsequent hetero-cluster formation. Consistently in non-polarized MDCK cells, where GPI-APs do not form homo-clusters in the Golgi apparatus, they are unable to form hetero-clusters and remain in a monomeric and dimeric forms as they reach the plasma membrane. These data suggest a unique mechanism of molecular complex formation at the apical membrane of epithelial cells driven by the selective sorting of GPI-APs homo-clusters and show that the same players (e.g., cholesterol; protein-protein interaction) may have distinct roles in the supermolecular organization of GPI-APs in different cell types.

P3.18

CLN8 interactors: a possible function of the CLN8 protein in the lipid metabolism and autophagy

R. Passantino, C. Cascio, I. Deidda, G. Galizzi, D. Russo, P. Guarneri
CNR Institute of Biomedicine and Molecular Immunology, Neuroscience Unit, Palermo, Italy

The NCLs are a group of neurodegenerative lysosomal storage diseases mainly occurring in childhood. They are characterized by lipopigment inclusions and visual loss, motor and cognitive dysfunction and epilepsy. Diagnosis relays upon the identification of mutations of at least ten genes (CLN1-CLN10), leading to distinct forms. Pathogenic mechanisms are still unknown and no cure is available. We have recently showed that in the *mnd* (motor neuron disease) mouse, which is a model of the CLN8 form of the late-infantile NCL (CLN8-vLINCL), oxidative stress, lipid peroxidation, inflammation and ER stress occur in certain neuronal types and are likely associated to autophagy and mitochondrial dysfunction in the progression of the disease (Guarneri et al., 2004; Galizzi et al., 2011). The CLN8 gene encodes a transmembrane protein with unknown function that is located at ER and Golgi compartment. Due to the high homology with the TCL-domain (TRAM- LAG1- CLN8), the CLN8 protein is presumed to be involved in the lipid synthesis and transport (Winter and Ponting, 2002). Here, by screening a human adult brain cDNAs library using the yeast two-hybrid split ubiquitin system, we identified interesting binding partners of the CLN8, such as the VAP-A protein, which is involved in the lipid synthesis and transport, and three other proteins - GABARAPL2, BNIP3 and BNIP3L - that belong to the autophagic pathway. These interactions were confirmed by co-immunoprecipitation and co-localization studies using Cos-1 and Hela cells transiently transfected. Our findings highlight the potential function of the CLN8 in the lipid homeostasis and endosomal-lysosomal pathway and provide clues to understanding the pathomechanism(s) of the CLN8-vLINCL.

P3.19

Thapsigargin alters gene expression within the secretory pathway and influences the retention kinetics of the COPII vesicles at the endoplasmic reticulum exit sites

G. Amodio[1], O. Moltedo[1], S. Bonatti[2], P. Remondelli[1]
[1]Dept Pharmaceutical and Biomedical Sciences, Univ. of Salerno, Italy
[2]Dept Biochemistry and Medical Biotechnology, Univ. of Naples Federico II, Italy

Thapsigargin (TG) is an inhibitor of the endoplasmic reticulum (ER) calcium transporters, which generates Ca2+-store depletion within the ER and a simultaneous increase of Ca2+ level in the cytosol. This imbalance drive cells to cope with stressful conditions, which affect folding of newly synthesized proteins and induce the accumulation of unfolded polypeptides, via activation of the unfolded protein response (UPR) pathway. Several evidences suggest a link between the UPR and the vesicular trafficking within of the early secretory pathway. In mammalian cells the UPR controls the expression of cargo receptors involved in the vesicular transport between the ER and the Golgi complex, such as ERGIC-53, MCFD2 and VIP36. We also reported that the ER stress rapidly reduces the transport to the Golgi complex of reporter proteins and alters the morphology of post-ER compartments. Most recently, we analysed the proteome changes in human cells following acute treatment with TG revealing a peculiar pattern of proteins that have a function within the secretory pathways, depend on calcium for their functions or participate to the control of protein synthesis or to the regulation of cell survival. Interestingly, treatment with TG results also in a rapid reduction of the COPII intermediates formed at the ER exit sites (ERES), suggesting that the ER stress alters the ER export by targeting the ERES. By analysing the contribution of TG on the control of the COPII formation, we found that thapsigargin, similarly to other UPR inducers, such as DTT and MG132, alters the distribution of the ERES and the assembly of the COPII complex at the ER membranes. More interestingly, the amount of COPII retained at the ER membranes in MEF IRE1/knock-out cells is markedly reduced compared to MEF/wild-type cells, suggesting that the UPR, through the IRE1 pathway, might modulate the formation of COPII-coated transport intermediates.

P3.20

Mechanism of transport through the Golgi complex

R. Rizzo[1], S. Parashuraman[1], C. Puri[1], A. Egorova[1], A. Luini[1,2]
[1]Telethon Institute of Genetics and Medicine, Napoli, Italy
[2]Institute of Protein Biochemistry, CNR, Naples, Italy

Background
The Golgi apparatus arranged as a stack of cisternae is a central sorting station in the secretory pathway. A main question in the transport field is how proteins are transported through the Golgi: whether anterograde transport of cargo mediated by carriers or by maturation of the entire cisternae? To address this, we are studying the dynamics of the Golgi residents to determine how these are coupled to the dynamics of the cargoes that are transported through the Golgi.
Observations
First, to investigate the dynamics of the Golgi enzymes, we have engineered a Golgi-resident glycosylation enzyme that can be polymerised in a regulated way, to obtain a polymer that is large enough not to enter retrograde carriers. We are also studying if and how this polymerization affects the localisation of the Golgi-resident proteins and the functioning of the Golgi apparatus. First, we find that the polymerisation of a cis Golgi resident enzymes (ManI-FM) induces its shift to the trans-Golgi, a displacement that is not apparently mediated by vesicles or tubules. Second, the depolymerisation of the enzyme, once this has reached the trans side of the Golgi, induces its recycling back to its steady state position (cis/medial) in a few minutes.
Conclusions
The polymerised ManI traverses the Golgi without entering the carriers. The depolymerisation of the enzyme at the trans level induces its recycling to the steady state position. Our results strongly fit with cisternal maturation model.

P3.21

Activation of mitochondrial biogenesis as a strategy to improve the energetic function of Leber's hereditary optic neuropathy cybrids

L. Iommarini[1], V. Carelli[2], C.T. Moraes[3], M. Rugolo[1]
[1]Dipart. di Biologia Evol. Sperimentale and [2]Dipart. di Scienze Neurologiche, Univ. di Bologna, Bologna, Italy
[3]Dept of Neurology and Cell Biology and Anatomy, Univ. of Miami, Miami, USA

Leber's Hereditary Optic Neuropathy (LHON) is characterized by rapid loss of central vision and optic atrophy, due to selective degeneration of retinal ganglion cells. This disease is caused by mutations in the mitochondrial DNA (mtDNA) genes encoding respiratory complex I subunits, the most common being 11778/ND4, 14484/ND6 and 3460/ND1. As reported for other mitochondrial diseases, the energetic dysfunction caused by LHON mutations stimulates a compensatory mitochondrial proliferation, as indicated by higher levels of mtDNA in LHON patients compared to controls. This increase resulted more consistent in carriers (individuals harboring LHON mutations but not clinically affected), suggesting that subjects able to induce a more efficient mitochondrial biogenesis were less prone to develop the disease.
Mitochondrial biogenesis is a complex process, finely tuned by specific transcription factors and coactivators, involving the coordinated expression of mitochondrial and nuclear genes. Recently, it has been shown that treatment with bezafibrate (BF) can improve mitochondrial function in a myopathy mouse model and in cells with moderate respiratory chain defects, via activation of mitochondrial biogenesis.
In this preliminary study we tested the effect of two PPARs agonists, bezafibrate (BF) and rosiglitazone (RGZ), on LHON cybrids, attempting to stimulate mitochondrial biogenesis and rescue their defective phenotype. Treatment of control and LHON cybrids with both drugs markedly increased mtDNA copy number and mitochondrial proteins content. Interestingly, both BF and RGZ significantly improved the viability of 11778/ND4 cybrids incubated in galactose medium. Accordingly, PPARs agonists may represent novel potential treatments in clinical trials enrolling LHON patients. A detailed dissection of the mitochondrial biogenesis pathways at work in unaffected LHON carriers compared to the affected individuals may further shed light on other therapeutic strategies.

P3.22

Rac1 controls cell polarity in FRT thyroid epithelial cells

M. Santoriello[1], M. Nitti[1], A.Corteggio[1], A. Mascia[2], G. Calì [2], S. Paladino[1], L. Nitsch[1], C. Garbi [1]

Polarization of epithelial cells is a complex process directed by external stimuli (such as cell-cell and cell-extracellular matrix adhesion) and involves the coordinated action of several proteins able to decrypt and translate these external signals. Our goal is to unravel how signaling transduced via Rac1 plays a role in the acquisition and maintenance of the polarized phenotype in the FRT thyroid epithelial cells. To this aim, we studied the effects on cell polarization of a specific inhibitor of Rac1, NSC23766, and of an inducible, dominant-negative form of Rac1, ER-RacN17, which was stably expressed in FRT cells.
Directional migration, transepithelial resistance acquisition by confluent monolayers grown on filters, cell aggregation and formation of polarized follicles in suspension culture were investigated and found to be affected both by the pharmacological inhibition of Rac1 activity and by the expression of dominant negative form of Rac1. Cells expressing Rac1-GFP showed that GFP fluorescence overlapped that of E-cadherin suggesting that Rac1 concentrated at the plasma membrane in association with the adherens junctions. Strikingly, we found a progressive loss of E-cadherin from the plasma membrane upon inhibition of Rac1 activity. Furthermore the loss of plasma membrane E-cadherin parallelled that of plasma membrane-associated Rac1 endogenous molecules. All these data indicate that Rac1 plays a crucial role in the control of cell polarization and that this effect can be exerted at least in part by the control of adherens junctions stability. We are currently investigating the molecular mechanisms by which these processes occur and we are testing the hypothesis that Rac1 might modulate the endocytic turnover of E-cadherin.

P3.23

The kinetics and trajectories of chromatin-related proteins

L. Stixová, E. Bártová, P. Matula, S. Kozubek
Institute of Biophysics, Academy of Sciences of the Czech Republic, v.v.i., Brno, Czech Republic

Protein exchange kinetics correlate with the level of chromatin condensation and, in many cases, with the level of transcription. We used fluorescence recovery after photobleaching to analyze the kinetics of heterochromatin-specific heterochromatin protein 1β (HP1β) and nucleolus-related proteins, upstream binding factor (UBF) and RNA polymerase I large subunit (RPA194). The relationship between protein nuclear arrangement and recovery kinetics was determined. Cellular trajectories of HP1β foci were analyzed by real-time optical monitoring. We considered whether the trajectories and kinetics of particular proteins change in response to histone hyperacetylation by histone deacetylase inhibitors or after suppression of transcription by actinomycin D.
In this study, we show that protein dynamics are influenced by many factors and events, including nuclear pattern and transcription activity. A slower recovery after photobleaching was found when proteins, such as HP1β were accumulated at specific foci. In identical cells, proteins that were evenly dispersed throughout the nucleoplasm recovered more rapidly. Distinct recovery kinetics for HP1β, UBF and RPA194 were observed after hyperacetylation or suppression of transcription. We observed that HP1β is located in both the chromocenters and fibrillarin-positive nucleoli interiors. The relationship between trajectory of HP1β in chromocenters and transcription level was not confirmed. Moreover, heterogeneity of foci movement was especially observed when we made distinctions between centrally and peripherally positioned foci.
Based on our results, we propose that protein kinetics are likely influenced by several factors, including chromatin condensation, differentiation, local protein density, protein binding efficiency, and nuclear pattern. These factors and events likely cooperate to dictate the mobility of particular proteins.
This work was supported by: LC535, LC06027, ME 919, AVOZ50040702, AVOZ50040507, LD11020

P3.24

Organelle-autonomous regulation of size and number: OA1 receptor sustains *Pmel17* expression

P. Falletta[1], M. Bono[1], P. Bagnato1, M. Monticone[2], D.C. Bennett[3], M.V. Schiaffino[4], C.R. Goding[5], C. Tacchetti[1,4], C. Valetti[1]
[1]MicroSCoBiO Research Center and IFOM Center of Cell Oncology and Ultrastructure, Dept of Experimental Medicine, Human Anatomy Section, Univ. of Genoa, Genoa, Italy
[2]Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy
[3]Division of Basic Medical Sciences, St. George's, Univ. of London, Cranmer Terrace, London, UK
[4]San Raffaele Scientific Institute, Milan, Italy
[5]Ludwig Institute for Cancer Research, Nuffield Dept of Clinical Medicine, Univ. of Oxford, Headington, Oxford, UK

One of the main themes of cell biology is the regulation of intracellular organelles number and the correct size preservation. Melanosome biogenesis is a good example of organelle maturation, and the Ocular Albinism type I, that displays a specific morphological phenotype characterized by enlarged melanosomes in the Retinal Pigment Epithelium and in the skin melanocytes, is an useful model to clarify some of these aspects. In this disease, the macromelanosome represents the abnormal growth of a single organelle. Our studies provide a potential molecular mechanism about the role of OA1 protein concerning the control of melanosome size and number.
OA1 influences *Mitf* transcription amount. In *Oa1* knockout melanocytes, as well as in silenced cells, the considerable decrease of *Mitf* does not determine a pigmentation gross alteration, but it provokes an abnormal expression of PMEL17, a key protein of the melanosome biogenesis. With these results, we found evidences that the control of melanin synthesis and of the rate of new melanosome biogenesis rely on different independent control factors.

P3.25

Tetanus and botulinum toxins need double anchorage to the membrane and intact disulfide bond for low pH induced entry into neurons

M. Pirazzini[1], O. Rossetto[1], P. Bolognese[1], M. Vono[1], S. Ashraf[1], C.C. Shone[2], C. Montecucco[1]
[1]Dept Biomedical Sciences and CNR Neuroscience Institute, Univ. of Padua, Padua, Italy
[2]Health Protection Agency, Porton Down, Salisbury, Wiltshire, UK

Tetanus and botulinum neurotoxins are di-chain proteins that cause paralysis by inhibiting neuroexocytosis. These neurotoxins enter into nerve terminals via endocytosis inside synaptic vesicles, whose acidic pH induces a structural change of the neurotoxin molecule that becomes capable of translocating its L chain into the cytosol, via a transmembrane protein-conducting channel made by the H chain. This is the least understood step of the intoxication process primarily because it takes place inside vesicles within the cytosol. We describe how this passage was made accessible to investigation by making it to occur at the neurons surface. The neurotoxin, bound to the plasma membrane in the cold, was exposed to a warm low pH extracellular medium and the entry of the L chain was monitored by measuring its metalloprotease activity with a ratiometric method. We found that the neurotoxin has to be bound to the membrane via at least two anchorage sites for a productive low-pH induced structural change to take place. Moreover, this process can only occur if the single inter-chain disulfide bond is intact. We determinate that the conformational change pH range of neurotoxin B, C and D is similar (4.5-6). Furthermore, using PROPKA3.0 software we found that tetanus and botulinum neurotoxins share a pool of conserved acid residues, that are predicted to protonate in the pH range 4.5-6. These residues could be involved in the initial steps of the pH dependent conformational change. We propose a stepwise sequence of events that lead from toxin binding to membrane insertion.

POSTER SESSION B

(presenting authors are shown underlined)

Topic 2
Stem Cells, Development and Regenerative Medicine

Topic 4
Cell Stress: Survival and Apoptosis

P2.1

Early changes of dendritic cells in human skin wounds

S. Bacci, B. De Fraia, A. Bonelli, P. Romagnoli
Dipartimento di Anatomia Umana, Istologia e Medicina Legale, Univ. of Florence, Italy

Dendritic cells (DC) primarily control immune responses and can interact to this aim with mast cells (MC) [Dudeck et al. Eur. J. Immunol. 2011; doi: 10.1002/eji.201040994.1], besides lymphocyes and other cell types; MC in turn respond to skin wounding in vivo since early after wounding [Bacci et al. J. Forensic. Sci. 2011 (in press)]. The response of DC early after wounding and their relationships to MC in these conditions have not yet been investigated. To address this issue, cryosections of skin, wounded since 0-24 h and taken at autopsy, were stained with fluorescent avidin (MC), UEA-1 (Ulex europaeus-1 lectin: endothelium), or antibodies for MHC-II (DC), CD1a (Langerhans cells) and PDGF (endothelium). Photomicrographs were subjected to computerized image analysis. As compared with intact skin, Langerhans cells increased significantly in number 5 min after wounding then slowly decreased. The labeling intensity and relative volume of MHC-II+ cells in the dermis were significantly increased since 5 min after wounding, for several hours. These cells, and MC, became part of a perivascular mononuclear cell infiltrate in the subpapillary dermis. Upon wounding the number of capillaries increased markedly. The findings and their time course suggest that presumably immature DC come to express CD1a quickly upon injury, while DC recruitment and redistribution in the dermis occurs quickly upon injury and is coordinated with modifications of MC and capillaries. Therefore DC, together with MC, may be candidate to regulate early injury response in human epidermis and dermis.

P2.2

Chromatin structure and epigenetics of embryonic stem cells

E. Bártová, G. Šustá?ková, S. Legartová, L. Stixová, S. Kozubek
Institute of Biophysics, Academy of Sciences of the Czech Republic, Brno, Czech Republic

Human embryonic stem cells (hESCs) are characterized by their capacity for sustained self-renewal and their have ability to differentiate into the specific cell types of all three germ layers. These characteristics make hESCs promising therapeutic tools in regenerative medicine. Here, we have studied chromatin structure, nuclear radial arrangement of selected genetic elements and histone epigenetic marks in human embryonic stem cells (hESCs) before and after retinoic acid-induced differentiation. The genome and epigenome of hESCs underwent differentiation-specific structural changes, characterized by the redistribution of centromeric heterochromatin, as evidenced by a perinuclear and perinucleolar accumulation of the centromeric markers CENP-A and H3K9 trimethylation. Pluripotency marker Oct3/4 gene was located on greatly extended chromatin loops in un-differentiated hESCs, however, nuclear radial distribution of Oct3/4 was similar in both pluripotent and differentiated cells.
ChIP-on-chip analysis revealed that hESC differentiation resulted in a genome-wide decrease in promoter H3K9 acetylation. Among the 24,659 promoters analyzed, only 117 were likely involved in pluripotency, while 25 significantly acetylated promoters were likely responsible for endoderm-like differentiation. Our analyses showed that especially chromosomes 11, 12, 17, and 19 are more prone to differentiation-specific changes in hESCs. We additionally suggest that a global decrease in H3K9 acetylation is an important epigenetic mark distinguishing pluripotent and more differentiated stages of hESCs.
This work was supported by the following grants: AVOZ50040507, AVOZ50040702, LC535, and LC06027, ME 919 and COST-CZ project LD11020.

P2.3

Zinc finger protein 521 (ZNF521): critical regulatory role in medulloblastoma initiating cells

R. Spina[1], G. Filocamo[2], L. Barbarossa[1], S. Scicchitano[1], F. Bernaudo[1], M. Lupia[1], E. Chiarella[1], T. Mega[1], D. Pelaggi[1], M. Mesuraca[1], C. Steinkülher[2], H.M. Bond[1], G. Morrone[1]
[1]Laboratory of Experimental Haematopoiesis and Stem Cell Biology, Univ. Magna Graecia, Catanzaro, Italy
[2]Istituto Superiore di Sanità, Rome, Italy

Zinc finger protein 521 (ZNF521), is a transcription co-factor containing 30 zinc fingers and an N-terminal motif that binds to members of the NuRD complex, initially identified for its striking differential expression in human CD34+ cells compared to mature leukocytes. In addition to haematopoietic stem cells, this gene is highly expressed in brain. Very high ZNF521 mRNA levels are observed during development in cerebellar granule neuron precursors, regarded as the cells-of-origin of medulloblastoma (MB), as well as in a subset of MBs with high tendency to metastasize.
We analysed its expression in cells from MBs spontaneously occurring in mice with heterozygous mutations of the inhibitory Sonic Hedgehog receptor, Ptc1. Transduction of these cells with two shRNAs for Zfp521 resulted in a strong decrease of growth and tumourigenicity. Enforced expression of ZNF521 in the human MB cell line, DAOY, that displays a moderate expression of this gene, leads to an increase in cell growth both in high and low adherence conditions, as well as sphere formation and clonogenicity in limiting diluition assays and enhanced wound healing capacity. Xenotransplant experiments in nude mice demonstrated a much higher frequency of tumourigenic cells in the ZNF521-transduced DAOY cells but not in those transduced with a deletion mutant lacking of NuRD-binding sequence. Thus, enforced expression of ZNF521 in DAOY cells appears to induce the expansion of a sub-population with stem-like features in a process that depends on the integrity of the NuRD-binding motif.
In conclusion, our data indicate that ZNF521 is likely to play a relevant role in the control of the immature cell compartment in medulloblastoma, and that its ability to recruit members of the NuRD complex is essential for its action. The identification of additional molecular interactors and target genes of ZNF521 will help to define its role in the regulation of normal and neoplastic cerebellar progenitors.

P2.4

Rosiglitazone stimulates dendritic cells generated from human CD133 positive precursors to acquire a highly efficient immunostimulatory phenotype

M.I. Bonetti[1], M. Santosuosso[2], B. Mazzanti[2], A. Aldinucci[3], C. Ballerini[3], R. Saccardi[2], A. Bosi[2,4], P. Romagnoli[1]
[1]Dept Anatomy, Histology and Forensic Medicine, Univ. Florence, Italy
[2]Hematology Unit, Careggi University Hospital, Florence, Italy
[3]Dept Neurological Sciences, Univ. Florence, Italy
[4]Dept Critical Care Medicine and Surgery, Univ. Florence, Italy

AIMS: Dendritic cells (DC) can differentiate in vitro from CD133+ cells of the human cord blood [1]. DC participate to the injury response in the arterial wall [2] and treatment with a PPAR-gamma stimulant (rosiglitazone) hampers this response in the rat, also affecting DC number and DC-lymphocyte interactions [3]. We have addressed here the effect of rosiglitazone on the differentiation of DC, starting from cord blood CD133+ precursors.
MATERIAL AND METHODS: CD133+ cells purified by immunomagnetic separation were cultured for 18 days with fetal calf serum, cytokines [2], and with or without rosiglitazone, 1 μmol/ml. They were analyzed by flow cytometry, immunofluorescence, electron microscopy and mixed lymphocyte reaction.
RESULTS: Culture led to the appearance of DC expressing MHC-II antigens and the differentiation markers CD80, CD83 and CD86; many expressed CD207/langerin and some also CD1a; the differentiated cells were potent stimulators of mixed lymphocyte reactions. With rosiglitazone fewer DC were generated in culture, but they expressed differentiation and maturation-related antigens in higher percentages and were better stimulators of CD4 positive cells than those generated without the PPAR-gamma agonist.
CONCLUSION: In apparent contradiction with the results in vivo in the rat, the direct effect of rosiglitazone in vitro is to promote the maturation of human cord blood derived cells into DC with a highly efficient immunostimulatory phenotype, although negatively affecting the number of the harvested cells.REFERENCES: [1] Bonetti et al., Blood 117: 3983, 2011. [2] Pieri et al., Histol. Histopathol. 23: 19, 2008. [3] Rinaldi et al., Shock 32: 638, 2009.

P2.5

[WITHDRAWN]

P2.6

Turning stem cells into retina: possible strategies for the cure of retinal degenerations

A. Messina[1], L. Lei[2], T. Incitti[1], R. Vignali[2], F. Cremisi[3], S. Casarosa[1]
[1]CIBIO, Univ. of Trento, Italy
[2]Dept of Biology, Univ. of Pisa, Italy
[3]Scuola Normale Superiore, Pisa, Italy

The main aim of regenerative medicine is to replace degenerating cells, and its success depends on the capability of the donor cells to correctly replace all the missing cell types. We have recently shown that in *Xenopus* embryos, proper doses of a single secreted molecule, Noggin, can drive animal cap embryonic stem (ACES) cells toward retinal cell differentiation without additional cues. This encourages further studies on the role of Noggin in the retinal differentiation of mammalian stem cells. Our aim is thus to gain further knowledge into the developmental mechanisms and cues necessary for the differentiation of stem cells into retinal neurons. We propose to initially address this issue in *Xenopus*, and to subsequently transfer the acquired knowledge to the differentiation of mammalian stem cells. The elucidation of the pathway through which Noggin elicits retinal fates in ACES cells will be a first step toward the setup of ameliorated protocols for the differentiation of stem cells toward retinal neurons.

P2.7

Unraveling the role of the G-protein coupled receptor APJ in cardiac lineage specification and differentiation

C. D'Aniello[1], S. Iaconis[1], G. Andolfi[1], G. Cobellis[2], G. Minchiotti[1]
[1]Institute of Genetics and Biophysics "A. Buzzati-Traverso", CNR, Naples, Italy
[2]Dept of General Pathology, Second Univ. of Naples, Naples, Italy

Mammalian cardiogenesis starts early during embryogenesis and requires different extracellular signals that are regulated precisely in time and space, intersecting with intracellular genetic programs that confer the ability of the cells to respond. Unmasking the essential signals for cardiac lineage specification has absolute importance for development and regenerative medicine, including the differentiation of progenitors/stem cells to a cardiac fate. We have recently identified the G-protein-coupled receptor APJ and its ligand Apelin as novel regulators of cardiac differentiation in embryonic stem cells (ESCs). To get mechanistic insight into the role of APJ in the early events of cardiomyogenesis, we used both loss and gain of function approaches. Loss of function experiments revealed a central role for APJ in the gene regulatory cascade promoting mesoderm patterning and cardiac specification and differentiation in mESCs. Notably, while expression of the early mesoderm marker Brachyury and the pre-cardiac marker Mesp1 was delayed in the absence of APJ, expression of terminal cardiac differentiation markers was dramatically reduced or absent. FACS analysis suggests that proliferating progenitor cells failed to exit the cell cycle and eventually to differentiate into cardiomyocytes. Accordingly, expression of the cyclin-dependent kinase inhibitors p57Kip2 and p21Kip2 was dramatically reduced. On the contrary, a time-controlled APJ overexpression, promoted cardiovascular differentiation and increased the number of ESC –derived cardiomyocytes by 3-fold. All together our data point to a central role for APJ signaling in controlling the balance between proliferation and differentiation of mesoderm progenitors and open the way to unravel the molecular codes essential for mesoderm patterning towards the cardiac lineage.

P2.8

Fibroin and poly-lactic acid scaffolds may be used in cardiac tissue engineering to drive the differentiation of cardiac progenitor cells: in vitro and in vivo studies

V. Di Felice[1], C. Serradifalco[1], L. Rizzuto[1], A. De Luca[1], A. Marino Gammazza[1], P. Di Marco[2], G. Cassata[2], R. Puleio[2], L. Verin[3], A. Motta[3], A. Guercio[2], G. Zummo[1]
[1]BioNeC Dept, Univ. of Palermo, Palermo, Italy
[2]Istituto Zooprofilattico Sperimentale della Sicilia, Palermo, Italy
[3]BIOtech Laboratories, Univ. of Trento, Trento, Italy

Introduction: Some of the main problems in translating preclinical studies based on cell-therapy for cardiac tissue engineering are to find the best stem cell population for transplantation, the best delivery route and the best time of cell injection into the myocardium (1).
Hypothesis: We wanted to understand if cardiac progenitor cells (CPCs) may improve their degree of differentiation into oriented compared to random porosity scaffolds both in vitro and in vivo.
Methods: We designed porous and partially oriented PLLA and fibroin scaffolds to deliver CPCs in an infarcted area of the heart, we isolated and characterized c-Kit+ positive CPCs and we tested the host reaction to scaffolds, CPCs, and CPCs cultured inside the scaffolds.
Scaffolds with different porosities, pore sizes, and properties were made by freeze-drying and salt-leaching. Silk fibroin nets were made by electrospinning. To test the foreign body reaction to scaffolds or CPCs, samples were implanted into the subcutaneous dorsal region of athymic Nude-Foxn1nu mice.
Results: CPCs isolated as previously described (2) were tested for the expression of c-Kit, MDR-1 and Sca-1 by flow cytometry. These cells were able to differentiate towards the cardiac phenotype in vitro. The degree of differentiation, the expression of extracellular matrix and integrin proteins, and the expression of several proteins of the sarcomere were dependent on the type of scaffold and the polymer used. In vivo, all the used scaffolds induced a foreign body reaction, apart from fibroin nets. Cardiac stem cells alone implanted in nude mice were also degraded by a T cell-mediated immune response.
Conclusions: Scaffolds are useful devices to deliver CPCs in the site of implantation, but more research is needed to study the host response to this population of cells and to find non-reactive biomaterials.
1: Di Felice V, et al. Anat Rec (Hoboken). 2009 Mar;292(3):449-54.
2: Di Felice V, et al. J Cell Physiol. 2009 Dec;221(3):729-39.

P2.9

S1P induces differentiation of human mesoangioblasts towards smooth muscle cells. A role in vascular regeneration

C. Donati[1,2], F. Cencetti[1,2], G. Marseglia[3], A. Magi[3], S. Serrati[4], G. Fibbi[4], C. Bernacchioni[1,2], S. Brunelli[5,6], M. Del Rosso[4], F.Torricelli[3], G.Cossu[5,7], P. Bruni[1,2]
[1]Dipartimento di Scienze Biochimiche, Univ. di Firenze, Firenze, Italy
[2]Istituto Interuniversitario di Miologia (IIM),[3]SOD di Diagnostica Genetica, Azienda Ospedaliero Universitaria, Firenze, Italy
[4]Dipartimento di Oncologia e Patologia Sperimentali, Univ. di Firenze, Firenze, Italy
[5]Divisione di Medicina Rigenerativa, Istituto Scientifico H San Raffaele, Milano, Italy
[6]Dipartimento di Medicina Sperimentale, Univ. di Milano-Bicocca, Monza, Italy
[7]Dipartimento di Biologia, Univ. di Milano, Milano, Italy

Smooth muscle cells (SMCs) control fundamental functions such as arterial tone and airway resistance. Circulating SMC progenitor cells can contribute to repair following vascular injury. Mesoangioblasts (Mb) are a new type of progenitor mesenchymal cells, capable of differentiating into many mesoderm cell types. The sphingolipid sphingosine 1-phosphate (S1P) regulates fundamental biological processes. We previously demonstrated that S1P acts as mitogen and anti-apoptotic agent in murine and human Mb. A microarray study performed to establish transcriptional profiles of human Mb treated with 1 μM S1P highlighted GATA6 as target gene regulated by this sphingolipid. Quantitative mRNA and protein analysis corroborated the microarray results demonstrating enhanced expression of the transcription factor GATA6 together with its co-regulator, LMCD1, and smooth muscle marker proteins. Importantly, GATA6 up-regulation induced by S1P was responsible for the enhanced expression of SM-specific contractile proteins. Moreover, by specific gene silencing experiments, GATA6 appeared to be critical for the pro-differentiating activity of the cytokine TGFβ. Inhibition of endogenous S1P formation in response to TGFβ abrogated GATA6 up-regulation, supporting the view that the S1P pathway plays a role in mediating the pro-myogenic effect of TGFβ. Co-cultures of human Mb with endothelial cells differentiated into "neovascular-like" networks, more resistant over time compared to the capillary structures formed by the endothelial cells alone. Interestingly, conditioned medium of endothelial cells significantly stimulated Mb migration at the same extent of S1P alone. When endothelial cells were pre-treated with the SphK inhibitor SKI-2, the chemo-attractant properties of the conditioned medium was significantly reduced. This study individuates novel players in the transcriptional regulation of Mb differentiation into SM cells and highlights a role for S1P to favour vascular regeneration.

P2.10

Tbx1 mutation causes neurodevelopmental abnormalities that may underly sensorimotor gating impairment in a mouse model of 22q11.2 Deletion Syndrome

G. Flore[1,2,] M. Bilio[1,2] ,S. Sannino[2], F. Russo[2], E. De Leonibus[2] ,E. Illingworth[2,3]
[1]Telethon Institute of Genetics and Medicine (TIGEM), Naples, Italy
[2]CNR Institute of Genetics and Biophysics "Adriano Buzzati Traverso", Naples, Italy
[3] Universita' degli Studi di Salerno, Fisciano, Italy

TBX1 belongs to the T-BOX family of DNA-binding transcription factors. TBX1 is the major gene for 22q11.2 deletion syndrome, also known as DiGeorge syndrome, but it's contribution to the behavioral disorders and psychiatric diseases that characterize this syndrome is unknown. In humans and mice, mutation of TBX1 is associated with behavioural deficits (reduced pre-pulse inhibition, PPI) that are indicative of impaired sensorimotor gating. We are attempting to identify the molecular and anatomic basis of this phenotype in mice. To date, in mice, Tbx1 expression has been revealed solely in brain endothelial cells, thus our study posits that Tbx1 contributes to sensorimotor impairment by exerting a non-cell autonomous effect on neurons during development. To begin to characterize the neurodevelopmental defects underlying reduced PPI in Tbx1 mutant mice we have evaluated a variety of neuronal markers in regions of the brain that are associated with pathways involved in PPI. We have found that Tbx-/- embryos have an abnormal and excessive accumulation of dopaminergic neurons, which is the main neuronal population involved in PPI, and an expansion of nearby axonal tracts. Moreover, pharmacological treatment with a D2-receptor antagonist had a blunted effect in Tbx1+/- mutants compared with controls, suggesting dopaminergic hyperactivity. Furthermore, abnormalities in neural proliferation and differentiation timing were revealed in Tbx-/- mice, which resulted in an increased neuronal output. Finally, we have found that brain expression of several genes in the Delta-Notch pathway is altered in Tbx-/- embryos. The Notch-Delta signaling pathway is required in neuronal precursors to establish a proper balance between cell proliferation and differentiation, thus abnormal Notch-Delta signaling might account for the excess of mature neurons revealed in these mutants.

P2.11

Hepatocyte growth factor modulates blood-testis barrier in adult rats

A. Catizone[1], G. Ricci[2], M. Caruso[1], F. Ferranti[1], R. Canipari[1], M. Galdieri[2]
[1]Dept of Anatomy, Histology, Forensic Medicine and Orthopedics, Section of Histology and Embriology, School of Pharmacy and Medicine, "Sapienza" Univ. of Rome, Rome, Italy
[2]Dept of Experimental Medicine, School of Medicine, Second Univ. of Naples, Naples, Italy and National Institute of Biostructures and Biosystems Interuniversitary Consortium–Unit of Sections of Naples

In adult mammalian testes Sertoli cells form junctional complexes with the adjacent Sertoli cells consisting of tight junctions and anchoring junctions (AJs), including a testis-specific cell-cell actin-based AJ type, which participate to the "blood–testis" barrier (BTB) formation. The BTB separates mitotic spermatogonia from meiotic germ cells and, in stages VII-VIII, the barrier is disassembled to allow the passage of preleptotene spermatocytes across the barrier. We have studied the effects of HGF on the regulation of the BTB in the adult rats. We demonstrate that HGF is maximally expressed in stages VII-VIII of the seminiferous epithelium cycle whereas its levels fall in the subsequent stages IX-VI. In stages II-VI a low but clearly detectable amount of HGF was present with spots of positivity mainly localized in the periphery of the tubular wall. We also demonstrate that in stages VII-VIII of the epithelium wave HGF modulates the tight junctions decreasing the levels of occludin, influences its distribution pattern and assembling. Moreover, HGF decreases the amount of the actin present in the tight junctions at the level of the maximal intensity of occludin, influences actin position and modifies the morphology of the actin cytoskeleton network. In conclusion we report that in the adult rat testis in stages VII-VIII of the seminiferous epithelium cycle HGF is highly secreted and strongly involved in the regulation of BTB when physiological disassembling of the tight junctions occurs.

P2.12

The role of ArhGAP15, a negative regulator of the small-GTPase Rac1, in neuronal migration and neuritogenesis during cortical development

D. Garzotto, C. Costa, G. Germena, G. Sarò, E. Hirsch, G.R. Merlo
Molecular Biotechnology Center, Univ. of Torino, Torino

Normal development of the central nervous system depends on the correct migration, neuritogenesis and synaptogenesis of neuronal progenitors. A large number of extracellular signals and intracellular regulators have being discovered that impact these processes at various levels. The Rho-type GTPases signalling enzymes are among the best known players, as they directly modulate cytoskeleton dynamics and organization in response to signals. Their enzymatic activity is finely regulated by GAP (inactivating) and GEF (activating) proteins, whose effect on cell motility is beginning to be characterised. ArhGAP15 is a Rac1-specific GAP expressed in olfactory, hippocampal and cortical neurons of the adult animal. During development and at early postnatal ages its spatio-temporal distribution overlaps with migratory routes of cortical interneurons and with differentiating neurons. To explore the function of ArhGAP15 on brain development we have generated ArhGAP15 knock-out mice. These animals are viable and fertile and show an apparently normal brain gross morphology. However, at embryonic day E15, immature interneurons show altered distribution on cortical tangential migratory pathway and show misoriented leading processes, suggesting possible defects on directional migration. These defects are confirmed in the cortex of newborn animals: immature GABAergic interneurons have an altered distribution and an increasing propensity to remain in tangential versus radial migration. Finally, primary neuronal cultures obtained from the KO cortex show a significant reduction in neurite elongation and branching. These data point to ArhGAP15 as an interesting new player in the modulation of cell motility, specifically during neuronal migration and maturation.

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P2.13

Exploiting dystrophin gene-dosage compensation by multiple HAC transfer into mesoangioblast stem cells

M.F.M. Gerli[1,*], H. Hoshiya[1,*], R. Tonlorenzi1, E. Ueno2, D. Covarello1, M. Oshimura2, G. Cossu1, F.S. Tedesco[1]
[1]Division of Regenerative Medicine, Stem Cells and Gene Therapy, San Raffaele, Scientific Institute, Milan, Italy
[2]Dept of Biomedical Science, Institute of Regenerative Medicine and Biofunction, Tottori Univ., Yonago, Japan
*These authors contributed equally to this work

Human artificial chromosomes (HACs) are episomal vectors able to carry large genomic sequences, such as entire loci (Ren X et al., 2006). Recently, the entire human dystrophin locus (whose mutations cause Duchenne muscular dystrophy) has been inserted into a HAC (DYS-HAC) and then successfully transferred to different target cells (Hoshiya H et al., 2009; Kazuki Y et al., 2010). Moreover, we have recently transferred the DYS-HAC into mesoangioblast vessel-associated stem cells (MABs; Minasi M et al., 2002) derived from the dystrophic mdx mouse (mdx(DYS-HAC)MABs): upon transplantation into scid/mdx mice, mdx(DYS-HAC)MABs gave rise to large clusters of dystrophin-positive myofibers, which significantly ameliorated morphology and function of dystrophic muscles (Tedesco FS et al., under revision). The precise dosage regulation of the native dystrophin has been poorly studied for gene/cell therapy purposes, mainly because of the very large size of the dystrophin gene (2.4Mb) and cDNA (14Kb), which cannot be accommodated into most of viral vectors. In this work we investigate the possibility of a dosage compensation effect of dystrophin gene, by means of multiple DYS-HAC transfer in mdxMABs. To address this issue we performed serial DYS-HAC transfers to obtain mdx(DYS-HAC)MABs containing different number of dystrophin genes (mdx(multiDYS-HAC)MABs). Different clones were then characterized by qPCR and FISH; furthermore, in vitro and in vivo differentiation assays are ongoing. Mdx(multiDYS-HAC)MABs can be an instrumental tool to unravel the mechanisms regulating dystrophin expression levels in skeletal muscle fibers and to determine if a cell containing multiple natively-regulated dystrophin genes can better compensate the genetic deficiency to optimize gene and cell therapies for Duchenne muscular dystrophy.

P2.14

Changes in DNA methylation patterns of the imprinted locus IGF2/H19 in monozygotic and dyzigotic twin pairs of different ages

C. Pirazzini[1,2,3], P. Garagnani[4,1,3], C. Giuliani[2], E. Marasco[3], E. Pini[1], V. Mantovani[3], M. Pierini[1,2], M. Capri[1,2], M.G. Bacalini[5], C. Franceschi[1,2]
[1]CIG-Interdipartimental Center L. Galvani, Bologna, Italy
[2]Dept of Experimental Pathology, Univ. of Bologna, Italy
[3]CRBA-Center for Applied Biomedical Research, S. Orsola-Malpighi Univ. Hospital, Bologna, Italy
[4]Dept of Hematology and Oncology "L. and A. Seragnoli", Bellaria Hospital, Bologna, Italy
[5]Dept of Cellular Biotechnologies and Hematology, Sapienza Univ., Roma, Italy

Epigenetic is a way to create new phenotypes by controlling gene expression architecture through different molecular mechanisms. Among these mechanisms, DNA methylation is the most studied. It is involved in several important mechanisms and genomic imprinting is one of these. Genomic imprinting is an epigenetic marking of genes in the parental germline that ensures the stable transmission of monoallelic gene expression patterns in a parent-of-origin-specific manner. Different studies highlight the need to clearly understand the role of DNA methylation on imprinted genes in the early stages of life and during the development until the last decades of life. First it is necessary to understand if this mechanism is inherited and how genetic and environment impact on this character. To this end twin studies are universally regarded as the most powerful tool.
This model is very useful to assess if changes in DNA methylation in MZ twins are influenced by sharing the same environment.In this study we want to address if methylation levels change within twin pairs according to aging. Further, we try to understand if there are differences in the DNA methylation pattern of regions located in the same gene but in different genomic position (island and shore).
In this study we evaluate the DNA methylation status of 47 couples of twins, aged 22-97 years in the imprinted locus IGF2/H19. We noted that the increase of proportion of lifetime in which twins do not share the same environment influences changes in DNA methylation level within pairs. It is possible to assess that beyond a certain age threshold, the probability that methylation levels vary inside the twin pairs increase. This could imply that DNA methylation adaptive response is more active in the second part of human life and this period coincides with a reduced reproductive capacity. Finally we have found that only the shore of Cpg islands produces differential signals related to the biological model considered.

P2.15

Type-1 (CB1) cannabinoid receptor promotes neuronal differentiation of neural stem cells

C. Compagnucci[1,2], S. Di Siena[1], M. B. Bustamante[1,2], D. Di Giacomo[1], M. Di Tommaso[3], M. Maccarrone[3,4], C. Sette[1,2], P. Grimaldi[1]
[1]Dept of Public Health and Cell Biology, Section of Anatomy, Univ. of Rome "Tor Vergata," Rome, Italy
[3]Dept of Biomedical Sciences, Univ. of Teramo, Teramo, Italy
[2]Institute of Experimental Neuroscience, Laboratory of Neuroembryology, and [4]Laboratory of Lipid Neurochemistry, Fondazione Santa Lucia IRCCS, Rome, Italy

Neurospheres are cellular aggregates that can grow in culture as free-floating clusters of neural precursor cells. We isolated neuronal precursor cells, or "neural stem cells" (NSCs), from the cortex of 13.5 dpc embryos. When cultured in medium supplemented with Epidermal Growth Factor (EGF) and b-Fibroblast Growth Factor (b-FGF), NSCs express nestin and maintain pluripotent features and markers of stemness. However, addition of serum and adhesion to substrates induce their differentiation into neurones, astrocytes and oligodendrocytes, with reproducible efficiency. Given the relevance of the endocannabinoid system in the CNS and its implication in a wide range of neurobiological processes (neurogenesis included), we set out to investigate the expression and function of type-1 and type-2 (CB1 and CB2) cannabinoid receptors in NSCs. Real-time PCR and Western blot analyses indicated that CB1 is present at higher levels than CB2 in NSCs. Activation of the former receptor with ACEA, a CB1-specific agonist, was not sufficient to induce differentiation of NSCs when they were grown as free-floating clusters in EGF/b-FGF-containing medium. However, when the cells were cultured under differentiating conditions, ACEA promoted neuronal differentiation, as revealed by increased number of ?III–tubulin positive cells with respect to untreated cells. In contrast, differentiation towards astocytes and oligodendrocytes was not affected by ACEA. The pro-neuronal differentiation effect was prevented by addition of the selective CB1 antagonist AM251, whereas the CB2-specific agonist JWH133 did not exert any effect, corroborating the specificity of the CB1-mediated response. Characterization of intracellular signalling pathways involved in ACEA-induced neuronal differentiation is currently under investigation. These results suggest that activation of CB1 by endogenous agonists (endocannabinoids) may represent a pro-neuronal differentiation signal for NSCs.

P2.16

Role of EGF-CFC cripto in skeletal muscle regeneration

O. Guardiola[1], P. Lafuste[2], S. Brunelli[3], S. Iaconis[1], G. Andolfi[1], G.Cossu[3], P. Carmeliet[2], G. Minchiotti[1]
[1]Institute of Genetics and Biophysics "A. Buzzati-Traverso", CNR, Naples, Italy
[2]VIB Vesalius Research Center, K.U.Leuven, Leuven, Belgium
[3]Division of Regenerative Medicine, Stem Cells and Gene Therapy DIBIT H San Raffaele, Milan, Italy

Cripto is a key player in embryogenesis and in the multiple signalling networks that orchestrate the differentiation of Embryonic Stem Cells (ESCs). We have identified Cripto as a new, unpredicted player of skeletal muscle regeneration. We show that Cripto is re-expressed during early phases of adult skeletal muscle regeneration. We detect high levels of Cripto expression during muscle regeneration and in newly formed myotubes whereas Cripto expression in normal non-injured muscle is undetectable. In vitro and in vivo data demostrated that Cripto is expressed by myogenic precursors and preliminary results show that Cripto is also expressed in F4/80 positive cells in early phases of skeletal muscle regeneration after CTX-injury. This quite unexpected result, prompted us to perform molecular studies, both in vitro and in vivo, to get insight into the molecular mechanisms that underlay Cripto activity in the early phases of skeletal muscle regeneration. By using gain of function and loss-of function approaches through viral-mediated gene transfer, we provide evidences that Cripto overexpression accelerates regeneration leading to muscle hypertrophy, whereas conditional loss of cripto causes muscle regeneration defects. Moreover, we provide novel evidence that Cripto is mitogenic for muscle stem cells (satellite cells) acting as a natural antagonist of the TGF-β ligand Myostatin and is chemotactic through activation of the TGF-β -related Nodal pathway. All together our data provide new insights in TGF-β ligand-specific signaling by Cripto on satellite cells and contribute in the understanding of the complex network of signaling pathways operating in skeletal muscle regeneration, disclosing new scenarios for the therapy of muscle disorders.

P2.17

Numb expression contributes to the undifferentiated state maintenance in human epithelial stem cells

G. Iannolo[1], S. Buccheri[2°], M.R. Sciuto[1°], C. Colarossi[2], S. Sinha[5], M. Gulisano[1,4], L. Memeo[2], R. De Maria[3]
[1]IOM Ricerca srl, Viagrande, Catania, Italy
[2]Dept Experimental Oncology, Mediterranean Institute of Oncology (IOM), Viagrande, Catania, Italy
[3]Dept of Hematology, Oncology, and Molecular Medicine, Istituto Superiore di Sanita` (ISS), Rome, Italy
[4]Dept of Physiological Sciences, Univ. of Catania (UNICT), Catania, Italy
[5]Dept of Biochemistry, State Univ. of New York at Buffalo, Center for Excellence in Bioinformatics and Life Sciences, Buffalo, New York, United States of America
°The authors equally contributed to this work

The epidermis is formed by several layers of cells maintained by a mitotically active basal stratum enriched in stem cells. During the life span epithelial stem cells in the basal layer undergo symmetric and asymmetric division cycles generating a dividing progenitor population. Skin homeostasis is maintained through a complex interplay between developmental signals and other cell regulatory pathways. In this context, Notch pathway plays a crucial role in cell fate determination and differentiation. This receptor and their ligands (Delta and Serrate/Jagged families) act in a cell specific manner (Artavanis-Tsakonas et al., 1999). Delta1 expression in basal layer induces commitment of neighboring epidermal stem cells expressing Notch. Its activation induces a keratinocyte differentiation, while the p53 tumor suppressor protein homolog p63 is required for stem cell self-renewal in various epithelial tissues (Yang et al., 2002). The importance of Notch signaling is well demonstrated in different cell compartments, such as the neural system, where its activity is inhibited by Numb (Fiuza and Arias, 2007). Numb proteins influence the cell fate by inhibiting the action of Notch by a still unknown mechanism (Guo et al., 1996). Numb has been shown to influence differentiation and proliferation in different neural models (Verdi et al., 1999). Numb is also expressed in most adult tissues, and its expression is diffused in mouse embryos. Therefore it is likely that Numb function is not restricted to neurogenesis. In our study, we evaluated the role of Numb in normal skin homeostasis and its involvement in stemness maintenance. We observed that Numb expression is lost during differentiation and that its exogenous expression promotes the maintenance of an undifferentiated state in epidermal stem cell.

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P2.18

Critical role of c-Myc in Acute Myeloid Leukemia involving direct regulation of miR-26a and histone methyltransferase EZH2

B. Salvatori[1], I. Iosue[4], N.D. Damas[1], A. Mangiavacchi[1], S. Chiaretti[5], M. Messina[5], F. Padula[4], A. Guarini[5], I. Bozzoni[1,2], F. Fazi[3], A. Fatica[1]
[1]Dept of Biology and Biotechnology "Charles Darwin" and Institute Pasteur Cenci-Bolognetti, Sapienza Univ., Rome, Italy
[2]Institute of Molecular Biology and Pathology, Consiglio Nazionale delle Ricerche, Rome, Italy
[3]Dept of Medical-Surgical Science and Biotechnologies, Sapienza Univ., Latina, Italy
[4]Section of Histology and Medical Embriology, DAHFMO, Sapienza Univ., Rome, Italy
[5]Division of Hematology, Dept of Cellular Biotechnologies and Hematology, Sapienza Univ., Rome, Italy

Hematopoiesis is a life-long, highly-regulated multistage process where a pluripotent self-renewing hematopoietic stem cell (HSC) gives rise to all blood cell lineages. In early hematopoietic progenitor cells, growth and maturation of erythroid, granulocytic, monocytic and megakaryocytic lineages are largely controlled by unique combinations of transcription factors that cooperatively regulate promoters and enhancers present on specific target genes. Acute myeloid leukaemia (AML) represents the clonal expansion of hematopoietic precursors blocked at different stages of differentiation. The pathogenesis of AMLs is correlated to recurrent genetic alteration. Increased expression or aberrant activation of c-Myc plays an important role in leukemogenesis. Here, we show that in acute myeloid leukemia (AML) c-Myc directly controls the expression of EZH2, a component of the Polycomb Repressive Complex 2, and miR-26a. MiR-26a is downregulated in primary blasts from AML patients and, during myeloid differentiation of AML cells, is induced together with decrease in c-Myc and Ezh2 levels. Previously, EZH2 was shown to be regulated by miR-26a at the translational levels in lymphomas. However, we demonstrate that in AML the variation of EZH2 mainly depends on c-Myc transcriptional control. We also show that enforced expression of miR-26a in AML cells is able to inhibit cell cycle progression by downregulating cyclin E2 expression. In addition, increased levels of miR-26a potentiate the antiproliferative effects of 1,25-dihydroxyvitamin D3 (VitD) and stimulate myeloid differentiation. Our results identify new molecular targets of c-Myc in AML and highlight miR-26a attractiveness as a therapeutic target in leukemia.

P2.19

A newly identified Galectin-8 from sea urchin embryos

K. Karakostis[1,2], C. Costa[1], F. Zito[1], H.C. Schröder[2], W.E.G. Müller[2], V. Matranga[1]
[1]Consiglio Nazionale delle Ricerche Istituto di Biomedicina e Immunologia Molecolare "Alberto Monroy", Palermo, Italy
[2]Institut für Physiologische Chemie Angewandte Molekularbiologie Universitätsmedizin der Johannes Gutenberg-Universität, Mainz, Germany

Galectins are carbohydrate binding proteins that specifically bind beta-galactoside derivatives (Jun Hirabayashi et al, 2002; Karin Pfeifer et al, 1993; Liu et al, 2011). The members of the galectin super-family interacting with cell-surface glycoconjugates regulate diverse cellular events, including signaling pathways, apoptosis, innate immune and inflammatory responses (Liu and Rabinovich 2005). Some galectins involved in biomineralization are found in mammalian osteoblasts and osteocytes (Tanikawa et al. 2010). In sponges, the matrix guided formation of silicatein-mediated silica spicules is strongly increased when associated with a galectin (Schröder et al, 2006; Müller et al 2009).
This study was undertaken to isolate and characterize galectin cDNAs from the *Paracentrotus lividus* sea urchin embryo. By RT-PCR and 3' RACE we amplified a putative galectin family member and cloned it in *pGEM-T-Easy* vector. The 1309nt clone includes a 933nt coding sequence, encoding a 34.7kDa protein, containing two tandem carbohydrate-recognition domains. The sequence homology, obtained by Blast analysis, suggested *Pl*-galectin as a novel member of the Galectin-8 family. We characterized the deduced aminoacid sequence by *in silico* analysis and modeling based on the high structural similarity of the N-Terminal domain of Human Galectin-8 crystal structure. The expression levels of the *Pl*-galectin-8 mRNAs were monitored during the development of the *P. lividus* embryo by whole mount *in situ* hybridization and comparative Q-PCR. In order to perform functional assays in view of future biomedical applications we cloned the CDS in the pCOLD-TF expression vector and isolated the recombinant *Pl*-Galectin protein expressed in *E.* coli (BL21.AI). As potential calcium binding sites were identified by similarity on the tridimensional model, the calcium binding activity was assayed using the isolated recombinant protein.
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P2.20

Regulation of GDNF, a paradigm of spermatogonial stem cell niche-derived factor.

D. Lamberti, M. Grasso, M. Stefanini, E. Vicini
Dept of Anatomical, Histological, Forensic & Orthopaedic Sciences, Univ. of Rome "La Sapienza", Rome, Italy

Spermatogenesis guarantees the continuous production of sperm from a small population of Spermatogonial stem cells (SSC).The great potential of SSC to self-renew or to give rise a population committed to differentiation is a key point for a correct spermatogenesis. The right balance between self-renewal and differentiation is guaranteed by the proper environment in the stem cell niche, through the production of several factors. One of the most studied factor is Glial cell Derived Neurotrophic Factor (GDNF) produced by Sertoli cells, the supporting cells of spermatogenesis. In the testis regulation of GDNF is poorly understood, but FSH seems to control the GDNF levels in Sertoli cells. We have previously demonstrated that GDNF mRNA levels and production is regulated during spermatogenesis in a stage-specific fashion. Maximal GDNF production is found in those stages of seminiferous epithelium when the Sertoli cells are more responsive to FSH. In order to understand GDNF regulation in this study we set out to characterized the regulation of GDNF promoter in Sertoli cells. Firstly, we demonstrated by qRT-PCR that in isolated seminiferous tubules the GDNF mRNA accumulates after stimulation with FSH probably through cAMP intracellular signalling. Next, we cloned different fragments of the putative GDNF promoter containing three different putative start sites of transcription in a luciferase reporter vector. Sertoli cells were transiently transfected with the different promoter regions and stimulated in presence and absence of 1mM (Bu)2cAMP, an analog of cAMP. We found a cAMP-dependent upregulation in the fragment containing the putative cAMP-responsive element (CRE) sites. These results suggest that the GDNF promoter is regulated by the cAMP pathway and probably by FSH. This study may shed light on the extracellular cues that impact on the spermatogonial stem cell niche-derived factors.

P2.21

Differentiation of ES cells and deficiency of Suv39h histone methyltransferases is accompanied by distinct levels of A- and B-type lamins

S. Legartova, G. Sustackova, L. Stixova, S. Kozubek, E. Bartova
Dept of Molecular Cytology and Cytometry, Institute of Biophysics, Academy of Sciences of the Czech Republic, Brno, Czech Republic

Nuclear lamins are major architectural elements of the mammalian cell nucleus, and they have been implicated in the functional organization of the nuclear interior, possibly by providing structural scaffold for nuclear compartments. Mutations in LMNA gene have been shown to cause a whole range of human disorders, called laminopathies. Here, we have studied, by the use of western blots, changes in the levels of A- and B-type lamins in human embryonic stem cells (hESCs) undergoing differentiation and after siRNA to LMNA mRNA. In addition, we have analyzed in which extent the levels of lamins can be influenced by deficiency of histone methyltransferases (HMTs) Suv39h1 and Suv39h2. Our preliminary experiments confirmed that interaction between lamins and histone modifiers could be important for higher-order chromatin arrangement. Moreover, differentiation of hESCs, characterized by distinct epigenetic profiles, was also accompanied by changes in the levels of A- and B-type lamins. Especially an absence of A-type lamins is of functional significance in hESCs.
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P2.22

PTHrP isoform expression in adipo- and osteo-differentiating human mesenchymal stem cells

A. Longo[1], I. Catanzaro[1], F. Caradonna[1], E. Tobiasch[2], C. Luparello[1]
[1]Dept STEMBIO (Sez. Cellular Biology), Univ. of Palermo, Palermo, Italy
[2]Dept of Natural Sciences, Bonn-Rhine-Sieg Univ. of Applied Sciences, Rheinbach, Germany

Multipotent mesenchymal stem cells (MSC) can differentiate in vitro towards osteoblasts and adipocytes, and although the early steps of the differentiation process have been examined thoroughly, still specific markers for the characterization of defined differentiation steps are lacking. To search for stemness/differentiation markers, we examined the expression of the splicing isoforms of parathyroid hormone-related peptide (PTHrP), a regulator of proliferation, differentiation and apoptosis. The PTHrP gene, coding for three protein variants of 139, 141 and 173 aa, has a complex organization with three transcriptional start sites, i.e. two TATA promoters, P1 and P3, and a GC-rich promoter, P2, and nine exons undergoing to alternative splicing. In the undifferentiated MSC we have found four transcripts encoding for the 139 and 173 aa isoforms, whereas osteo-differentiating cells produced only two transcripts encoding for the same protein isoforms, and adipo-differentiating cells only one transcript encoding for the 173 aa isoform. Therefore, our results strongly suggest that during osteo- and adipo-differentiation, the expression of PTHrP isoforms by MSC becomes increasingly selective and P2 is always silenced. Consequently, PTHrP isoform expression could be considered a putative marker of MSC differentiation. We examined the methylation state of PTHrP P2 promoter in undifferentiated and osteo-differentiating MSC, to investigate the possible correlation between methylation and silencing of this promoter in some cell preparations. In agreement with the gene expression data, three CpG island internal sites of this promoter were found to be hypermethylated in DNA preparations from undifferentiated and differentiated cells. Further studies will be performed to underline these findings also for P3 promoter. We conclude that PTHrP plays a role in the differentiation of MSC through both the selective regulation of these isoforms and the mechanism of promoter methylation.

P2.23

Modelling eye specification in embryonic stem cells

G. Lupo[1,2], C. Novorol[1], J. Smith[3], L. Vallier[3], E. Miranda[2], M. Alexander[3], R. Pedersen[3], W. Harris[1]
[1]Dept of Physiology, Development and Neuroscience, Univ. of Cambridge, Cambridge, UK
[2]Dipartimento di Biologia e Biotecnlogie, Univ. di Roma "La Sapienza", Rome, Italy
[3]Anne McLaren Laboratory for Regenerative Medicine, Univ. of Cambridge, Cambridge, UK

Eye specification results from three steps of embryonic inductive interactions. First, the neuroectoderm is induced in the dorsal embryonic region. Subsequently, antero-posterior patterning of the neuroectoderm specifies the forebrain at its rostral end, while progressively posterior regions are specified as midbrain, hindbrain and spinal cord. Finally, a subset of the forebrain is specified as eye-forming region (eye field), while neighbouring regions take on different forebrain fates, such as telencephalon or diencephalon. A network of eye field transcription factors (EFTFs), including Pax6, Six3, Lhx2, Six6 and Rax, becomes active in the eye field and controls its specification to eye fates. However, the mechanisms controlling expression of this EFTF network remain unclear. By employing embryonic stem cells (ESCs) as a developmental model system, we show that the first two steps leading to eye specification, namely neuroectoderm induction and forebrain specification, can be successfully reconstructed in ESCs grown in chemically defined, feeder-free, adherent conditions by means of Nodal, Wnt and BMP antagonism in the absence of exogenous FGF. However, these conditions appear to be insufficient for robust Rax expression and full eye fate specification. Experiments are underway in order to elucidate the molecular signals controlling Rax expression within the anterior neuroectoderm.

P2.24

Hypoxia-regulated expression of β-dystrobrevin and miRNA-143 targeting during Ntera-2 neuronal differentiation

P. Macioce[1], M.T. Quaranta[2], I. Spinello[2], G. Macchia[1], L. Gaddini[1], C. Labbaye[2]
[1]Dept of Cell Biology & Neuroscience, Istituto Superiore di Sanità, Rome, Italy
[2]Dept of Hematology, Oncology & Molecular Medicine, Istituto Superiore di Sanità, Rome, Italy

Traditionally viewed as a pathological condition, hypoxia has also physiological roles in normal tissues. It has recently been shown that reduced oxygen levels maintain neuronal stem cells (NSC) in a state of quiescence while mild hypoxia favors NSC proliferation and differentiation. During hypoxia, cells activate a number of adaptive responses, including gene regulation by hypoxia-inducible factors (HIFs). HIFs are heterodimers consisting of an O_2-labile α subunit and a stable β subunit. To date, three HIF-α isoforms have been described: HIF-1α, HIF -2α and HIF-3α. Hypoxia allows HIF-α to escape from normoxia-mediated degradation, translocate into the nucleus and bind hypoxia-response elements (HREs) in the promoter of target genes and miRNAs. β-Dystrobrevin (DTNB), a member of the Dystrophin-associated Protein Complex, has recently been described to interact with the chromatin remodeling proteins iBRAF and BRAF35 and suggested to be involved in neuronal differentiation. As mild hypoxia favors neuronal differentiation, we planned to study the expression of DTNB and its putative regulation by miR-143 in Ntera-2 cells induced to neuronal differentiation by retinoic acid (RA), in normoxic (20% O_2) and hypoxic (5% O_2) condition. Our results suggest that during RA-induced neuronal differentiation of NTera-2 cells in normoxia, miR-143 may control DTBN expression. We are currently analyzing a putative HRE in the promoter of DTNB gene to investigate whether DTBN may function as a hypoxia responsive gene. We have also assessed HIF-1α, -2α and -3α mRNA and protein expression during RA-treatment of normoxic NTera-2 cells. Our results show that in normoxia all HIF-α mRNAs are upregulated, and that nuclear proteins HIF-2α and HIF-3α but not HIF-1α are expressed, implying that HIF-1α may be involved in the regulation of specific hypoxia-responsive target genes. We are now analyzing DTBN and HIF-α expression under hypoxic conditions.

P2.25

Protein kinase C theta is a key determinant of the inflammatory response involved in the pathogenesis of muscular dystrophy

L. Madaro[1], A. Pelle[1], C. Nicoletti[1], G. Bossi[2], S. Soddu[2], M. Bouché[1]
[1]Dept of Anatomy, Histology, Forensic Medicine and Orthopedics, Unit of Histology and Med. Embryology, CE-BEMM and Interuniversity Institute of Myology, Sapienza Univ. of Rome, Rome, Italy
[2]Dept of Experimental Oncology, Molecular Oncogenesis Laboratory, Regina Elena Cancer Institute, Rome, Italy

Inflammation is a key pathological characteristic of dystrophic muscle lesion formation, limiting muscle regeneration and resulting in fibrotic and fatty tissue replacement of muscle, that exacerbates the wasting process in dystrophic muscles. Limiting inflammation and immune response is thus one of the therapeutic options to improve healing, and a few downstream molecular targets have been already proposed. PKCθ is a member of the PKCs family highly expressed in both lymphocytes and skeletal muscle; given its crucial role in T-cell activation and proliferation, it is being proposed as a valuable pharmacological target for T-cell dependent immune disorders. We here show that lack of PKCθ expression, in mdx mice (the mouse model of Duchenne muscular dystrophy), obtained in the bi-genetic mouse model mdx/θ-/-, greatly prevented muscle wasting, improving muscle regeneration, maintenance and strength. This phenotype was associated to reduction in inflammatory infiltrate, pro-inflammatory gene expression and pro-fibrotic markers, as compared to mdx mice. Moreover, bone marrow transplantation experiments demonstrated that the phenotype observed was primarily dependent on lack of PKCθ expression in hematopoietic cells. Our findings reveal that PKCθ signaling pathway plays a crucial role in the progression of muscular dystrophy and can be proposed as a new pharmacological target to counteract the disease.

P2.26

Prognostic Impact of MGMT Promoter Methylation in 79 Glioblastoma Patients Treated with Temozolamide

G. Perazzoli[2], P. Tolu[1], Y. Asara[1], R. Ortiz[2], C Melguizo[2], PJ. Álvarez[2], O. Caba[3], J. Prados[2], B. González[4], R. Luque[4], R. López[5], C. Bueno[5], F. Tanda[6], MA. Bella[6], A. Aranega[2], R. Madeddu[1,7]
[1]Dept of Biomedical Sciences, Univ. of Sassari, Italy
[2]Biopathology and Medicine Regenerative Institute (IBIMER), Dept of Human Anatomy and Embriology, School of Medicine, Univ. of Granada, Spain
[3]Dept of Health Sciences, Univ. of Jaén, Spain
[4]Oncology Service, Virgen de las Nieves Hospital, Granada, Spain
[5]Anatomy and Pathology Service, Virgen de las Nieves Hospital, Granada, Spain
[6]Institutes of Anatomic Pathology and Histopathology, Univ. of Sassari, Italy
[7]National Institute of Biostructures and Biosystems, INBB, Italy

Glioblastoma (GMB), the most common primary brain tumor in adults, is a rapidly progressive and fatal disease as indicated by a median overall survival of less than 1 year in a population-based study. Assessment of promoter methylation of the O 6-methylguanine DNA methyltransferase (MGMT) gene has recently gained importance in molecular profiling of this pathology. Although epigenetic silencing of the MGMT gene promoter has been associated with prolonged survival in glioblastoma patients it is unclear if their determination will be an important prognostic marker and/or a predictive marker for response to temozolomide in patients with newly diagnosed glioblastoma. In order to help determine this possible connection we analyze MGMT promoter and survival in 79 patients with GMB.
Seventy-nine patients with GMB, treated with TMZ were analyzed for MGMT promoter methylation. All patiens were treated with TMZ followed with radiotherapy. Genomic DNA was isolated from paraffin embed tissue samples obtained previous to treatment. MGMT promoter methylation was determined by methylation-specific polymerase chain reaction after bisulfite treatment. Progression survival was calculated according to the Kaplan–Meier method.
Results and Conclusion: MGMT promoter was methylated in 34 patients (43%) and unmethylated in 45 patients (57%). Significant correlation was observed between MGMT promoter methylation and patients survival treated with TMZ (P = 0.002 by the log-rank test). The results of the present study suggest that the determination of MGMT promoter methylation status might be a predictive and prognostic biomarker in the treatment of patients with GBM and that the methods employed for its assessment could be used to therapeutic decision.

P2.27

Organizational principles of NuMA/LGN/Gαi complex, and its relation with cellular polarity in asymmetrically dividing stem cells

M. Mapelli, S. Culurgioni, A. Alfieri, V. Pendolino, F. Laddomada
European Institute of Oncology, Milano, Italy

The asymmetric outcome of a cell division requires the alignment of the mitotic spindle with the cellular polarity. In Drosophila melanogaster, impairment in any of the genes involved in spindle orientation or cortical polarity alters the balance between symmetric and asymmetric divisions, and hence the daughter cells' fate, with detrimental effects on tissue development and homeostasis. In line with the recently proposed cancer stem cell hypothesis, mistakes in asymmetric stem cell divisions also pertain the development and perpetuation of human cancers.
The correct placement of the mitotic spindle within the cell is orchestrated by cortically localized force generators acting on astral microtubules. Force generators are macromolecular machines localized at spatially restricted cortical sites in conjunction with polarity cues, and able to exert pulling forces on microtubules emanating from the spindle poles. The core components of the force generators are the evolutionary conserved NuMA/LGN/Gαi complexes, that genetically and physically interact with the polarity proteins Par3/Par6/aPKC via the bridging molecule Inscuteable. I will present data obtained from biochemical analyses on the in vitro reconstituted NuMA/LGN/Gαi assemblies. In line with the proposed conformational switch model, apo LGN in kept in a closed conformation by intra-molecular interactions between the N-terminal TPR containing domain and the C-terminal region, coding for four GoLoco motifs. Binding studies between a battery of TPR and GoLoco LGN fragments unveiled a parallel arrangement of the two LGN portions, and mapped the minimal contact surface between the two. SEC analyses performed on full-length LGN suggest that loading of four Gαi molecule is semi-cooperative, and that NuMA binding induces a conformational transition. I will also report on the organizational principles underlying the interaction of LGN with Inscuteable.

P2.28

How AML1-ETO and CDKN1A expression influences the transcriptional program of murine hematopoietic stem cells and early progenitors

M. Mazza, A.U. Cammarata, L. Luzi, P.G. Pelicci
European Institute of Oncology, Department of Experimental Oncology, Milan, Italy

Acute myeloid leukemia (AML) is an aggressive neoplasm characterized by the accumulation of immature myeloblasts in the perypheral blood and the bone marrow of patients. A genetic lesion often found in M2 leukemias and shown to start the leukemogenic process in animal models of the disease is the translocation t(8;21)(q22;q22). This translocation generates an aberrant transcript by fusing the gene AML1 to ETO and leads to the onset of an M2 type of leukemia (overall survival 35-60%). Expression of the fusion transcript in mice subsequently treated with the mutagenic agent N-ethyl-N-nitrosourea (ENU) induces leukemia onset. A recent study has established the essential role of the gene CDKN1A (P21) in the leukemogenic process mediated by AML1-ETO (Viale et al. , Nature, 2009).
The goal of the present study is to establish which genes are regulated by AML1-ETO in the hematopoietic stem cell compartment (LT-HSC, ST-HSC) and in early progenitors (MPP, CMP) and to establish what is the contribution of P21 to leukemogenesis.
Preliminary results show that: i) AML1-ETO expression severely affects gene expression in the cells where it's expressed; ii) P21 is an important determinant of the stem cell like signature of HSCs and MPPs expressing AML1-ETO; iii) once myeloid commitment has started, CMP stage, this transcriptional program dominates over the differences induced by the expression of AML1-ETO.
This study should uncover which pathways are crucial for the onset of the leukemia induced by AML1-ETO. Future experiments, aimed to interfere with the expression of specific genes, will establish their contribution to the leukemic phenotype.

P2.29

The role of transcription factors for the development of anterior brain and olfactory connection: a profiling approach

G. Garaffo[1], P. Provero[1], D. Tomaiuolo[1], P. Peano[2], P. Pinciroli[2], C. Battaglia[2], F. Francalanci[1], M. Santoro[1], G.R. Merlo[1]
[1]Molecular Biotechnology Center, Univ. of Torino
[2]CNR-Istituto Tecnologie Biomediche, Milano

The development of the olfactory sensory system entails the coordinated morphogenesis and differentiation of the olfactory epithelium and of the anterior forebrain. At early stages, immature olfactory neurons (ON) extend their axons to contact and form synapses with secondary projection neurons. Axon elongation is accompanied by migration of the GnRH+ neurons - a process impaired in the Kallmann syndrome, comprising anosmia and severe hypogonadism.
Axon elongation and connection is controlled by signals and interactions between the ON and adjacent cells, via morphogens, guidance molecules, ECM. Recent evidence point to a cell-autonomous regulation by transcription factors (Dlx5, Fez and Emx2). Our aim is to investigate on the transcriptional network controlling this developmental program, and identify genes and miRNAs essential for axon connectivity. We focus on mice null for Dlx5, a homeogene expressed early in ONs, essential for their differentiation and connectivity.
We compared profiles of expressed genes and miRNAs between normal and Dlx5-/- ON, and combined these data with the compilation of predicted Dlx sites on conserved regions of the genome. Our data suggest that Dlx5 controls ON differentiation via regulation of the miR200 family (and other miRs), and controls axon connectivity via changes in gene expression. Relevant categories include: signalling adapters, control of intracellular calcium, membrane and vesicle trafficking. We are functionally testing putative Dlx5 targets on axon elongation-connection, using the zebrafish reporter lines Trpc2:Venus and OMP:CFP.
The value of studying this embryonic process stems from the peculiarity of the peripheral olfactory system to renew neurons and connections, physiologically as well as following lesions. Investigations on the regulatory network may have more general implications for the biology of nerve regeneration and repair. This work is supported by the Telethon Foundation, project TCR07004.

P2.30

Activation of the Sonic Hedgehog signaling pathway is necessary for Noggin-mediated retinal induction of *Xenopus* pluripotent cells

A. Messina[1], L. Lei[2], T. Incitti[1], R. Vignali[2], F. Cremisi[3], S. Casarosa[1]
[1]CIBIO, University of Trento
[2]Dept of Biology, University of Pisa
[3]Scuola Normale Superiore, Pisa

The main aim of regenerative medicine is to replace degenerating cells, and its success depends on the capability of the donor cells to correctly replace all the missing cell types. We have recently shown that in *Xenopus* embryos, proper doses of a single secreted molecule, Noggin, can drive animal cap embryonic stem (ACES) cells toward retinal cell differentiation without additional cues. We performed a transcriptome analysis to identify the molecular effectors of Noggin, and our data show a possible involvement of the sonic hedgehog (SHH) signaling pathway in the Noggin-mediated retinal induction of ACES cells. Functional validation experiments show that blocking SHH signaling by cyclopamine (a well-known inhibitor of the SHH receptor) significantly inhibits the retina-forming properties of high-Noggin expressing ACES cells, as seen both by transplantation experiments and by qRT-PCR. Further validation experiments are ongoing. We are also carrying out experiments to investigate the possible role of other signaling molecules. The elucidation of the pathways through which Noggin elicits retinal fates in ACES cells will be a first step toward the setup of ameliorated protocols for the differentiation of stem cells toward retinal neurons.

P2.31

The role of Prep1 in regulating hematopoietic stem cell maintenance

L. Modica, G. Iotti, F. Blasi
IFOM, Istituto FIRC di Oncologia Molecolare, Milano

Prep1, a homeodomain transcription factor, belongs to the TALE family and plays an essential role during early development. A hypomorphic mutation of the Prep1 gene ($Prep1^{i/i}$) causes embryonic lethality between E17.5 and P0 with a pleiotropic embryonic phenotype that includes defects in all hematopoietic lineages. Deficiency in hematopoietic stem cells (HSCs) might be responsible for the downstream hematopoietic phenotype observed.

We observed that Long-Term Hematopoietic Stem Cells (LT-HSCs), identified as $Lin^-Sca1^+ckit^+CD150^+CD48^-CD41^-$ in E14.5 fetal livers (FLs), are strongly reduced in $Prep1^{i/i}$ FLs compared to wild type (wt), and show strongly impaired ability to form colonies in LTC-IC assays *ex vivo*. *In vivo* limiting dilution experiments have shown a reduced frequency of LT-HSCs in $Prep1^{i/i}$ FLs compared to wt. Furthermore, in long-term competitive transplantation assays, $Prep1^{i/i}$ HSCs are considerably less efficient than wt cells in establishing HSC and progenitor compartments in lethally irradiated repicients. Importantly, the $Prep^{i/i}$ stem cell pool shows defects in self-renewal ability.
Interestingly FL $Prep1^{i/i}$ LT-HSCs also showed a strongly reduced G0 pool suggesting a role of Prep1 in the maintenance of the self-renewing quiescent HSC pool. Moreover, we could observe the activation of proliferative pathways in $Prep1^{i/i}$ LT-HSCs as demonstrated by the increased phosphorilation of Stat1 and by the overexpression of Sca1. This feature might force HSCs towards the active proliferating state.
All these data suggest a crucial role of Prep1 in regulating critical features of HSCs.

P2.32

Mesenchymal stem cells induce tumors in an ectopic bone formation model

E.Molino, R.Tasso
Laboratory of Regenerative Medicine, DOBIG-Dept Oncology, Biology and Genetics, Genova Univ., Genova, Italy

Due to their differentiative and immunosuppressive properties, Bone marrow (BM)-derived mesenchyaml stem cells (MSC) are presently considered, among all the other adult stem/progenitor cells, the ideal candidate for regenerative medicine and tissue engineering applications. Despite the promising initial results obtained in clinical trials, emerging experimental data indicate that MSC can undergo or induce a tumorigenic process in determined circumstances.
Recently, we have reported that, in the murine model of ectopic bone formation under unloaded conditions, the implant of combinations of mouse MSC/scaffold constructs in immunocompetent syngenic or immunocompromised recipient mice led to the formation of a host-derived sarcoma. Using the same experimental model no tumorigenic processes were observed, implanting both more committed mouse cells, such as osteoblasts, and MSC derived from other species.
Searching for mechanisms involved in the tumor formation, we found retroviral particles in the cells extracted from the formed tumor masses, calming an involvement of endogenous viruses into the cancerous degeneration. We postulated that grafted MSC are able to promote slatentization of silent transforming endogenous viruses, in a paracrine fashion.
The work points out the ability of transplanted donor MSC to interact with the recipient surrounding tissues, underlying that these cells can contribute to the microenvironmental changes that can influence not only the oncogenic potential of implanted MSC, but also the host response to the cell graft.

P2.33

Quiescence-keeper microRNAs dampen oncogene-induced transcriptional reprogramming

N. Francesco
Dept of Experimental Oncology, IEO - European Institute of Oncology, Milan, Italy

Under physiological conditions proliferation and differentiation are coupled, with differentiated cells exhibiting irreversible cell-cycle arrest ("post-mitotic block"). This tightly regulated equilibrium is subverted in cancer, with tumor cells exhibiting unrestricted growth and de-differentiation phenotype as consequence of an altered gene expression program.
By exploiting the ability of the E1A oncogene to overcome the proliferative block of terminally differentiated myotubes, we characterized the transcriptional regulation of messages and microRNAs and their network of interactions during proliferation and differentiation. We identified two genetically independent mechanisms that cooperate to ultimately regulate the post-mitotic state: while the transcription of cell cycle and DNA replication genes is controlled by the Retinoblastoma (Rb) tumor suppressor pathway, a group of differentiation-associated microRNAs (DA-miRNAs), an Rb-independent component, promotes differentiation and, at the same time, targets the Rb pathway at post-transcriptional level, thus stabilizing the gene expression program towards the cell-cycle arrest. Individual expression or knock-down of DA-miRNAs is sufficient to regulate quiescence in proliferating myoblasts and upon E1A induced cell-cycle re-entry on differentiated cells, respectively. Of note, DA-miRNAs jointly affect cell cycle and Rb-dependent gene expression, even at their physiological expression levels, in order to limit unscheduled proliferation and oncogene-induced gene expression program, thus acting as "quiescence-keepers".
A number of genetic and molecular evidences suggests that loss of microRNAs is involved in tumorigenesis and confers to cancer an aggressive phenotype. Our results support a model in which differentiation-associated microRNAs (DA-miRNAs) could act as a tumor suppressor pathway by controlling both quiescence and differentiation at post-transcriptional level.

P2.34

Sox2 molecular functions in neural stem cells maintenance and Shh-dependent brain patterning: insights from conditional mouse mutants

R. Favaro, A. Ferri, P. Pagella, C. Verzeroli, J. Bertolini, S. Mercurio and S.K. Nicolis
Dept Biotechnology and Biosciences, Univ. of Milano-Bicocca, Milano, Italy

Sox2 encodes a transcription factor essential for pluripotent stem cells, and its knock-out is early embryonic lethal. Sox2 is later expressed throughout the developing nervous system and in neural stem cells (NSC), and its heterozygous mutation in humans causes central nervous system pathology, including hippocampal abnormality and epilepsy.
We are using conditional mutagenesis in mouse to explore the role of Sox2 in the development of NSCs, both in their in vivo environment (the developing brain) and in vitro, in NSC cultures. Late deletion of Sox2 (with nestin-Cre) leads to loss of postnatal hippocampal stem cells, and of NSC self-renewal in culture. Early loss of Sox2 (through Bf1-Cre) leads to impairment of brain development already during embryogenesis, particularly severe in the ventral brain. The functions of Sox2, a "panneural" transcription factor, are thus stage-and region-specific. We find that Sox2-mutant NSC (late and early) are defective in long-term self-renewal, because they fail to produce diffusible substances normally required for their maintenance through an autocrine mechanism. Among these, Sonic hedgehog (Shh, a Sox2 direct target) plays a prominent role; in vivo, Shh agonists can rescue brain abnormalities of Sox2 deletion mutants (late and early). At early stages, Sox2 is required for the activation of Nkx2.1, a central regulator of ventral brain development, and it directly regulates this gene. Nkx2.1 is also (as Sox2) a direct activator of Shh, explaining the early impairment in Shh expression in the early mutants. Early Sox2-deletion mutants indeed bear similarity to mouse models of holoprosencephaly, a human disease that can be caused by mutations in Shh, and of its downstream genes. Finally, we are comparing normal and Sox2-deleted in vitro NSCs by functional genomics approaches (ChIP-seq, ChIA-PET; RNA-seq) to get further insight into Sox2 neural target genes and Sox2 molecular modes of action.

P2.35

Serotonin 2B receptor signaling is required for craniofacial and ocular morphogenesis in Xenopus

E. Reisoli, G. Marras, I. Nardi, M.Ori
Unità di Biologia Cellulare e dello Sviluppo, Dipartimento di Biologia, Univ. di Pisa, Italy

Serotonin (5-HT) is a neuromodulator that plays many different roles in adult and embryonic life. Among the 5-HT receptors, 5-HT2B is one of the key mediators of 5-HT functions during development. We used Xenopus laevis as a model system to investigate the role of 5-HT2B in embryogenesis. By means of gene gain and loss of function approaches and tissue transplantation assays, we demonstrated that 5-HT2B modulates, in a cell-autonomous manner, postmigratory skeletogenic cranial neural crest cell (NCC) behavior. 5-HT2B overexpression induced the formation of an ectopic visceral skeletal element and altered the dorsoventral patterning of the branchial arches. Loss-of-function experiments revealed that 5-HT2B signaling is necessary for jaw joint formation and for shaping the mandibular arch skeletal elements. In particular, 5-HT2B signaling is required to define and sustain the Xbap gene expression necessary for jaw joint formation. We also showed that the phospholipase C beta 3 (PLC) is the effector of the transduction pathway acting downstream of 5-HT2B. The in vivo experiments also revealed that serotonin signaling, via 5-HT2B receptors, results in the formation of defective eyes, characterized by irregular form, position and coloboma. Interestingly, we showed that the 5-HT2B gene is expressed in periocular mesenchyme that represents a key signaling center required for a correct eye morphogenesis. These results contribute to the understanding of the interactive networks of patterning signals that are involved in the development of the vertebrate craniofacial and ocular structures.

P2.36

A new regulator of neural progenitor migration and glioblastoma invasion

B. Ortensi[1], D. Osti[1], S. Pellegatta[1,2], F. Pisati[1,2], P. Brescia[1], G. Finocchiaro[1,2], G. Pelicci[1]
[1]Dept Experimental Oncology, European Institute of Oncology (IEO), Milano, Italy
[2]Dept Molecular Neuro-Oncology, Fondazione I.R.C.C.S Ist. Neurologico C. Besta, Milano, Italy

Glioblastoma multiforme is an aggressive and highly invasive brain tumor, for which no effective therapy has been developed yet. Cancer stem cells, a sub-population of immature and infiltrative tumor cells, seem to be the main responsible and therefore they are considered the best candidate target for new therapies. We identified Rai protein, a member of the family of Shc-like adaptor proteins, as a new regulator of migration of normal and cancer stem/progenitor cells. Rai knock-down in the mouse impairs neuroblast migration and Rai silencing in human glioblastoma cancer stem cells impairs their invasion ability. Rai depletion is associated with alteration of multiple signaling pathways, yet it always leads to reduced expression of proinvasive genes, providing new insights in the mazy process of tumor infiltration.

P2.37

Knock-down of lysosomal cathepsin D negatively impacts on development and life-span of zebrafish

M. Ozzano[1], C. Follo[1], R. Castino[1], V. Mugoni[2], M. Santoro[2], C. Isidoro[1]
[1]Laboratorio di Patologia Molecolare del Dipartimento di Scienze Mediche and Centro di Biotecnologie per la Ricerca Medica Applicata dell'Univ. del Piemonte Orientale, Novara, Italy
[2]Molecular Biotechnology Center, Univ. of Torino, Torino, Italy

Cathepsin D (CD) is an aspartic protease resident in endosomal and lysosomal compartments of all eukaryotic cells. CD activity is essential to accomplish the extensive or partial proteolysis of protein substrates within acidic compartments therein delivered via endocytosis, phagocytosis or autophagocytosis. Accumulating evidence point to a role of CD in various steps of development in vertebrates, from oocyte maturation to histogenesis, morphogenesis and remodeling of embryonic organs. Here we report the first phenotypic description of the lack of CD expression during zebrafish (Danio rerio) development obtained by morpholino-mediated knock-down of CD mRNA. Since the un-fertilized eggs were shown to be supplied with maternal CD mRNA, only a morpholino targeting a sequence containing the starting ATG codon was effective. The main phenotypic alterations produced by CD knock-down in zebrafish were: 1. impairment of yolk adsorption; 2. absence of the swim-bladder; 3. microphtalmia and degeneration of the retinal pigment epithelium; 4. skin hyper-pigmentation; 5. slow-down of body growth; 6. shortened lifespan. Rescue experiments confirmed the involvement of CD in the developmental processes leading to these phenotypic alterations. Our findings add to the list of CD functions in organ development and patho-physiology in vertebrates. Given the similarity of organ development, organization and function between zebrafish and mammals, these data suggest that defective CD-mediated lysosomal proteolysis may contribute to several pathologies in humans also, including blinding diseases.
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P2.38

Modulating the teratogenic potential of the mouse Embryonic Stem Cells (ESCs)

M. Palmisano, R. Molteni, B. Clissi, R. Pardi
Leukocyte Biology Unit, Vita-Salute San Raffaele Univ., Milan, Italy

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 for treatment of diseases by ESC genetic manipulation, in order to preserve their potential to differentiate into specific cell types, while escaping a development into teratoma.
We established a mouse ESC culture protocol enabling mESCs to grow in absence of both FBS and feeder cells, allowing easier manipulation and usage of mESCs. The mESCs 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. Indeed, recent papers demonstrate a central role of selected miRNAs in the ESC cell cycle, suggesting that they promote indirectly the G1/S transition. In particular, during differentiation, the expression level of the miR-290 family is downregulated, and the G1 phase, regulated by p21 and p27, is elongated. p21, in turn, is a direct target of miR-294, belonging to the miR-290 family. On this basis, we demonstrated, in vitro, that the anti-miR-294 blocks the downregulation of p21, and the indirect upregulation of c-Myc, leading to a reduction of proliferation, without interfering with the ESC differentiation.
To analyze in vivo the effect of the miR-294 loss-of-function phenotype, we are switching to a vector-based approach, by generating a "sponge vector" using a lentiviral backbone. Then, we will perform the teratoma-formation assay, by subcutaneous injection of engineered ESC in NOD/SCID mice, to analyze the teratogenic potential of downreguleted-miR-294 ESC.

P2.39

Nuclear receptor ligands are potent tumor specific anti-inflammatory agents in breast cancer stem cells model

A. Papi[1], M. Orlandi[1], T. Guarnieri[1,3,6], M. Taffurelli[4], C. Ceccarelli[5], D. Santini[5], G. Storci[2,3], M. Bonafe,[2,3]
[1]Dept of Experimental Biology, Univ. of Bologna, Bologna, Italy
[2]Dept of Pathology, University of Bologna, Bologna, Italy
[3]Center for Applied Biomedical Research (CRBA), St. Orsola-Malpighi Hospital, Univ. of Bologna, Bologna, Italy
[4]Dept of Surgical and Anesthesiological Sciences, Univ. of Bologna, Bologna, Italy
[5]Dept of Gastroenterology and Pathology, Univ. of Bologna, Bologna, Italy
[6]National Institute of Biostructures and Biosystems (INBB), Rome, Italy

The presence of a population in breast tumor tissues denominated "breast cancer stem cells" has several biologic and clinical implications. Nowadays, "breast cancer stem cells" can be studied in vitro by expanding multicellular spheroids, named mammospheres (MS), from breast cancer specimens.
We studied the effects on MS originated from specimens of normal mammary gland (N-MS) and from breast cancer tissues (T-MS) of three drugs: the pioglitazone (PGZ), a ligand of Peroxisome Proliferator Actived Receptor-gamma, the all-trans-retinoic acid (RA), a ligand of retinoic acid receptor-alpha and the 6-OH-11-O-hydroxyphenantrene (IIF), a ligand of retinoic X receptor-gamma. We used also the MS obtained from the tumorigenic cell line MCF7 (MCF7-MS) and from the non-tumorigenic cell line MCF10 (MCF10-MS), as cellular model counterparts for T-MS and N-MS, respectively.
We demonstrated that PGZ, IIF and RA reduced the number of T-MS and MCF7-MS but not that of N-MS and MCF10-MS in normoxia and in hypoxia condition. We observed the reduction of the Interleukin-6 and SLUG (two enhancers of MS formation) expression and activities through the inhibition Nuclar Factor-kappa B pathway in T-MS but not in N-MS. The drugs also induced differentiation by increasing expression of the estrogen receptor-alpha and cytokeratin-18 and reduction of Notch-3 and Jagged-1, markers on MS.
In conclusion, we demonstrated that the drugs used and in particular the rexinoid IIF may be a promising therapeutic tool for the treatment of breast cancer because reduced the inflammation in stromal micro-environment cells and inhibited survival only of cell with "breast cancer stem cell" phenotype.

P2.40

Metabolic control of pluripotent stem cell metastability

S. Comes, L. Casalino, A. Cimmino, E.J. Patriarca, G. Minchiotti
Institute of Genetics and Biophysics "A. Buzzati-Traverso", Naples, Italy

Current evidence indicates that the phenotypic, functional, and molecular heterogeneity of stem cells is not only arising from the coexistence of several stem cell populations *in vitro*, but also from stem cells' ability to interconvert between different pluripotent states in response to extracellular signals. At the extreme of this plastic phenotypic variation, cells displayed identifiable features of the two types of mouse embryo -derived pluripotent stem cells; i.e. ESCs and EpiSCs, which derive from the inner cell mass and late epiblast or primitive ectoderm, respectively. To date, factors and molecular mechanisms controlling mESC metastability are not fully resolved. In order to identify modulators of this phenotypic transition, we screened a library of metabolites and identified two metabolically related amino acids, namely L-Proline (L-Pro) and L-Ornithine, as key regulators of mESC proliferation and phenotypic transition. Both compounds, but mainly L-Pro, force ESCs toward a novel epiblast stem cell (EpiSC) –like metastable state. However, unlike EpiSCs, L-Pro–induced cells (PiCs) rely on leukemia inhibitor factor to self-renew, colonize blastocyst, rapidly revert to ESC state after L-Pro removal. Most remarkably, PiC generation depends on L-Pro metabolism and is counteracted by L-Ascorbic acid. Interestingly, L-Pro derives from the degradation of extracellular matrix (ECM) through matrix metalloproteinases activity, both during embryo implantation and tumor invasion. We hypothesize that L-Pro metabolism may have a role in these complex biological processes. Based on our data and these considerations, the ESC→PiC transition represents a unique model system to get insight into the molecular mechanisms controlling stem cell plasticity both in development and disease. Genome wide expression analysis reveals a peculiar molecular signature of PiCs, shedding light on the mechanisms controlling L-Pro –induced ESC metastability.

P2.41

Molecular imaging of NF-Y transcriptional activity maps proliferation sites in live animals

F. Goeman[1,5,6], I. Manni[1,5], S. Artuso[1,5], B. Ramachandran[2], G. Toietta[3], G. Rando[2], C. Cencioni[3], S. Germoni[4], S. Straino[3], M. Capogrossi[3], S. Bacchetti[1], A. Maggi[2], A. Sacchi[1], P. Ciana[2], G. Piaggio[1]
[1]Experimental Oncology Dept, Istituto Regina Elena, Rome, Italy
[2]Dept Pharmacological Sciences, Milan Univ., Milan, Italy
[3]Vascular Pathology Lab, Istituto Dermopatico dell'Immacolata, Rome, Italy
[4]SAFU, Istituto Regina Elena, Via delle Messi D'Oro 156, 00158 Rome, Italy
[5]equal contribution
[6]present address: Translational Oncogenomics Unit, Istituto Regina Elena, Rome, Italy

In vivo imaging involving the use of genetically engineered animals is an innovative powerful tool for the noninvasive assessment of the molecular and cellular events that are often targets of therapy. Based on the knowledge that the activity of the Nuclear Factor-Y (NF-Y) transcription factor is restricted to proliferating cells, we have generated a transgenic reporter mouse, called MITO-Luc, in which luciferase expression is controlled by a NF-Y-dependent promoter, that represents an unprecedented tool for monitoring cell proliferation in living animals. In these mice bioluminescence imaging of NF-Y activity visualizes areas of cell proliferation, both physiological and during regeneration in response to injury. Using this tool, we highlight for the first time an unknown function of NF-Y activity in liver regeneration. The MITO-Luc reporter mice should facilitate investigations on the involvement of genes in cell proliferation as well as provide a useful model for studying aberrant proliferation in disease pathogenesis. It should be also useful in the development of new anti/pro-proliferative drugs and the assessment of their efficacy and side effects on non target tissues.

P2.42

From zebrafish (*Danio rerio*) to mouse, the function of Nfix in skeletal muscle development is partially conserved during the evolution

A. Pistocchi[1,2], G. Gaudenzi[1], A. M. Fortuny[1,3], A. Pianca[1], E. Foglia[1], G. Cossu[1,2], F. Cotelli[1], G. Messina[1,2]
[1]Dept of Biology, Univ. of Milan, Italy
[2]Division of Regenerative Medicine, DiBiT, San Raffaele Scientific Institute, Milan, Italy
[3]Facultat de Biologia,Universitat de Barcelona, Spain

The transcription factor Nfix regulates fetal-specific transcription in developing murine skeletal muscle, as previously demonstrated by our group. Zebrafish and murine muscle development is similar, however, zebrafish develops at a much faster rate. By 24 hours post fertilization (24 hpf), a functional myotome is formed with primary slow and fast fibers. At 48 hpf, secondary slow fibers differentiate in several body locations. These distinct primary and secondary myogenic waves in zebrafish are reminiscent of specific murine muscle developmental stages (embryonic and fetal). Knock-down of the Nfix ortholog in zebrafish (*nfixa*) alters fiber type development, similar to what has been observed previously in the mouse. In contrast, whereas Nfix mutant mice are motile, nfixa knock-down in zebrafish results in impaired motility with disruption of the sarcoplasmic reticulum. These results demonstrate a conserved role for Nfix and *nfixa* in governing secondary myogenesis, however *nfixa* plays a non-conserved role during myofiber maturation in the zebrafish.

P2.43

Human term placenta and stem cells: morphological localization of stem cells "niche" in fetal membranes

E. Resca[1], M. Riccio[1], M. Zavatti[1],T. Maraldi[1], A. Pisciotta[1], G. Carnevale[1], G. B. La Sala[2], A. Nicoli[2], S.S.W. Shaw[3], P. De Coppi[3], A. De Pol[1]
[1]Dept of Laboratories, Pathological Anatomy and Forensic Medicine, Modena and Reggio Emilia Univ., Modena, Italy
[2]Dept of Obstetrics and Gynecology, Arcispedale Santa Maria Nuova, Reggio Emilia, Italy
[3]Surgery Unit, UCL Institute of Child Health and Great Ormond Street Hospital, London, UK

Chorionic and amniotic membranes of human term placenta are a rich source of stem cells. Stem cell "niche" is a highly balanced microenvironment that allows stem cells to survive and remain quiescent and then respond by replicating, migrating and differentiating to replace or repair tissue, when needed. We have tried to identify the "niche" within the tissue, which has not been well defined yet. The stem cell marker profile has been investigated both in tissue sample and in cultured cells in vitro.
Samples were processed for morphological observations to detect the localization of stem cells "niche" by using immunohistochemical and TEM analysis. Hence, starting from these observations, stem cells were isolated from HTP fetal membrane, after enzymatic digestion, using MACS® selection against c-Kit. The isolated subpopulations were then analyzed by immunofluorescence and western blot in order to check the preservation of stem cell marker during in vitro culture. Immunohistochemical and/or ultrastructural investigations revealed different morphological localizations of stem cells. In the chorionic membrane, c-Kit positive cells were mostly localized in perivascular region and in the connective tissue, while cells close to the syncitiotrophoblast layer had a nuclear positivity for Oct-4 and Stella, markers of pluripotency, identifying the stem potentiality of these cells. In the amniotic membrane, only few amniotic epithelial cells were positive for c-Kit. *In-vitro* cultured placental cells rapidly adhered on plastic culture: interestingly c-Kit positive cells express at the same time Oct-4 marker. These observations suggest that chorionic and amniotic membranes from HTP may retain a reservoir of stem cells throughout pregnancy, and that spatial localization of cells with different expression of stem cell markers may indicates the presence of more than one "niche", depending of the grade of stemness.

P2.44

Spatio-temporal distribution of R-Spondin1 (RSPO1) and its antagonist Dickkopf-1 (DKK1) during prenatal development of the ovary

G.Ricci[1], F.Ferranti[2], M.Caruso[2], R.Canipari[2], S.Majore[3], S.Valentini[3], M.Stefanini[2], P.Grammatico[3] and A.Catizone[2]
[1]Dept of Experimental Medicine, Second Univ. of Naples
[2]Dept of Anatomy, Histology, Forensic Medicine and Orthopedics, "Sapienza" Univ. of Rome
[3]Medical Genetics "Sapienza" Univ. of Rome and Medical Genetics Lab. S.Camillo-Forlanini Hospital of Rome

The embryonic development of the genital apparatus is a complex process whose molecular regulation has been only partially clarified. Since 1990 SRY was identified as the master gene of testis determination while only recently RSPO1 has been demonstrated as the best candidate responsible for ovary determination. RSPO1 belongs to a protein superfamily that comprises four distinct secreted proteins. These ligands bind LRP6, induce its phosphorylation, and promote β-catenin stabilization, similarly to WNTs ligands effects. DKK1 is considered an antagonist of R-spondins and WNTs pathway since it is able to induce LRP6 endocytosis via the association with the co-receptor Kremen.
At present the precise role exerted by RSPO1 in ovary differentiation is still not well known and only few and incomplete expression studies have been carried out on RSPO1/DKK1/Kremen-1 during ovarian development. In this study we report for the first time the distribution pattern of RSPO1 protein and its antagonist DKK1 in the mouse ovary during its organogenetic period (from 11.5 dpc to 18.5 dpc). In particular we found that RSPO1 is clearly detectable since 12.5 dpc as a diffuse signal in the whole ovary and become gradually restricted to the cortical part of the organ starting from 13.5 dpc. The distribution pattern of DKK1 appears similar in the early stages of ovary development however, in addition to the described positivity, several DKK1 positive cells localized in the medullary region become well evident starting from 15.5 dpc to 18.5 dpc. By western blotting analysis we found that the amount of RSPO1 decreases during ovary development from 13.5 dpc to 18.5 dpc while Kremen1 increases. These observations suggest that in the latest developmental stages RSPO1 function is gradually regulated in the ovary by the coordinated action of Kremen-1 and DKK1 and that an appropriate dosage balance of these molecules could be responsible for a proper control of ovary organogenesis.

P2.45

Rosiglitazone stimulates dendritic cells generated from human CD133 positive precursors to acquire a highly efficient immunostimulatory phenotype

M.I. Bonetti[1], M. Santosuosso[2], B. Mazzanti[2], A. Aldinucci[3], C. Ballerini[3], R. Saccardi[2], A. Bosi[2,4], P. Romagnoli[1]
[1]Dept Anatomy, Histology and Forensic Medicine, Univ. Florence, Italy
[2]Hematology Unit, Careggi University Hospital, Florence, Italy
[3]Dept Neurological Sciences, Univ. Florence, Italy
[4]Dept Critical Care Medicine and Surgery, Univ. Florence, Italy

AIMS: Dendritic cells (DC) can differentiate in vitro from CD133+ cells of the human cord blood [1]. DC participate to the injury response in the arterial wall [2] and treatment with a PPAR-gamma stimulant (rosiglitazone) hampers this response in the rat, also affecting DC number and DC-lymphocyte interactions [3]. We have addressed here the effect of rosiglitazone on the differentiation of DC, starting from cord blood CD133+ precursors.
MATERIAL AND METHODS: CD133+ cells purified by immunomagnetic separation were cultured for 18 days with fetal calf serum, cytokines [2], and with or without rosiglitazone, 1 μmol/ml. They were analyzed by flow cytometry, immunofluorescence, electron microscopy and mixed lymphocyte reaction.
RESULTS: Culture led to the appearance of DC expressing MHC-II antigens and the differentiation markers CD80, CD83 and CD86; many expressed CD207/langerin and some also CD1a; the differentiated cells were potent stimulators of mixed lymphocyte reactions. With rosiglitazone fewer DC were generated in culture, but they expressed differentiation and maturation-related antigens in higher percentages and were better stimulators of CD4 positive cells than those generated without the PPAR-gamma agonist.
CONCLUSION: In apparent contradiction with the results in vivo in the rat, the direct effect of rosiglitazone in vitro is to promote the maturation of human cord blood derived cells into DC with a highly efficient immunostimulatory phenotype, although negatively affecting the number of the harvested cells.REFERENCES:
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P2.46

The role of Nfix in post-natal myogenesis and muscle regeneration

G. Rossi[1], S. Antonini[2], S. Monteverde[1], G. Cossu, [1,2], G. Messina[1,2]
[1]Division of Regenerative Medicine, Stem Cells and Gene Therapy, San Raffaele Scientific Institute, Milan, Italy
[2]Dept of Biology, Univ. of Milan, Milan, Italy

Nfix is part of a family of highly conserved proteins that function as transcriptional activators and/or repressors of cellular and viral genes. Members of this family are widely expressed in different organs, and *Nfix* is the most expressed in muscle. Recently, the central role of Nfix in driving the transcriptional switch between embryonic and fetal myogenesis has been highlighted. The aim of of this work is to establish if and how Nfix plays a role in post-natal muscle development, focusing on physiopathological processes such as muscle homeostasis, growth, and regeneration after injury. To address this issue, we have analyzed *Nfix* expression in Satellite Cells both *in vivo* and *in vitro*, characterized a *Nfix* null mouse model, and evaluated the regeneration capacity after injury of *Nfix* null mice, compared with their *wt* littermates. The results have shown that, in the absence of *Nfix*, muscle morphology is altered; moreover, it has been observed a general conversion to a slower-twitching phenotype. Finally, the regeneration capacity of *Nfix* null mice is delayed when compared with their *wt* littermates. In addition to that, *Nfix* is expressed in all the regenerated fibers of the *wt* mouse, reinforcing the hypothesis that its presence is crucial for regeneration. All together, the data obtained demonstrate that Nfix is not only fundamental for the switch from embryonic to fetal myogenesis, but may also be a regulator of adult muscle histogenesis and regeneration. We are now working with two muscle-specific *Nfix* null models, in order to address the question of the specificity of the phenotype observed.

P2.47

Satisfactory performance of hepatocytes in three-dimensional culture on innovative biopolymeric scaffolds

A. Stampella[1], M. Massimi[2], A. Barbetta[3], G. Rizzitelli[3], M. Dentini[3], L. Conti Devirgiliis[1]
[1]Dept Biology and Biotechnology Charles Darwin, Sapienza Univ. of Rome, Italy
[2]Dept Basic and Applied Biology, Univ. of L'Aquila, Italy
[3]Dept Chemistry, Sapienza University of Rome, Italy

The introduction in recent years of innovative biomaterials as scaffolds to replace physiological matrix components has led to significant advances in culture techniques, in terms of cell survival, quantitative expansion, maintenance of differentiated phenotype and specific functions of cells. These bio-artificial tissues can be used as functional substitutes for damaged organs, as models to study complex biological processes, or for tissue-specific toxicology tests. The aim of our research was to identify the most suitable biomaterial for technological applications with hepatocytes, e.g. tissue therapy, cellular transplantation, metabolic analysis of new drugs or screening for potentially toxic substances. Since the possibility to improve the performance of these systems depends strongly on the methods used to create the scaffolds, here we analyzed porous matrices made of gelatin or blends of gelatin and glycosaminoglycans, obtained with different methods for the culture of the C3A cell line, considered a good model of human hepatocytes. Scaffolds were obtained using either a concentrated emulsion-templating technique known as high internal phase emulsion (HIPE) or a gas foaming technique; the latter method uses an inert gas instead of the internal liquid phase toluene, avoiding the use of organic solvent and allowing the creation of scaffolds with larger pores and interconnections. By analyzing cell adhesion, viability, ultrastructural morphology, production of albumin and urea and induction of cytochrome P450-3A4 as the main metabolic markers of hepatocyte functions, it was possible to compare the performances of matrices prepared with the different methods. Taken together our results suggest that the morphology of the scaffolds (surface porosity, void/interconnection size) is less crucial than the utilization of potentially harmful molecules during their creation.

P2.48

Differentiation-independent fluctuation of pluripotency-related transcription factors and other epigenetic marks in embryonic stem cell colonies

Gabriela Sustackova[1], Sona Legartova[1], Stanislav Kozubek[1], Lenka Stixova[1], Jiri Pachernik[2], and Eva Bartova[1]
[1]Dept of Molecular Cytology and Cytometry, Institute of Biophysics ASCR, v.v.i., Brno, Czech Republic
[2]Faculty of Sciences, Masaryk Univ., Brno, Czech Republic

Embryonic stem cells (ESCs) maintain their pluripotency through high expression of pluripotency-related genes. Here, we show that differing levels of Oct4, Nanog, and c-myc proteins among the individual cells of mESC colonies and fluctuations in these levels did not disturb mESC pluripotency. Cells with strong expression of Oct4 had low levels of Nanog and c myc proteins, and vice versa. In addition, cells with high levels of Nanog tended to occupy interior regions of mESC colonies. In contrast, peripherally positioned cells within colonies had dense H3K27 trimethylation (H3K27me3), especially at the nuclear periphery. We also observed distinct levels of endogenous and exogenous Oct4 in particular cell cycle phases. The highest levels of Oct4 occurred in G2 phase, which correlated with the pKi-67 nuclear pattern. Moreover, the Oct4 protein resided on mitotic chromosomes. We suggest that there must be an endogenous mechanism that prevents the induction of spontaneous differentiation, despite fluctuations in protein levels within a mESC colony. Based on the results presented here, it is likely that cells within a colony support each other in the maintenance of pluripotency.

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P2.49

Cholinergic contribution to Schwann cell differentiation in myelinating phenotype

C. Uggenti[1], A. Pisano[1], M.E. De Stefano[1], V. Magnaghi[2], C. Talora[3], J. Wess[4], A.M. Tata[1],
[1]Dept of Biology and Biotechnology Charles Darwin, Research Center of Neurobiology Daniel Bovet, La Sapienza Univ. of Rome, Italy
[2]Dept of Endocrinology, Physiopathology and Applied Biology, Univ. of Milan, Italy
[3]Dept of Experimental Medicine, La Sapienza Univ. of Rome, Italy
[4]NIDDK, National Institutes of Health, Bethesda, USA

ACh, independently on its function to cholinergic synapses, appear involved in the control of nervous system development and can contribute to axon-glia interaction. ACh receptors have been found in glial cells such as astrocytes, oligodendrocytes and Schwann cells suggesting a role for ACh in the maturation and physiology of glial cells.
Rat Schwann cells express different muscarinic receptor subtypes. M2 receptor is the most abundant subtype in and its activation causes a reversible arrest of cell cycle in G1 phase. The negative effect of M2 agonist on cell proliferation suggest an its possible effect on Schwann cell differentiation. Recently we have demonstrated in cultured Schwann cells that the M2 agonist arecaidine causes an increased expression of myelin proteins such as P0, PMP22 and MBP. Moreover we report that the M2 activation causes an up-regulation of transcription factors involved in the induction of myelinating phenotype such as krox 20 and sox 10 and a down-regulation of the genes involved in the maintainance of undifferentiated state such as c-jun and Notch-1. Electron microscopy and morphometric analysis of the sciatic nerve of M2/M4 KO mice have shown that the KO fibers have an altered myelin organization, increased myelin thickness and several degenerating axons. These data suggest that ACh may contribute to Schwann cell differentiation in myelinating phenotype and in the stabilization of compact myelin organization. This appears relevant during fiber maturation and may be an interesting field of investigation in the de-myelinating diseases where ACh may play a strategic relevance for re-myelination.

P2.50

The BH3-mimetic ABT-737 induces death of chemoresistant non small lung cancer stem cells: potential for therapeutics.

G. Zapparelli, F. Pedini, M. Patrizii, F. Francescangeli, M. Biffoni, A. Eramo, F. Lotti, R. De Maria, A. Zeuner
Dept of Hematology, oncology and molecular medicine, Istituto Superiore di Sanità, Rome, Italy

Increasing evidence suggests that tumors arise from a rare subpopulation of cells known as cancer stem cells (CSCs). CSCs possess the ability to self-renew and to undergo asymmetric divisions giving rise to a more differentiated progeny which represents the bulk tumor mass. CSCs have been shown to be resistant to conventional chemotherapy. Therefore it is essential to find CSCs-specific targeted therapies to obtain tumor eradication. The high expression of antiapoptotic proteins frequently found in tumors and its correlation with chemotherapy resistance, encouraged the development of molecules designed to induce apoptosis specifically in cancer cells. BH3 mimetics are a new class of pro-apoptotic anticancer agents that showed efficacy in preclinical animal models and in early stage clinical trials. These agents act by inhibiting the pro-survival function of Bcl-2 and Bcl-XL proteins. Here we demonstrate that the BH3 mimetic ABT-737 has a higher toxicity against non small lung cancer stem cells (NSCLC-SCs) than commonly used antineoplastic agents. In vitro ABT-737 was able to reduce LCSCs self-renewal and induce death of LCSCs and differentiated cancer cells by activating the mitochondrial apoptotic pathway. We found significant mitochondrial depolarization in LCSCs intact spheroids, cytochrome c and AIF release from mitochondria and activation of caspases 3 and 9. In vivo treatment of mice bearing LCSC-derived tumor xenografts with ABT-737 determined a complete arrest of tumor growth as compared with tumors of mice treated with chemotherapy alone. A double staining for CD133 and TUNEL revealed that TUNEL-positive cells overlapped with stem cells identified by CD133 positivity. Caspase 3 and PARP were also activated in CD133+ cells within ABT-737-treated xenografts. Taken together, these results indicate that ABT-737 is able to target the LCSC population present in lung tumors and may represent an effective treatment in lung cancer.

P4.1

New therapeutic strategies in the treatment of Non Small Cell Lung Cancer

R. Alfieri, M. Galetti, S. La Monica, A. Cavazzoni, C. Fumarola, M. Bonelli, S.Tramonti, P.G. Petronini
Dept Experimental Medcine, Univ. of Parma, Parma, Italy

Epidermal Growth Factor Receptor (EGFR) is an established new target for the treatment of epithelial tumors, including non-small cell lung cancer (NSCLC). Small molecules inhibitors, such as erlotinib and gefitinib, have proven to be a useful addition to standard therapy in advanced NSCLC. However, tumor cells often acquire resistance to these EGFR inhibitors. The mechanisms that mediate resistance to gefitinib treatment include secondary mutations in EGFR i.e. the point mutation T790M, the amplification of MET and constitutive or alternative activation of downstream pathways.
In this study we have investigated in a panel of NSCLC cell lines new therapeutic approaches to circumvent acquired gefitinib resistance.
Clear evidence exists for the involvement of constitutive activation of the PI3K/AKT signaling pathway in lung carcinogenesis and in resistance to tyrosine-kinase inhibitors. We assessed the effects of combining the mTOR inhibitor everolimus (RAD001) with gefitinib on a panel of NSCLC cell lines characterized by gefitinib-resistance and able to maintain p70S6K phosphorylation after gefitinib treatment. Everolimus plus gefitinib induced a significant decrease in the activation of MAPK and mTOR signaling pathways downstream of EGFR, however, resulted in a growth-inhibitory effect rather than in an induction of cell death. Indeed, the inhibition of mTORC1 by rapamycin and analogues resulted in the release of the negative feedback loop between p70S6K and IRS1, leading to sustained activation of the survival AKT pathway. Taking advantage from this compensatory pathway, combined therapies designed to block both mTOR and PI3K/Akt pathways may provide a superior efficacy.
We evaluated the potential of combining the gefitinib with a new dual PI3K and mTOR inhibitor in those cell lines showing EGFR-independent activity of the PI3K/AKT pathway, in order to induce both an antiproliferative and a proapoptotic effect.

P4.2

Protein expression in MCF7 cells: Cd-5Fu effects

Y. Asara[1], J.A. Marchal[2], E. Carrasco[2], H. Boulaiz[2], M.A. Garcia[2], A. Montella[1], A. Aranega[2], R. Madeddu[1,3]
[1]Dept of Biomedical Sciences, Univ. of Sassari, Italy
[2]Dept of Human Anatomy and Embriology, Univ. of Granada, Spain
[3]National Institute of Biostructures and Biosystems, INBB, Italy

Cadmium (Cd) is known as a highly toxic metal wich represents a major hazard to the environment. The toxicity of this metal contributes to a large variety of health conditions, including major diseases such as heart disease, cancer and diabetes. The fluoropyrimidines, especially 5-Fluorouracil (5-Fu) are antimetabolite inhibitors of the novo purine and pyrimidines syntheses. 5-Fu plays an important role in standard chemotherapy protocols for a range of solid tumors. Breast cancer represent the most common tumor of female in many industrialized countries. The MCF7 human breast cancer cell line had been used as an excellent experimental model to improve the efficacy of different therapies before use in patients. We studied the synergic or antagonist effect of the antineoplastic agent 5-FU and the environmental carcinogenic agent Cd. Our analysis was done to demonstrated modifications in protein expression related with cell cycle and apoptosis. Cells were plated on 6-well plates in their respective medium. After treatment during 6, 12, 24 and 48 h with 5-Fu and the Cadmium Chloride (CdCl2), with different condition of concentration and time point. Medium was removed and the cells were lysed. The protein sample was subjected to electrophoresis, transferred onto nitrocellulose membranes, and blocked in PBS containing 5% non-fat dry milk for 1 h at room temperature. Primary antibodies were used for Bcl-2, Caspase 8, c-myc, cyclin-D1, cyclin A, p53 and β-actin at 1:100 dilution.
Secondary antibodies used included antirabbit IgG peroxidase conjugate and anti-mouse IgG peroxidase conjugate.
Bands were visualized using the ECL system (Amersham Pharmacia Biotech, UK). Results were concordant with gene expression by qRT-PCR assay. In contrast to 5-FU treatment the addition of Cadmium induced an increase of anti-apoptotic bcl-2 and both proliferative cyclins and c-myc proteins.
Our findings suggest that Cd prevents the cytotoxic effect of 5-FU on breast cancer cells.

P4.3

Involvement of CtBP1-S/BARS in tumour progression: a specific arget for pharmacological intervention

I. Ayala, A. Colanzi, C. Valente, D. Corda
Institute of Protein Biochemistry, National Research Council, Naples

The C-terminal binding protein (CtBP) family includes five proteins that have been implicated in both fission of intracellular membranes and transcriptional repression: CtBP1-L, CtBP1-S/BARS (BARS), CtBP2-L, CtBP2-S and RIBEYE. As a component of fission-inducing machineries, BARS regulates trafficking of membranes in endocytosis and exocytosis and Golgi partitioning during the G2 phase of the cell cycle, a step that also controls cell entry into mitosis. As transcriptional co-repressors, the CtBPs have been linked to tumorigenesis and tumour progression through promotion of epithelial-mesenchymal transition, actions as apoptosis antagonists, and repression of several tumour-suppressor genes. To determine whether CtBP loss of function can provide an anti-tumoral strategy, we depleted CtBP1 and CtBP2 in cells by transfection with siRNAs. This knock-down reduced cell growth and increased cell sensitivity to apoptotic stimuli.
In addition, we have recently seen that BARS is localised at invadopodia, specialised plasma-membrane protrusions formed by invasive cells that are associated with degradation of the extracellular matrix, a basic process for metastasis formation. Transfection of BARS dominant-negative mutants significantly inhibited the invasive ability of these cells, measured as extracellular-matrix degradation. However, although these data indicate that depletion of CtBPs would be beneficial in treatment of cancers, in some cases, such as in melanoma cells, overexpression of CtBPs reduces cell migratory and invasive potential. Therefore, approaches applied to prevent tumour spreading might depend on the origin of a tumour, or its degree of differentiation. These data support the involvement of BARS in tumour formation and spreading. Moreover, they suggest that BARS is a target for the design of anti-cancer therapies. To this end, we are developing newly synthesised small molecules that can differentially modulate the diverse actions of BARS.

P4.4

Dexamethasone inhibits Fas-induced apoptosis in osteoclasts

L. Babini[1], F. Prattichizzo[1], L. Graciotti[1], F. Tomassoni-Ardori[1], G. Borghetti[1], A. Procopio[1,2], M. R. Rippo[1,2]
[1]Laboratory of Experimental Pathology, Dept of Clinical and Molecular Sciences, Polytechnic Univ. of Marche, Ancona, Italy
[2]Center of Clinical Pathology and Innovative Therapies, Italian National Research Center on Aging (INRCA, IRCCS), Ancona, Italy

Dexamethasone is a corticoid anti-inflammatory drug frequently used in autoimmune or inflammatory diseases. It is known that long therapy with this drug or its analogues is often associated with bone loss and it causes the most prevalent form of secondary osteoporosis. This is due, at least in part, to the increase of osteoclastogenesis and osteoclasts (OC) activity and the induction of osteoblasts apoptosis.
Here we show that Fas receptor triggering induces apoptosis of human in vitro differentiated osteoclasts obtained from PBMC of young male healthy donors cultured in presence of the osteoclastogenic factors RANK-L and M-CSF. Pre-treatment of OC with dexamethasone protects cells from Fas-induced apoptosis and this effect could be mediated, in part, by the up-regulation of the anti-apoptotic proteins FLIP, Bcl-2, and survivin. Moreover, immuno-cytochemical staining revealed that dexamethasone induced a cytosolic de-localization of survivin which correlates with its anti-apoptotic role. Surprisingly, the pan-caspase inhibitor zVAD, doesn't protect OC from Fas-induced cell death but has the opposite effect: it enhances Fas-induced apoptosis and has a pro-apoptotic effect per se, suggesting a basal caspase activity role for osteoclasts survival.
In conclusion our experimental data suggest that dexamethasone could favour bone erosion not only by increasing osteoclastogenic activity but also promoting OC survival in presence of the pro-apoptotic cytokine FasL.

P4.5

Activation of stress genes in response to UV-B radiation in sea urchin embryos

R. Bonaventura, R. Russo, F. Zito, V. Matranga
Istituto di Biomedicina e Immunologia Molecolare "A. Monroy", Consiglio Nazionale delle Ricerche, Palermo, Italy

We report the effects on development and stress response proteins/genes of a single dose of UV-B radiation (400 J/m2) on *Paracentrotus lividus* embryos exposed at the cleavage stage (3 hours post-fertilization). We observed a significant impairment of development in 90% of the irradiated embryos, which developed abnormally with major defects in gastrulation and skeleton formation. Results correlated with the delocalization and reduction in number of the skeletogenic cells, called the Primary Mesenchyme Cells (PMCs), expressing the skeleton-specific protein *Pl*-msp130, as measured by indirect immunofluorescence on whole mount embryos. In previous studies we have shown that irradiated embryos increased the levels of the heat shock protein 70 (hsp70) (Bonaventura et al 2005) and of the *Pl*14-3-3ε transcript (Russo et al 2010). Here, we extended our studies to the expression of other stress response proteins, including BAG-3, XPB-ERCC3, NF-kB and Bcl2. Transcripts coding for XPB-ERCC3 and NF-kB were also analyzed by relative Q-PCR. We found an increase in the XPB-ERCC3 transcript levels at 2 hours after irradiation, while no changes were observed in NF-kB expression. By Western blotting, we found an increase in the levels of all the measured proteins 24 hours after UV-B irradiation, except for NF-kB, as compared to controls. In summary, sea urchin embryos have been proven to possess protective anti-apoptotic machinery activated at the transcriptional and translational levels at different times in response to ionizing radiation.
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P4.6

AKTIP, a new lamin interacting protein, is involved in telomere metabolism and DNA replication

R. Burla[1], M.T. Carcuro[1], G.D. Raffa[1], A. Galati[1], S. Cacchione[1], M. Crescenzi[3], G. Cenci[1], L. Ciapponi[1], M. Gatti[1,2], I. Saggio[1,2]
[1]Dept of Biology and Biotechnology Sapienza
[2]IBPM CNR
[3]ISS

Proper telomere maintenance is a crucial process needed to protect the genome against instability and telomere dysfunction that has been linked to tumorigenesis and premature aging. Driven by results assigning a telomeric role to its Drosophila homologue peo, we have collected a set of data on a new human gene that can be linked to telomere metabolism, AKTIP. AKTIP down regulation triggers proliferation impairment, premature senescence and DNA damage response activation. AKTIP KD causes telomere dysfunction, as indicated by the presence of DNA damage foci at telomeres (TIFs) and by that of aberrant telomeres in AKTIP KD p53-/-MEFs, including multiple telomeric signals at the ends of chromosomes, also known as fragile telomeres, indicative of telomere replication impairment. The mechanistic role of AKTIP appears, indeed, to be linked to replication: AKTIP can interact with DNA and with crucial components of the replisome (RPA and PCNA), furthermore, AKTIP KD cells display an intra-S block. A particularly seducing aspect of AKTIP comes from its localization, characterized by a typical punctate signal at the nuclear rim. This pattern is consistent with the interaction of AKTIP with nuclear lamins, which we have assessed by GST-pull down and mass spectrometry, and also with that with components of the replication forks (e.g. PCNA), which typically situate at the periphery of the nucleus in the final part of S-phase. Taken together, our data suggest that AKTIP could become a new important player of the mechanistic scenarios of different human diseases linked to "telomeraging" including cancer and laminopathies.

P4.7

MiRNA characterization in Malignant Mesothelioma tissues and cells

L. Graciotti[1], A. Ceka[1], R. Lazzarini[1,2], S. Di Nuzzo[1], F.B. Aiello[3], A.D. Procopio[1,2]
[1]Laboratory of Experimental Pathology, Dept of Clinical and Molecular Sciences, Polytechnic Univ. of Marche, Ancona, Italy
[2]Center of Clinical Pathology and Innovative Therapies, Italian National Research Center on Aging (INRCA, IRCCS), Ancona, Italy
[3]Dept of Oncology and Neuroscience, Univ. of Chieti, Chieti, Italy

Most patients diagnosed with Malignant Mesothelioma (MM) have poor prognosis and poor response to conventional chemotherapy. As a consequence, there is a compelling need to identify new approach for the treatment of MM patient. A growing body of evidence suggests that miRNAs play crucial roles in tumorigenesis. To date some miRNAs has been recognized altered in many neoplasm including Mesothelioma and some of them has been proposed as marker for diagnosis or prognosis.
Regarding MM various past researches pointed to different miRNA and we suspect that the reason of such variability can be ascribed to different samples origin.
Therefore we analysed miRNAs content in surgical samples taken from patients diagnosed for pleural MM, processing them using a laser-capture microdissection in order to reduced as much as possible the surrounding tissue and compared the results with the original un-dissected sample. A further analysis was undertaken on primary cultured cells obtained from some of the MM patients.
Data obtained from the profiling was further analysed if the miRNAs was up or down-regulated more than three fold respect the appropriate control.
We found one down-regulated miRNA, mir-214, that was altered in all sample of MM, the putative targets of mir-214 were determined and interestingly some of the over-expressed proteins in MM already reported in literature were target of mir-214.
Mesothelin a well known protein typically associated with MM was one of the target of mir-214. Mesothelin was also up regulated in primary cell lines of MM. Transfection of the cells with mir-214 induced a reduction in mesothelin expression thus confirming the role of this miRNA in the pathogenesis of MM.

P4.8

Relationship between autophagy and apoptosis in *Paracentrotus lividus* embryos cadmium exposed

M. Agnello, R. Chiarelli, G. Morici, M.C. Roccheri
Dept STEMBIO (Biologia Cellulare) Univ. of Palermo, Palermo

Sea urchin embryo is a developmental model that offers an excellent opportunity to investigate the possible adaptive response of cells exposed to different stress during differentiation. We previously demonstrated that cadmium treatment triggers the accumulation of metal in embryonic cells and the activation of defense systems depending on concentration and exposure time, through the synthesis of heat shock proteins and/or the initiation of apoptosis.
Using several techniques to detect autophagy (neutral red, acridine orange and LC3-detection) we demonstrated that Cd-exposed P. lividus embryos adopt this process as an additional stratagem to safeguard the developmental program. In particular we observed that embryos treated at subletal Cd concentration activate a massive autophagic response after 18 h, which decreases between 21 and 24 h, in the opposite of apoptotic process.
In order to investigate a possible temporal relationship between autophagy and apoptosis, we tested apoptotic signals by TUNEL and immunofluorescence in situ assays of cleaved caspase-3. Quantitative analysis has shown that embryos activate a massive apoptosis after 24 h of Cd-exposure. Therefore a functional relationship between autophagy and apoptosis was estimated evaluating apoptotic signals in Cd-exposed embryos, upon treatment with the autophagic inhibitor 3-methyladenine. We found that the inhibition of autophagy produced a contemporaneous reduction of apoptotic signals, suggesting that the two phenomena are functionally related. In effect using methylpyruvate, a cell-permeable substrate for ATP production, apoptotic signals were substantially restored.
These data could be explained considering that autophagy could energetically contribute to apoptotic execution through its catabolic role.

P4.9

Long non-coding antisense RNA controls UCHL1 translation through the 5' overlapping region and an embedded SINEB2 repeat

C. Carrieri[1], M. Biagioli[1,2], L. Cimatti[1], A. Beugnet[3], I. Ferrer[4], C. Santoro[5], S. Fedele[1], S. Zucchelli[1,6], A. Forrest[7], P. Carninci[7], S. Biffo[3,8], E. Stupka[9], S. Gustincich[1,2]
[1]Neurobiology Sector, International School for Advanced Studies (SISSA), Trieste, Italy
[2]The Giovanni Armenise-Harvard Foundation Laboratory, Trieste, Italy
[3]Laboratory of Molecular Histology and Cell Growth, DIBIT, San Raffaele Scientific Institute, Milano, Italy
[4]Institute of Neuropathology, IDIBELL-University Hospital Bellvitge, Barcelona Univ., Llobregat, Spain
[5]Dept Medical Sciences, Eastern Piedmont Univ., Novara, Italy
[6]Istituto Italiano di Tecnologia IIT, SISSA Unit
[7]Omics Science Center, RIKEN Yokohama Institute, Japan
[8]Dept Environmental and Life Sciences, Eastern Piedmont Univ., Alessandria, Italy
[9]UCL Cancer Institute, Paul O' Gorman Building, Univ. College London, London, UK

The majority of the mammalian genome is transcribed. This generates a vast repertory of transcripts that includes protein-encoding mRNAs, long non coding RNA (lncRNA) and repetitive sequences, such as SINEs (short interspersed nuclear element). A large percentage of ncRNAs are nuclear-enriched with unknown function. Antisense (AS) lncRNAs may participate in sense/antisense pairs (S/AS) overlapping with a protein coding gene on the opposite strand and regulate epigenetic silencing, transcription and mRNA stability. Here we identify AS Uchl1 as a nuclear-enriched lncRNA AS to Uchl1, a gene involved in brain function and neurodegenerative diseases. AS Uchl1 increases UchL1 protein synthesis at post-transcriptional level, identifying a new functional class of lncRNAs. AS Uchl1 activity depends on the presence of a 5' overlapping sequence and an embedded inverted SINEB2 element. In addition, mTORC1 inhibition by rapamycin causes an increase in UchL1 protein that is associated to shuttling of AS Uchl1 RNA from the nucleus to the cytoplasm and recruitment of the overlapping sense protein-encoding mRNA to active polysomes for translation. Thus, AS Uchl1 is the first lncRNA able to stimulate translation of specific mRNAs, in conditions in which CAP-dependent translation is reduced.

P4.10

SERPINB3 increases resistance to cisplatin-induced cell death in hepatoma cells through inhibition of the Permeability Transiton Pore

F. Ciscato[1], M. Sciacovelli[2], M.G. Ruvoletto[1], P. Bernardi[2], A. Rasola[2], P. Pontisso[1]
[1]Dept of Clinical and Experimental Medicine and [2]Dept of Biomedical Sciences, Univ. of Padua, Italy

SERPINB3, a member of the ovalbumin protease inhibitor family, is overexpressed in epithelial tumors and in hepatocellular carcinoma (HCC), wherein it increases invasiveness and proliferation. Chemotherapeutic resistance is crucial to poor prognosis in advanced HCC, in which the cells become refractory to several noxious stimuli. Mitochondria are key regulators of cell death and opening of the mitochondrial permeability transition pore (PTP) constitutes a point of no-return in cell commitment to death. Reduced PTP sensitivity to diverse stress stimuli has been observed both in vitro and in vivo cancer models, and could play a primary role in tumor cell resistance to anticancer drugs. To determine the molecular mechanisms through which SERPINB3 confers resistance to chemotherapeutic-induced apoptosis, HepG2 hepatoma cells were stably transfected with human SERPINB3. Cell death was induced with the first-line chemotherapeutic Cisplatin and PTP inducer EM 20-25; then it was evaluated by FACS and MTT analyses. In parallel, PTP sensitivity was determined by the fluorimetric Ca2+ retention capacity assay in the presence of Calcium Green-5N, and the subcellular localization of SERPINB3 was assessed by partial tryptic digestion of purified mitochondria lysates. HepG2 cells expressing SERPINB3 showed a significant and dose-sensitive increase in the resistance to cell death induced by Cisplatin and EM 20-25 compared to control cells, as documented by both MTT and FACS experiments. Moreover, we found that a fraction of SERPINB3 was located in mitochondria and after treatment this fraction increased. SERPINB3 can inhibit PTP opening after Cisplatin treatment and after direct PTP induction. Taken together, these results indicate that SERPINB3 protects from apoptosis acting on core components of the mitochondrial apoptotic machinery.

P4.11

Interplay between genetic and epigenetic modifications in a model of inflammation-driven cancer

A. Collino[1], S. Ghisletti[1], F. Iannelli[1], S. Sinha[1], P. Nicoli[1], I. Barozzi[1], P. Carninci[2], A. Tanay[3], F. Ciccarelli[1], G. Natoli[1]
[1]Dept of Experimental Oncology, European Institute of Oncology, Milan, Italy
[2]Functional Genomics Technology Team, Omics Resource Development Unit, Omics Science Center, Riken Yokohama Institute, Yokohama, Japan
[3]Dept of Computer Science and Applied Mathematics, Weizmann Institute, Rehovot, Israel

Increasing evidence is suggesting the importance of epigenetic alterations in tumor development and progression. Currently, the epigenetic modifications that have most often been associated to cancer are aberrations in DNA methylation and histone modifications. However, the relationship between epigenetic changes and genetic mutations in tumorigenesis is still poorly understood. Indirect data from patients suggest that polyclonal epigenetic changes often precede, and favor, the emergence of clonal mutations. However, this has never been investigated and directly verified in an experimentally tractable cancer model that provides access to multiple intermediate phases of tumor development.
Despite lacking intrinsic mutagenic potential, chronic inflammation is acknowledged to be one of the main risk factors for many tumors, as demonstrated by a plethora of epidemiological and molecular observations. Therefore, tumorigenic activity of chronic inflammation might reside in its ability to stimulate epigenetic changes that would lay the ground for subsequent genetic alterations.
Our work aims at identifying inflammation-derived epigenomic modifications in the early steps of tumor development and to assess their correlation with, and impact on, DNA mutations. Specifically, mutations and epigenetic variations will be measured using large-scale genomic approaches at multiple stages of tumorigenesis in *mdr2*-knockout mice, a model of purely inflammatory hepatocellular carcinoma (HCC). The comparative analysis mutations and epigenetic changes along the progression from chronic inflammation to dysplasia and eventually carcinoma will clarify their chronological relationship and mutual interplay.

P4.12

Quantitative and functional analysis of polycystins in T-lymphoblasts from ADPKD patients and in PKD2-downregulated Jurkat cells

S. Missiroli[1], A. Mangolini[1], G. Aguiari[1], D. Lugli[2], L. Palmieri[2], A. Zuccalà[3], L. Cagnoli[4], R. Mignani[4],G. Russo[5], L. Catizone[5], R. Magistroni[2], L. del Senno[1]
[1]Biochemistry and Molecular Biology, Univ. of Ferrara, Nephrology units from Imola[3], Rimini[4], Ferrara[5] and Modena[2], Italy

BACKGROUND: Autosomal dominant polycystic kidney disease (ADPKD) is caused by mutations in PKD1 (85%) and PKD2 (15%) genes. Complex gene analysis and several missense changes limit pre-symptomatic DNA testing, and data from functional studies are required. Polycystin-1 (PC1) is a receptor-membrane protein which can interact with the Ca2+-permeable channel polycystin-2 (PC2). This study aims to define whether defects in PC1 and/or PC2 expression may be detected in peripheral blood-derived T-lymphocytes (TL) from ADPKD subjects.
METHODS: IL2-stimulated TL were obtained from 31 dialyzed control and 34 ADPKD patients from 4 Nephrology Units. PC1 and PC2 were analyzed by immunoblotting. Functional studies included TL cell proliferation, clumps size and Ca2+ measurements in Fura-2AM-loaded cells stimulated with platelet-activating factor (PAF). Variations in Ca2+ levels were preliminarily investigated in Jurkat T-cells, wt and expressing a PC2-GFP, before and after PC2 depletion by PKD2-siRNA.
RESULTS: Differently from PC1, PC2 expression was easily detectable by immunoblotting in TL. No differences were found from ADPKD and non ADPKD samples. However, TL from genetically characterized ADPKD2 had less PC2 compared to controls. Cell proliferation, death and size of clumps were increased in ADPKD TL. Functional activity of PC2 was shown in Jurkat cells by reduction in PC2-GFP and in evoked Ca2+ levels (~ 25%) after PKD2 downregulation. Consistently, evoked Ca2+ levels were lower in ADPKD than in control TL (85,18±6,3 vs 125±20).
CONCLUSIONS: A functional PC1/PC2 channel complex should exist in TL cells because mutations in PKD1 or PKD2 induce a reduction in PAF-evoked Ca2+ levels and an increase in cell survival and clump size. These features could identify the PKD phenotype. TL-cells, therefore, may contribute to pre-symptomatically identification of ADPKD subjects.
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P4.13

P63 role in breast cancer stem cells

S. Di Franco[1], A. Benfante[1], M.L. Colorito[2], F. Iovino[1], D. Barcaroli[3], M. Todaro[1], V. De Laurenzi[3], G. Stassi[1,4]
[1]Dept of Surgical and Oncological Sciences, Cellular and Molecular Pathophysiology Laboratory, Univ. of Studies of Palermo, Italy
[2]Laboratorio di Tecnologie Oncologiche (LATO), Fondazione Istituto San Raffaele - G. Giglio di Cefalù, Palermo, Italy
[3]Dept of Biomedical Sciences, Univ. "G. d'Annunzio" Chieti–Pescara, Chieti, Italy
[4]Cellular and Molecular Oncology, IRCCS Fondazione Salvatore Maugeri, Pavia, Italy

Since their discovery, p53 homologues, p63 and p73, have been investigated for their possible role in cancer development, precisely because of their homology with p53, known to be one of the most mutated genes in cancer. However, early studies showed that only a small part of the tumors bring mutations in these genes, suggesting that perhaps their main role was not in the development of cancer. Subsequent studies underlined that an altered expression, rather than a mutation in these genes, could be found in various cancers. In particular, p63, fundamental in the development of stratified epithelia, was the subject of interest since it appears to play a key role in tumors of epithelial origin. p63 is normally expressed, at nuclear level, in a subset of basal myoepithelial cells surrounding the glandular cells. It 's been shown that in some cases of breast cancer the expression of p63 is altered, and usually this event correlates with a lower degree of cell differentiation and increased tumor aggressiveness. Here we present data, both in vitro and in vivo, on how the expression of different isoforms of p63 affects breast cancer progression, using the breast cancer stem cells as a predictive model.

P4.14

Identification of microRNAs targeting the DNA damage response in breast cancer

A. Bisso[1,2], M. Faleschini[1,2], F. Zampa[1], S. Piazza[1], L. Santarpia[3], V. Cappelletti[4], M.G. Daidone[4], R. Agami[5], G. Del Sal[1,2]
[1]LNCIB, Area Science Park, Padriciano, Trieste, Italy
[2]Dipartimento di Scienze della Vita, Univ. of Trieste, Trieste, Italy
[3]"Sandro Pitigliani" Medical Oncology Unit, Hospital of Prato, Istituto Toscano Tumori, Prato, Italy
[4]Dept of Experimental Oncology, Fondazione IRCCS Istituto Nazionale dei Tumori, Milano, Italy
[5]Division of Tumour Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands

Several in vivo and in vitro data demonstrated that the DNA damage response (DDR) plays a crucial role in tumorigenesis acting as an anti-cancer barrier. Indeed, cells respond to DNA breaks caused by both exogenous (e.g. chemicals, chemotherapeutics) and endogenous (e.g. deregulated function of oncogenes) sources of DNA damages by properly triggering the activation of the DDR. The latter involves the recruitment of DNA repair proteins to sites of damage and the activation of checkpoint mechanisms to slow down cell cycle progression, thus allowing DNA damage repair or, if the damage could not be repaired, leading to apoptosis or cellular senescence. As a consequence, alterations of DDR functionality have a deep impact on cell proliferation, survival, genomic instability and tumor progression.
microRNAs (miRNAs) are small non coding RNAs that finely regulate gene expression by binding the 3'UTR of their target mRNAs, thus altering their translation, stability and localization. It has been shown that several miRNAs modulate critical cellular processes deregulated in cancer, acting either as oncogenes or tumor suppressors.
In the present study, we focused on the identification of miRNAs as new modulators of DNA damage activated signaling in several normal and transformed breast cancer cells. In particular, we discovered that miR-181 family acts as a modulator of the levels of ATM (ataxia telangiectasia mutated) kinase, one of the most important proteins involved in sensing DNA double strand breaks and activating DDR. The role of miR-181 family in the DDR pathway, its involvement in the induction of cellular senescence triggered by oncogenic stress (OIS) and its relevance in breast cancer will be presented.

P4.15

An early impact of ER stress, inflammation and oxidative stress in the CLN8^{mnd} mouse model of neuronal ceroid lipofuscinosis

G. Galizzi[1], I. Deidda[1], D. Russo[1], C. Cascio[1], R. Passantino[1], G. Drago[1], P. Bigini[2], P. Guarneri[1]
[1]CNR Institute of Biomedicine and Molecular Immunology, Neuroscience Unit, Palermo, Italy
[2]Mario Negri Institute for Pharmacological Research, Milan, Italy

The CLN8^{mnd} or *mnd* (motor neuron degeneration) mouse is a naturally-occurring model of the CLN8-associated late-infantile form of neuronal ceroid lipofuscinoses (NCLs). The NCLs are a group of lysosomal neurodegenerative diseases characterized by epilepsy, progressive motor and cognitive decline, blindness, and by the lysosomal accumulation of autofluorescent lipopigment. The CLN8^{mnd} carries a frameshift mutation in the CLN8 gene, likely encoding a truncated protein at the endoplasmic reticulum (ER), and exhibits premature-onset retinopathy and adult-onset neurological and behavioural dysfunctions such as hyperactivity, aggressiveness, epileptic-like repetitive brain activity, cognitive deficit, mild ataxia and hind limb clutching progressing to severe spastic paralysis. The typical autofluorescent inclusions staining positive for the mitochondrial ATP synthase subunit c appear far before the development of *mnd* symptoms. The pathomechanisms of *mnd* disease as well as NCLs' diseases remain still unclear. We and others have demonstrated that changes in calcium levels, mitochondrial energy and lipid metabolism as well as the involvement of oxidative stress together with the formation of lipid peroxides and HNE-adducts occur in the progression of the *mnd* disease. By analyzing the ER-stress pathways in the *mnd* mice at the presymptomatic state, we observed that ER-stress responses differed within the CNS regions. However, all the *mnd* structures exhibited the typical autofluorescent inclusions and developed inflammatory responses integrated with ER-stress pathways. We here show that very early TNF-alpha-mediated responses and changes in the oxidative state, as detected by the ER oxidoreductin (ERO1alpha), may affect the autophagy-lysosomal degradation machinery.

P4.16

Hsp70 is involved in MMP2 level in mouse mesoangioblast stem cells

F. Geraci, G. Turturici, G. Sconzo
Dept STEMBIO sez Cellular Biology, Univ. of Palermo, Palermo, Italy

Mouse mesoangioblasts are vessel derived stem cells which are able to differentiate in almost all mesodermic tissue. They have the ability to migrate and to cross the endothelial barrier. We have previously demonstrated that they are able to release membrane vesicles containing matrix metalloproteinases (MMP2, MMP9), important in cell migration, and that they express Hsp70 under physiological growth condition. The aim of this study was to evaluate whether or not there is a link between Hsp70 and MMP 2 in these cells. For this reason we studied MMP2 both in mesoangioblasts and in one Hsp70 silenced cellular clone. Membrane vesicles were isolated from the two clones and the gelatinolytic activity of MMP2 was detected by zymography. Densitometric analysis showed a reduction in both proMMP2 and MMP2 bands in silenced clone. According to these data it is possible to hypothesize a link between MMP2 and Hsp70 level in these cells. Quantitative real time PCR demonstrated that this reduction was due to a decrease in MMP2 mRNA level and not to protein degradation. It is known that MMP2 is transcriptionally activated by NF-kB. The diminution of MMP2 level in silenced clone could be due to a physical interaction between NF-kB and Hsp70. This possibility was tested by immunoprecipitation assays. p65 was found to co-immunoprecipitate with Hsp70, while no co-immunoprecipitation was observed between Hsp70 and IkB. In addition, in silenced cells there is a small reduction in p65 cytoplasmic level, whereas a significant reduction was observed in the nucleus.
As MMP are involved in cell migration, we used an in vitro assay system to evaluate the effects of MMP2 quantity in cell migration and ECM invasion. The obtained results showed that Hsp70 silenced cells had a lower migrating capability.
These data let us to hypothesize that Hsp70 regulates MMP2 level and this process is probably due to its link with NF-kB. In conclusion, Hsp70 improve cell migration capability.

P4.17

Glycolysis regulates Ca^{2+} compartmentalization and the onset of stress-induced apoptosis

C. Cerella[1,2], E. Bruni[1], M. De Nicola[1], M. Diederich[2], L. Ghibelli[1]
[1]Dipartimento di Biologia, Univ. di Roma Tor Vergata
[2]Laboratoire de Biologie Moleculaire et Cellulaire du Cancer, Hopital Kirchberg, Luxembourg

Apoptogenic damaging treatments such as hydrogen peroxide, puromycin or etoposide immediately and transiently inactivate glyceralheyde-3-phosphate dehydrogenase (GAPDH) via ADP-ribosylation; the consequent block of glycolysis delays apoptosis until relapse of the glycolytic flux (at about 1.5 h post-stress), indicating that this is part of a stand-by mechanism aimed at inhibiting apoptosis until an eventual repair and recovery of the damaged cells occur [1]. The same treatments produce alterations of cytosolic and ER Ca2+, suggestive of transient sarco-endoplasmic reticulum Ca+ ATPases (SERCA) inhibition, with a timing similar to the alterations of glycolysis [2]. We analyze here if the two phenomena (SERCA and glycolysis inhibition) may be linked by a cause-effect relationship. We found that exogenous glycolysis inhibition inhibits SERCA, showing that in our cell systems (U937 and Jurkat leukemia cells) SERCA activity requires glycolytic ATP. This is accompanied by reduced apoptosis. The same is found with the SERCA inhibitor tapsigargin, indicating that a) apoptosis requires a Ca2+ filled ER and/or working SERCA; b) that glycolysis and SERCA works in a similar pro-apoptotic pathway induced by cell damage; c) that damaged cell spontaneously block this pathway.

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P4.18

The effects of Idebenone on mitochondrial bioenergetics

V. Giorgio[1], V. Petronilli[1], A. Ghelli[2], V. Carelli[3], M. Rugolo[2], P. Bernardi[1]
[1]Dept of Biomedical Sciences and CNR Institute of Neurosciences, Univ. of Padova, Padova, Italy
[2]Depts of Evolutionary and Experimental Biology, Univ. of Bologna, Bologna, Italy
[3]Neurological Sciences, Univ. of Bologna, Bologna, Italy

We have studied the effects of idebenone (IdB), a short-chain analog of coenzyme Q10, on mitochondrial function in situ. We have used cybrids generated by fusion of osteosarcoma 143B.Tk- cells with enucleated fibroblasts derived from one control (HQB17) and one LHON patient (RJ206, harboring the 3460/ND1 LHON mutation causing G3460A aminoacid substitution in the MT-ND1 subunit); and XTC.UC1 cells derived from a human thyroid carcinoma, bearing a C insertion at bp3571 in ND1, which generates a premature stop codon at aminoacid 101 of MT-ND1. We found that addition of IdB to HQB17 cybrids caused mitochondrial depolarization and NADH depletion, which were inhibited by cyclosporin (Cs) A, suggesting an involvement of the permeability transition pore (PTP), consistent with our previous work in isolated mitochondria. On the other hand, addition of dithiothreitol (DTT) together with IdB did not cause PTP opening, and rather allowed maintenance of the mitochondrial membrane potential in situ. Treatment of HQB17, RJ206 and XTC.UC1 cells with pre-reduced Idebenol sustains membrane potential (in the presence of CsA) and ATP synthesis in permeabilized cells, even in the presence of rotenone and malonate, consistent with earlier results. These findings suggest that Idebenol could be useful in Complex I deficiencies provided that it is maintained in the reduced state, a task that in some but not all cell types appears to be performed by dicoumarol-sensitive NAD(P)H:quinone oxidoreductase NQO1. The potential advantage of maintaining respiration at Complex III and allowing NADH oxidation to occur should be carefully weighed against the inhibitory effect of IdB on Complex I.

P4.19

The mitochondrial chaperone TRAP1 and the regulation of the respiratory chain in tumor cell models

G. Guzzo[1], M. Sciacovelli[1], F. Esposito[2], M. Landriscina[3], P. Bernardi[1], A. Rasola[1]
[1]Dept of Biomedical Sciences, Univ. of Padova, Padova, Italy
[2]Dept of Biochemistry and Medical Biotechnology, Univ. Federico II, Naples, Italy
[3]Dept of Medical Sciences, Univ. of Foggia, Foggia, Italy

Cancer is a highly heterogeneous and complex disease, whose development requires a reorganization of cell metabolism. Most tumor cells downregulate mitochondrial oxidative phosphorylation and increase the rate of glucose consumption and lactate release, independently of oxygen availability. This metabolic rewiring is believed to favour tumor growth and survival.
Tumor cells express high levels of TRAP1 (Tumor necrosis factor receptor associated protein 1), a mitochondrial chaperone endowed with anti-apoptotic functions and a protective role in the response to oxidative stress. Based on these premises, we decided to investigate if TRAP1 is involved in the energy metabolism dysregulation that characterizes the process of tumorigenesis.
To this aim, we have investigated the oxygen consumption rate (OCR) of tumor cell models where TRAP1 expression had been stably down-modulated by RNA interference, comparing it with cells expressing scrambled shRNAs, and we have found that TRAP1 expression correlates with a lower level of mitochondrial respiration, which diminishes cell OCR. To dissect the mechanism by which TRAP1 inhibits respiration, we have studied whether it interacts with respiratory chain (RC) complexes through blue native gels and immunoprecipitations. We have found that TRAP1 interacts with RC Complex I, II and IV. The in vitro activity of RC Complex I and Complex II is increased by the down-modulation of TRAP1, whereas Complex IV activity is unaffected. Similarly, TRAP1 inhibits RC Complexes I and II in colorectal tumor samples with respect to the surrounding normal mucosae. Chemical inhibition of TRAP1 rescued the activity of RC complexes, whereas 3-nitropropionic acid, a Complex II inhibitor, abrogated any difference in the OCR of cells with or without TRAP1. We therefore postulate that TRAP1 contributes to the reprogramming of tumor cell metabolism through its interactions with RC complexes, thus favouring the process of neoplastic transformation.

P4.20

Mitochondrial ROS drive autophagy and cell death in dopaminergic neuronal cells: essential role of lysosomal iron

M. Cagnin, I. Fiorentino, A. Giovia, R. Castino, C. Isidoro
Laboratorio di Patologia Molecolare and Centro di Biotecnologie per la Ricerca Medica Applicata, Univ. del Piemonte Orientale "A. Avogadro", Dipartimento di Scienze Mediche, Novara, Italy

Autophagy, a lysosomal degradation pathway, is triggered by oxidative stress as a defensive response. How autophagy and death pathways are coordinated in cells subjected to oxidative stress is still poorly understood. In human neuroblastoma SH-SY5Y cells hydrogen peroxide (H2O2, 200 μM) rapidly (<5 min) induced autophagy, as shown by processing and vacuolar relocation of LC3. Accumulation of autophagosome peaked at 30 min of H2O2 exposure. The continuous presence of H2O2 eventually (at >60 min) caused autophagy-dependent annexin V-positive cell death. Infection with a recombinant adenovirus expressing a dominant negative mutant of Vps34 or genetic silencing of beclin-1 prevented induction of autophagy and abrogated lysosome leakage, bax activation, caspase-dependent apoptosis and necrosis induced by H2O2. H2O2 rapidly (5 min) triggered the formation of dichlorofluorescein (DCF)-sensitive HO. free radicals within mitochondria, while the mitochondria-associated oxidoradicals revealed by MitoSoxTM (O2.-) became apparent after 30 min of exposure to H2O2. The lysosomotropic iron chelator deferoxamine (DFO) prevented the mitochondrial generation of both HO. and O2.- and suppressed the induction of autophagy and of cell death by H2O2. Upon exposure to H2O2, Akt was intensely phosphorylated in the first 30 min, concurrently with mTOR inactivation and autophagy, and it was dephosphorylated at 2 h, when >50 % of the cells were dead. DFO did not impede Akt phosphorylation, which therefore was independent of ROS generation, but inhibited Akt dephosphorylation. The present work establishes HO. as the autophagy-inducing ROS and highlights the need for free lysosomal iron for its production within mitochondria in response to hydrogene peroxide. Further, our data demonstrate the link between hyper-activation of autophagy and apoptosis in dopaminergic neuronal cells chronically exposed to oxidative stress.
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P4.21

Yeast models for unfolded protein response related disorders

F. Lari, E. van Anken
Dept Genetics and Molecular Biology, San Raffaele Institute, Milan

Secretory proteins fold in the endoplasmic reticulum (ER) assisted by ER chaperones. When the ER folding capacity is exceeded, the Unfolded Protein Response (UPR) is activated. The UPR is thus key to the development of secretory cells. The UPR is also implicated in a range of pathologies. Folding deficient mutant alleles of ER client proteins can lead to disease, either because: accumulation of misfolded proteins causes proteotoxic aggregates; misfolded proteins provoke a maladaptive UPR leading to apoptosis or ultimately they accumulate in the ER but the UPR is not activated and the cell architecture is severely compromised. Since many of the salient features of the UPR are conserved from yeast to man, we develop yeast models that recapitulate these UPR related disorders by inducible expression of mutant alleles coding for: Ig heavy chain ?CH1; P0glycoprotein S63del; Uromodulin C150S. We score for ER stress sensitivity and UPR signalling to stratify differences in outcome of ER homeostasis caused by the various misfolded proteins.

P4.22

Preliminary results on functional characterization of Bcl-2-β, a soluble Bcl-2 protein isoform

R. Loffredo, M. Mazzierli, I. Granucci, A. Lupia, F. Di Gesualdo, E. Witort, M. Lulli, S. Capaccioli
Dept of Experimental Pathology and Oncology, Univ. of Florence and Phoenix Onlus Stem Cell Foundation for Human Life

Introduction. Some years ago, we have discovered that *bcl-2* gene expression is also subjected to post-transcriptional control based on interactions among an Adenine and Uracil-Rich Element (ARE) of its mRNA and several ARE-Binding Proteins (AUBPs), which modulate *bcl-2* mRNA stability with impact on cell life and death. We then demonstrated that Bcl-2 protein itself plays also the additional role of *bcl-2* mRNA destabilizing AUBP. Two isoforms of Bcl-2 protein have been described: the Bcl-2-α antiapoptotic protein, endowed with the ARE and a hydrophobic domain allowing its anchorage to the mitochondrial membrane, and the shorter Bcl-2-β, avoided of both elements and therefore soluble and not subjected to post-transcriptional control, which function is unknown.
Aims. To unravel the function of Bcl-2-β, on the basis of the above premises but also of clue that very often, immunochemical analyses of a variety of tumors show that Bcl-2 is, paradoxically, an independent predictor of good prognosis
Results. By means of a highly specific nested RT-PCR and Western blot analyses we have identified *bcl-2-α* and *bcl-2-β* mRNA, as well as their relevant Bcl-2 protein isoforms, in Hek293, CaCo2, DLD-1, Karpas 422, M14, Mewo, A375, HeLa and Daudi cell lines. Evaluating the effects of either isoform-specific or nonspecific siRNAs on *bcl-2-α* and *bcl-2-β* mRNA and protein levels, we observed that silencing of Bcl-2-β resulted in an enhancement of *bcl-2-α* mRNA and protein. Furthermore, cell commitment to apoptosis by UV irradiation was paralleled by the lowering of *bcl-2-α* mRNA and the relevant protein, while the levels of *bcl-2-β* mRNA and protein were unaffected.
Conclusions. Our results confirm our working hypothesis: the two isoforms of Bcl-2 play completely different roles, being Bcl-2-α the well-known mitochondrial antiapoptotic protein and Bcl-2-β a cytoplasmic *bcl-2* mRNA destabilizing AUBP.
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P4.23

CSN5/JAB1 influences oncogene-induced responses in hepatocytes upon MYC induction

A. Lukacs, M. Panattoni, R. Pardi
Leukocyte Biology Unit, Vita-Salute San Raffaele Univ., Milan, Italy

The acute upregulation of an oncogene in non-transformed cells does not lead directly to transformation, it rather leads either to cell cycle arrest, apoptosis or senescence. The COP9 signalosome (CSN) is an evolutionarily conserved protein complex whose absence leads to embryonic death in mice. CSN is involved in the regulation of Cullin-based E3 ligases, influencing the degradation of a wide range of protiens that take part in all basic cellular processes.
The analysis of online microarray data reveals that CSN subunit transcript abundance shows an increase in the liver of patients that have hepatocellular carcinoma (HCC) compared to normal donors. This increase correlates with disease progression while the CSN-coding genes in general are mutated at a very low rate in cancer.
To observe the effect of the CSN on pathway regulation in the context of oncogene-induced responses, we performed real-time PCR based arrays in mice that lack the catalytic subunit of the CSN (CSN5/JAB1) in a liver specific manner. As a result of an adenoviral infection of MYC, a well characterized liver oncogene, selected cell cycle components show upregulation in the knockout versus wild type, while NF-kB signaling components are downregulated. Network analysis of these
genes identifies a core network. Gene set enrichment analysis (GSEA) reveals that our core network is composed of the most differentially expressed genes in human HCC when comparing patients having HCC to those without. These results suggest that our mouse model shares pathway dysregulation with human HCC. The members of the core network are involved in sensing replicative stress, thus high CSN5/JAB1 levels seem to be permissive for cancer progression because of the repression of physiological safeguard responses that sense oncogene overdose. We are currently working in vivo and in vitro on the mechanistic details to reveal the effectors of the process.

P4.24

Coenzyme Q10 applied to rat corneas as eye drops, reaches the retina where it protects retinal cells from apoptotic agents: possible application for apoptosis related retinal degenerations

M. Lulli[1], I. Granucci[1], R. Loffredo[1], F. Di Gesualdo[1], A. Lupia[1], M. Dal Monte[2], S. Capaccioli[1] , E. Witort[1]
[1]Dept of Experimental Pathology and Oncology, Univ. of Florence
[2]Dept of Biology, Univ. of Pisa

Premises. Exposure to environmental damaging agents, aging and metabolic diseases induce increased apoptotic death of retinal cells, often resulting in complete loss of sight and major cause for legal blindness worldwide. We previously discovered that, independently of its free radical scavenging property, Coenzyme Q10 dramatically reduced apoptosis by inhibiting mitochondrial depolarization in vitro (Papucci et al. J Biol. Chem. 2003; 278: 28220–28228).
Results. Here, we provide clear evidence that Coenzyme Q10 administered as eye-drops to cornea rapidly reached the choroid-retina area, and prevented both ganglion and retinal pigmented epithelial cells apoptosis in vivo, by hampering mitochondrial permeability transition that leads to apoptosis by the extrusion of cytochrome c to cytoplasm.
Conclusions. These experimental evidences strongly suggest that topically administered Coenzyme Q10 could be a simple, non-invasive, candidate for the prophylaxis and treatment in variety of severe retinal degenerative disorders - from glaucoma to AMD – in which pathogenesis, the excess of apoptosis plays a key role.
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P4.25

Role of COP9 signalosome in neoplastic progression in a model of hepatocarcinogenesis

L. Maiorino[4], M. Panattoni[1], F. Sanvito[2], L.G. Guidotti[3], C. Doglioni[2], R. Pardi[1,4]
[1]Leukocyte Biology Unit, San Raffaele Institute, Milan, Italy
[2]Dept of Pathology, San Raffaele Institute, Milan, Italy
[3]Immunopathogenesis of Liver Infections Unit, San Raffaele Institute, Milano, Italy
[4]Vita-Salute San Raffaele Univ. Medical School, Milano, Italy

The COP9 signalosome (CSN) is a highly conserved protein complex involved in multiple biological processes, including survival, cell cycle, differentiation and DNA damage response. The CSN modulates protein degradation through its catalytic subunit JAB1/CSN5 that removes the modifier Nedd8 from cullin component of E3 ubiquitin ligases.
JAB1/CSN5 has been found to be overexpressed in several cancers. In those cases high level of JAB1/CSN5 often correlated with reduced level of p27 and poor prognosis. In addition, coordinate amplification of MYC and JAB1/CSN5, which indeed lie on the same arm of chromosome 8, has been proved to induce the wound response signature: a poor-prognosis expression pattern of 512 genes in breast cancer.
Taken together these data suggest a close involvement of JAB1/CSN5 in carcinogenesis, however its specific role remains yet to be unravelled.
Preliminary data from our lab hint that JAB1/CSN5 could be a repressor of the oncogene-induced DNA damage response (DDR). By controlling turnover of critical substrates including both oncogenes and effectors of the response, JAB1/CSN5 could interfere with the balance between normal and aberrant proliferation induced by activated oncogenes. The inactivation of JAB1/CSN5 and the associated COP9 Signalosome should favour the DDR, thus inhibiting the transforming potential of several oncogenes and raising a higher barrier to tumorigenesis.
This project aims to address the role of JAB1/CSN5 in a murine model of chemically-induced hepatocellular carcinoma at early stages of tumor progression. To this purpose, we induce hepatocarcinogenesis in JAB1/CSN5flox/flox mice, and, during foci formation, we genetically inactivate JAB1/CSN5 by infection with a liver-directed adeno-associated vector, serotype 8, expressing Cre recombinase under the control of the hepatospecific transthyretin promoter. We expect the deletion of JAB1/CSN5 to impair neoplastic progression.

P4.26

Control of mitochondrial Ca^{2+} uptake by autophagy

C. Mammucari, A. Tosatto, R. Rizzuto
Dept of Biomedical Sciences, Univ. of Padova

Autophagy plays an essential role in the maintenance of cell homeostasis by removing misfolded proteins and damage organelles. We have shown that muscle-specific deletion of a crucial autophagy gene, Atg7, results in profound muscle atrophy and age-dependent decrease in force (Masiero et al., Cell Metabolism, 2009). Atg7 null muscles accumulate sarcoplasmic reticulum distension, disorganized sarcomeres and formation of aberrant concentric membranous structures. Most importantly, abnormal mitochondria accumulate in Atg7 null muscles. However, how these processes modulate Ca2+ signaling is still poorly understood. Mitochondrial Ca2+ homeostasis is an important component of the calcium-mediated cellular response to extracellular stimuli (Mammucari et al., Biofactors, 2011). It controls key organelle functions, such as aerobic metabolism and the induction of apoptotic cell death, and shapes the spatio-temporal pattern of the cytosolic [Ca2+] increase. Thus, a population of functional mitochondria is necessary to guarantee optimal control of mitochondrial Ca2+ - mediated responses. Nevertheless, how autophagy modulates mitochondrial-mediated Ca2+ signaling is still unknown. Our data show that mitochondrial Ca2+ uptake upon stimulation with inositol trisphosphate (IP3) mobilizing agents is higher in autophagy-incompetent cells compared to control cells and this is not due to differences in cytosolic Ca2+ transients. Our hypothesis is that lack of autophagy, by modulating mitochondrial Ca2+ entry, sensitizes mitochondria to Ca2+ overload caused by cell death stimuli. These results suggest that mitochondrial Ca2+ signaling is a fundamental component of autophagy-regulated cell homeostasis.

P4.27

The mitochondria-Akt axis as a key determinant in the regulation of autophagy in cancer cells

S. Marchi, C. Agnoletto, A. Bononi, M. Bonora, E. De Marchi, C. Giorgi, S. Missiroli, S. Patergnani, F. Poletti, A. Rimessi, J. Suski, P. Pinton
Dept of Experimental and Diagnostic Medicine, Section of General Pathology, Interdisciplinary Center for the Study of Inflammation (ICSI), Laboratory for Technologies of Advanced Therapies (LTTA), Univ. of Ferrara, Ferrara, Italy

The serine/threonine kinase Akt (also known as protein kinase B, PKB) is a potent inhibitor of apoptosis, hyper-activated in many human cancers, and plays a vital role in many cellular processes such as proliferation and survival. Akt is also described as a potent inhibitor of autophagy, a highly regulated machinery involved in the turnover of long-lived proteins and whole organelles. Once activated, Akt translocates to different intracellular compartments, and rapidly accumulates inside mitochondria. Although its kinase activity is well studied, the role of mitochondrial Akt remains elusive. Mitochondria are well known as fundamental actors in apoptosis, but recently their recycle in autophagic process seems to be important in regulating cell fate.
For this purpose, we have generated chimeras of Akt targeted to different mitochondrial sub-compartments (outer membrane, inner membrane and matrix). Surprisingly, two mitochondrial-Akt chimeras are strong inducers of autophagy, showed through different assays used for monitoring the autophagic process. Moreover, we have demonstrated how an autophagic stimulus, i.e. rapamycin, is able to induce Akt activation/accumulation into the mitochondrial compartment, underlining a putative "autophagic function" of this kinase in the organelle. Finally, mitochondrial Akt is able to affect Ca2+ homeostasis, suggesting a novel autophagic role of the kinase that concerns the mitchondria-Ca2+ axis.

P4.28

cFLIP involvement in AKT-mediated sensitivity to ER stress

E.S. Marini, C. Giampietri, S. Petrungaro, B. Esposito, S. Palchetti, A. Filippini, E. Ziparo
DAHFMO - Section of Histology and Medical Embryology Univ. of Rome "La Sapienza"

cFLIP is a DED-containing antiapototic protein that prevents procaspase-8 activation to the DISC upon death-receptor stimulation. The flip gene encodes both for short and long isoforms. Although short isoforms are mainly involved in the regulation of extrinsic apoptosis, cFLIP-L has also been described as a caspase-8 activator, depending on the ratio of cFLIP-L to caspase-8. Moreover, post-translational processing of cFLIP is known to promote ERK and NF-kB proliferative signaling pathways thus highlighting a role for this protein in regulating cell proliferation.
We have generated a novel transgenic mouse overexpressing cFLIP-L in skeletal muscle satellite cells. We have demonstrated that cFLIP-L overexpression stimulates proliferation of skeletal muscle precursors by activating ERK and NF-kB pathways. Further, analysis of intracellular signaling pathways indicates that cFLIP-L overexpression reduces phosphorylation of both GSK3β and AKT. By contrast, FLIP-/- mouse embryonic fibroblasts (MEFs) display an increase in AKT and GSk3β phosphorylation levels and a reduction in cell proliferation as compared with WT MEFs. It is known that cells with marked activated AKT show reduced sensitivity to several apoptotic stimuli, included ER stress-induced apoptosis. We have therefore investigated the sensitivity of cFLIP-/- MEFs to ER stress-mediated apoptosis, demonstrating that, upon treatment with the ER stress inducers thapsigargin and tunicamycin, cFLIP-/- MEFs show reduced caspase-3 activation as compared with WT. We thus conclude that cFLIP-L expression is involved in positively controlling cell sensitivity to ER stress-induced apoptosis and we hypothesize that such effect depends on cFLIP-L driven modulation of AKT phosphorylation.

P4.29

Myotonic dystrophy protein kinase protects cells from death by favoring hexokinase II association to mitochondria

B. Pantic, E. Trevisan, E. Berta, P. Bernardi, S. Salvatori, A. Rasola
Dept of Biomedical Sciences and National Research Council Institute of Neuroscience, Univ. of Padua, Padua, Italy

DMPK is a serine/threonine protein kinase that was initially proposed to be related to the pathogenesis of the most frequent adult muscular dystrophy, myotonic dystrophy 1 (DM1). Recently, it has been shown that DMPK is not the principal cause of the DM1, and yet very little is still known on its role in signal transduction pathways. We have examined the role of mitochondria-anchored isoform A, either by stably expressing it in cells lacking endogenous protein, or by stably silencing the endogenous one. DMPK presence dramatically decreased levels of mitochondrial superoxide and consequently increased cell survival, both in SAOS-2 and rhabdomyosarcoma cells. This cell death was prevented by antioxidants, permeability transition pore inhibitors, and hexokinase II inhibitor 5-thioglucose. At the molecular level, DMPK increased hexokinase II association to mitochondria, and detachment of HKII from mitochondria abolished differences in superoxide levels. HKII antiapoptotic effect seems to be independent of its enzymatic activity since a) it is evident in the conditions of prolonged glucose depletion and b) 5TG diminished levels of mitochondrial superoxide and prevented cell death by favouring HKII re-association to OMM.

P4.30

The Ewing Sarcoma protein (EWS) regulates DNA damage-induced alternative splicing

M.P. Paronetto[1,2], B. Miñana [1], I. Bernardis[3], E. Eyras[3,4], and J. Valcárcel[1,3,4]
[1]Centre de Regulació Genòmica; Barcelona, Spain
[2]Dipartimento di Scienze della Salute, Univ. di Roma "Foro Italico", IUSM, Rome, Italy
[3]Universitat Pompeu Fabra; Barcelona, Spain
[4]Institució Catalana de Recerca i Estudis Avançats, Barcelona, Spain

Alternative splicing allows cells to expand the coding potential of the genome and to respond to many different external stimuli and /or stresses. The Ewing Sarcoma protein EWS is a member of the TET (TLS/EWS/TAF15) family of DNA and RNA binding proteins, with proposed roles in transcription and RNA processing. Here we report that EWS knockdown generates changes in alternative splicing of genes related to DNA repair and DNA damage signalling pathways, including *ABL1*, *CHEK2* and *MAP4K2*. Chromatin immunoprecipitation, RNA crosslinking / immunoprecipitation (CLIP) and mobility shift experiments documented that EWS binds to its target genes and transcripts *in vitro* and *in vivo*. EWS HITS-CLIP revealed an enrichment of EWS clusters near 5' splice sites of constitutive and alternative exons, including some of the known EWS targets. Motif search analyses identified GGGTG as a sequence enriched in CLIP-tag sequences, a sequence which is bound by EWS in vitro.Interestingly, upon irradiation of cells with low intensity UV light, EWS transiently translocates to nucleoli, concomitant with decreased interaction with its pre-mRNA targets and with changes in alternative splicing that parallel those induced by EWS knockdown. For example, a reduction of c-ABL protein expression was observed upon UV light irradiation or EWS knock down. Consistent with the functional relevance of EWS-mediated regulation for DNA damage response, EWS knock down results in reduced cell viability and proliferation upon UV irradiation, an effect attenuated by increased expression of c-ABL.
Collectively, our results support the notion that EWS re-localization upon UV irradiation contributes to changes in alternative splicing that play a role in the cellular response to genotoxic stress.

P4.31

The Protein Kinase C β and mitochondria axis as a key regulator of autophagy

S. Patergnani[1], S. Marchi[1], C. Agnoletto[1], A. Bononi[1], M. Bonora[1], E. De Marchi[1], C. Giorgi[1], S. Missiroli[1], F. Poletti[1], A. Rimessi[1], J. Suski[1,2], P. Pinton[1]
[1]Dept of Experimental and Diagnostic Medicine, Section of General Pathology, Interdisciplinary Center for the Study of Inflammation (ICSI), Laboratory for Technologies of Advanced Therapies (LTTA), University of Ferrara, Ferrara, Italy
[2]Nencki Institute of Experimental Biology, Warsaw, Poland

Autophagy (cellular self-eating) – a lysosome-mediated catabolic process of eukaryotic cells to digest their own constituents – is a major route for the bulk degradation of aberrant cytosolic macromolecules and organelles. Autophagy principally serves an adaptive role to protect organisms against diverse pathologies, including cancer, neurodegeneration, and aging.
Recently, a number of works report Protein kinase C family (PKCs) as crucial mediators of the autophagic process. Starting from these observations, we found that the isoform β of the PKCs family significantly reduces autophagic levels. Furthermore, PKC-β also modulates mitochondrial physiology, lowering fundamental parameters, as like mitochondrial calcium uptake, mitochondrial ATP production and mitochondrial membrane potential, indicating a possible relationship between autophagy, mitochondria and PKC-β.
Moreover, here we show that mitochondrial membrane potential plays a crucial role in the regulation of autophagy and a pharmacological forced increase of this parameter not only leads to a raising in autophagic levels in control cells, but also contrasts the inhibitory effects of PKC-β on autophagy. In conclusion, our results indicate that PKC-β is a negative regulator of autophagy, and, moreover, the finding that a pharmacological modulation of mitochondrial membrane potential modifies autophagic levels may be useful in certain diseases (including various cancer types and neurodegenerative disorders) where a reduced level of autophagy is present.

P4.32

p53 centrosomal localization diagnoses ataxia-telangiectasia homozygotes and heterozygotes

A. Prodosmo[1], A. De Amicis[2], C. Nisticò[3], G. Di Rocco[1], E. Cundari[4], L. Chessa[2], S. Soddu[1]
[1]Dept of Experimental Oncology, Regina Elena Cancer Institute, Rome, Italy
[2]II Scholl of Medicine, La Sapienza Univ., Rome, Italy
[3]Dept of Clinicall Oncology, Regina Elena Cancer Institute, Rome, Italy
[4]Institute of Biology and Molecular Pathology, National Research Council, Rome, Italy

The risk for malignancy in individuals with Ataxia-Telangiectasia (A-T) is 38%. Individuals heterozygous for A-T disease-causing mutations (A-T-htz) are usually asymptomatic, but their cancer risk is approximately four times that of the general population, primarily because of breast cancer. Risk for cancer probably depends on multiple factors. However, the lack of rapid, efficient, and possibly not-expensive, carrier assays that would allow to easily identify ATM heterozygous in the general population has so far limited the screenings to obtain an accurate assessment of the relative contribution of ATM heterozygosity to cancer risk. Recently, we have identified a particular phenotype of lymphoblastoid cells expanded from A-T patients or their relatives that might offer a good opportunity to develop such a carrier assay. In particular, we have shown that the oncosuppressor p53 localizes at centrosomes at mitosis and monitors the mitotic spindle integrity. This p53 activity requires the integrity of ATM and cells from A-T patient present a p53 delocalization from almost 100% of their centrosomes in mitosis. Surprisingly, we observed that heterozygous consistently ($p<0.0001$) express an intermediate phenotype, with approximately 50% of their lymphoid cells having p53 delocalization from the centrosomes. Based on these observations, we have developed a simple and non-expensive test to identify the heterozygous carriers for A-T disease-causing mutations in the general population, by testing p53 centrosomal localization in cell-cycle stimulated peripheral blood lymphocytes. We are presently employing this carrier test in a pilot study for cancer risk association in breast cancer carrying patients

P4.33

Identification of new members of metallothionein gene family in sea urchin

M.A. Ragusa, S. Costa, M.C. Roccheri, F. Gianguzza
Dept Scienze e Tecnologie Molecolari e Biomolecolari (STEMBIO), Univ., Palermo, Italy

Heavy metals are common marine pollutants that emanate from such sources as industrial and sewage treatment discharges and anti-fouling paints. Cadmium ($Cd2^{+}$) serves no essential function in biological organisms and it is a highly toxic and carcinogenic metal. In *Paracentrotus lividus* sea urchin embryos cadmium causes development arrest or severe malformations. At lower doses, cadmium activates different responses (HSPs synthesis, autophagic or apoptotic processes) that can allow embryo survival.
This study was conducted to elucidate the gene transcription activation/upregulation or repression/ downregulation inducted in sea urchin embryos grown under cadmium stress.
Comparison between transcriptomes of 30 hours embryos treated or untreated with 100 mM cadmium chloride ($CdCl_2$) was conducted by RDA technique. Results showed increased transcription levels, in treated embryos, of metallothionein genes, and genes coding for detoxification enzymes.
Bioinformatic analysis of metallothionein sequences showed at least five different transcripts (MT-4 toMT -8) and only one of them was already new (MT-8 probably corresponds to Pl-MT). Quantitative real-time RT-PCR experiments confirmed RDA results and showed the specific activation of MT-4, MT-5 and MT-6 genes and the upregulation (or mRNA stabilization) of MT-7 and MT-8 genes. To investigate the threshold for gene activation, we are going to check the variations of transcript levels in embryos exposed to different doses of $CdCl_2$.

P4.34

HIPK2-mediated phosphorylation of H2B at midbody is critical for cytokinesis

C. Rinaldo[1], A. Moncada[1], F. Siepi[1], A. Prodosmo[1], A. Gradi[1*], G. M. Pierantoni[2], A. Giorgi[3], A. Fusco[2], E. Schininà[3], S. Soddu[1]
[1]Dept Experimental Oncology, Regina Elena Cancer Institute, Rome, Italy
[2]Institute of Experimental Oncology and Endocrinology CNR c/o Dept Cellular and Molecular Biology and Pathology, Univ. Federico II, Naples Italy
[3]Dept of Biochemical Sciences, Univ. of Rome La Sapienza, Italy
[*]Present address, Univ. of Sassari, Italy

HIPK2 is a multi-talented S/T kinase playing critical role in cell fate decision during development and in response to genotoxic damage. In the process of characterizing its functions, we found that HIPK2 depletion/inactivation lead to different signs of cytokinesis failure, such as bi- and multi-nucleation, long intercellular bridges, syncytia formed by cells that remain connected and reenter into mitosis.
In addition, by a mass spectrometry analysis we identified histone H2B as a HIPK2-interactor and by immunofluorescence we surprisingly observed that HIPK2 co-localizes with H2B at the midbody (MB) during cytokinesis. Based on these results, we focus our attention on the HIPK2-H2B interaction and their possible role in cytokinesis.
We showed that HIPK2 phosphorylates H2B at S14 and both proteins co-localize with α-tubulin and AURORA B kinase at the MBs in the absence of DNA bridges, indicative of chromosomal abnormalities, suggesting that HIPK2 and H2B have this localization independently from DNA damage. Furthermore, we demonstrated that HIPK2 is required for H2B-S14 phosphorylation at MB. Strikingly, the expression of S14-phosphomimetic H2B mutant, abolishes all cytokinesis defects and rescue the proliferation in HIPK2-depleted cells, indicating that HIPK2-mediated H2B phosphorylation at S14 is required for cytokinesis. These data point the HIPK2/H2B interplay as an important regulator of cytokinesis and uncover a novel essential function for these proteins in cell division.

P4.35

A role for LSD1 in acute promyelocytic leukemia

P.L. Rossi[1], O. A. Botrugno[1], Iros Barozzi[1], Gianmaria Frige'[1], C. Mercurio[2], M. Varasi[2], S. Minucci [1,3]
[1]European Institute of Oncology, Milan, Italy
[2]European Institute of Oncology, Drug Discovery Program, Milan, Italy,
[3]Dept of Biomolecular Sciences and Biotechnologies, Univ. of Milan, Milan, Italy

LSD1 was the first histone demethylase discovered. LSD1 catalyzes the demethylation of H3K4me1/2 and has been suggested to demethylate H3K9me2 when involved in the estrogen/androgen-receptor dependent transcriptional regulation. LSD1 is found in chromatin modifying complexes such as Co-REST and NURD, mainly involved in transcriptional repression. There are several evidences for the role of LSD1 in the hematopoietic development upon recruitment by specific transcription factors. In order to study the biological role of LSD1 several inhibitors have been used so far, like Paragyline and Tranylcipromine, mainly derived from MAO inhibitors. We recently developed a novel LSD1 inhibitor, more specific and acting at relatively low concentrations: 14e. LSD1 inhibition sensitizes NB4 cells (APL cell line) expressing the fusion protein PML-RAR to RA-induced apoptosis and differentiation. 14e, even given alone, has a strong effect on growth and differentiation in primary murine leukemic blasts and similar results were obtained upon suppression of LSD1 expression by RNA interference, ascertaining that the effect of the compound was due to the specific inhibition of LSD1 activity. Primary murine leukemic blasts treated with 14e lose their leukemogenic potential and the capability to transmit the disease when reinoculated in syngenic mice, suggesting a loss of leukemic stem cells (LSCs). In order to understand the molecular mechanism underlying the LSD1 inhibition induced apoptosis/differentiation we performed a ChIP-Seq assay with an anti-LSD1 antibody and we found that 55% of PML-RAR binding sites in NB4 cells overlap with LSD1. The PML-RAR/LSD1 overlapping sites show an enrichment of PU.1 binding motif, a master regulator of myeloid development. Our results suggest that the differentiation block in APL could result from an interplay between PML-RAR and LSD1 acting on a subset of PU.1 target genes and strongly support LSD1 as a novel potential target for the APL treatment.

P4.36

Extrasynaptic NMDA receptor stimulation induces CDKL5 cytoplasmic translocation and proteasomal degradation

L. Rusconi[1], P. La Montanara[1], C. Kilstrup-Nielsen[1], N. Landsberger[1,2]
[1]Dept of Structural and Functional Biology, Univ. of Insubria, Busto Arsizio Italy
[2]Division of Neuroscience, San Raffaele Scientific Institute, Milan, Italy

Mutations in the X-linked gene cyclin-dependent kinase like 5 (CDKL5) have been found in patients with epileptic encephalopathy characterized by early onset intractable epilepsy, including infantile spasms and other types of seizures, severe developmental delay and often the development of Rett syndrome (RTT)-like features. Despite its clear involvement for the proper brain development, CDKL5 function is still far from being understood. In this study, we analysed the subcellular localization of the endogenous kinase in primary murine hippocampal neurons. CDKL5 was localized both in nucleus and cytoplasm and, conversely to proliferating cells, did not undergo constitutive shuttling between these compartments. Nevertheless, glutamate stimulation was able to induce the exit of the kinase from the nucleus and its subsequent accumulation in the perinuclear cytoplasm. Moreover, we found that sustained glutamate stimulation promoted CDKL5 proteasomal degradation. Both events boosted by glutamate were mediated by the specific activation of extrasynaptic pool of NMDA receptors, which have been linked to cell death pathways. Proteosomal degradation was also induced by withdrawal of neurotrophic factors, a different paradigm of cell death. On the contrary, we have recently found that stimulation of synaptic activity by KCl and BDNF, which promote neuronal maturation and survival, induced an increase in CDKL5 expression level; this suggest that activation of antithetical death or survival pathways may result in opposite effects on CDKL5 expression level. All together, our data on subcellular localization and expression of CDKL5 suggest the involvement of this kinase within cell death and survival pathways.

P4.37

Circulating microRNAs as new biomarkers in age-related diseases

G. Santini[2], R. Lazzarini[3], A. Ceka[1], E. Ponzio[2], L. Babini[1], R. Antonicelli[4], F. Olivieri[1]
[1]Dept of Clinical and Molecular Science, Polytechnic Univ. of Marche, Italy
[2]Dept of Biomedical Science, Polytechnic Univ. of Marche, Italy
[3]Center of Clinical Pathology and Innovative Therapy, I.N.R.C.A. National Institute, Ancona, Italy
[4]Operative Unit of Cardiology-UTIC, I.N.R.C.A. National Institute, Ancona, Italy

Many chronic degenerative diseases, especially in the elderly have an insidious course, characterized by occurrence of complications and marked inter-individual differences in response to drug treatment. Cardiovascular disease, myocardial infarction (AMI) and chronic heart failure (CHF) are the most common age-associated diseases in industrialized countries, and the overall leading cause of death. New non-invasive biomarkers, specific and sensitive may be useful for diagnosis and monitoring of these diseases.
MicroRNAs (miRNAs) are a class of single-stranded RNA molecules, about 20-23 nucleotides long, non-coding, and highly conserved among different species, identified in humans. Their function is carried out preferentially in the cell, where they act as modulators of gene expression and have recently been measured in the circulation (both plasma and serum), defined as circulating miRNAs. They, being associated with Ago proteins or contained in microvesicles, are protected from degradation while remaining very stable in plasma, which is an important characteristic for circulating biomarkers.
Preliminary data suggest that some miRNAs may be useful circulating markers of acute myocardial infarction (AMI), especially in geriatric patients, characterized by atypical symptoms. However, there are few data on the diagnostic/prognostic clinical relevance of specific circulating miRNAs expression and there are no data regarding miRNAs expression in healthy subjects of different age.
We aimed to define the clinical relevance of specific circulating miRNA in the differential diagnosis of AMI, evaluating age-related changes of miRNAs expression in healthy subjects of different age, including very old people.

P4.38

Selection of mesoangioblast stem cells more resistant to hydrogen peroxide adverse microenvironments

G. Turturici, F. Geraci, G. Sconzo
Dept STEMBIO, Univ. of Palermo, Palermo, Italy

The therapeutic efficacy of stem cells injected or engrafted in a damaged tissue is greatly limited by their poor survival. In order to explorer this phenomenon and find a new strategy to enhance the survival after stem cell delivery, we reproduced in vitro, using mouse mesoangioblasts (mabs) as a model of stem cells, an oxidative stress similar to that produced in inflamed tissues. Mabs are known to improve the conditions of experimental muscular dystrophy in mice, dogs and a clinical trial is currently ongoing. To find some cell clones more resistant to oxidative stress we treated mabs with H_2O_2 using conditions corresponding to the maximum concentration (400 μM) found in inflamed tissues. This 24 h extreme treatment blocks the cell cycle in G_2/M phase for 3 days, and it gradually returns to normality in the following days. The cells do not proliferate and die slowly up to 70% in a week. The block at the G_2/M checkpoint depends on $p38^{MAPK}$ activation. At the beginning of the treatment the cells activate the autophagy as a defence mechanism as demonstrated by the presence of GFP-LC3II protein on autophagosomes, and by the formation of acidic compartments. Apoptosis and necrosis also begin immediately, remaining at low levels through the entire period of recovery, as verified by annessin V assay, caspase 3/7 activity assay, JNK and $p38^{MAPK}$ activation. After 8 days the proliferation recovers at low level and from these surviving mabs we selected cell clones. We calculated that only 3% of total treated cells is able to form clones. These clones are much more resistant to a second treatment either drastic (400 μM) or mild (200 μM) which is more frequent in inflamed tissues. Clones treated with 400 μM have a partial arrest in G_2/M phase for 1 day and only 15% die. On the contrary, clones treated with 200 μM do not block the cell cycle and only 5% die. This method allowed us to select stem cell clones that are resistant to an hydrogen peroxide adverse microenvironment.

P4.39

Quantification and localization of anti-apoptotic matrix metalloproteinase (MMP15) in human colorectal carcinogenesis

P. Sena,[1] F. Mariani,[2] L. Marzona,[1] L. Roncucci,[2] C. Palumbo[1]
[1]Dept Biomedical Sciences, section Human Morphology, Univ Modena and RE, Itlay
[2]Dept Internal Medicine, Univ Modena and RE, Itlay

Matrix metalloproteinases (MMPs) are capable of degrading all kinds of extracellular matrix proteins, but they also can process a number of bioactive molecules. They are known to be involved in the cleavage of cell surface receptors, in the release of apoptotic ligands and in chemokine/cytokine in/activation. MMPs also play a major role on cell processes like proliferation, migration, differentiation, apoptosis, as well as on angiogenesis and host defense. In the present study the attention was focused on the anti-apoptotic activity of MMP-15. This protease was hypothesized to increase both apoptosis resistance and metastasis progression by an unknown regulatory mechanism; thus, we have tried to elucidate its involvement in human colorectal cancer development. The expression profile of MMP-15 was assayed from samples of normal mucosa, microadenomas and cancer using confocal analysis, Western blotting and quantitative reverse transcription polymerase chain reaction (qRT-PCR). Western blot and qRT-PCR showed that MMP-15 expression level increases from normal mucosa to microadenomas, with a reduced level of expression in cancer, respect to microadenomas. The semiquantitative immunofluorescence analysis correlates with these data, also showing a stromal localization of MMP-15, thus indicating an important role of stromal compartment, especially in the early phases of neoplastic transformation. This up-regulation of MMP-15 during tumourigenesis suggests that the metastatic potential, which are thought to evolve late in cancer progression, could be acquired early by tumor cells through the selection of the anti-apoptotic phenotype. The identification of an expanding range of MMP substrates (not only matrix proteins) will provide the basis on which to consider the widening range of biological functions attributed to MMPs, in particular those correlated to cell proliferation/death, critical for tumour development and progression.

P4.40

Loss of activation loop-Y autophosphorylation of HIPK2 yields to an insoluble misfolded protein

F. Siepi[1], S. Camerini[2], V. Gatti[1], A. Prodosmo[1], C. Rinaldo[1], A. Moncada[1], M. Crescenzi[2] , S. Soddu[1]
[1]Dept of Experimental Oncology, "Regina Elena" Cancer Institute, Rome, Italy
[2]Dept of Cell Biology and Neurosciences, National Institute of Healt, Roma, Italy

HIPK2 is a S/T kinase involved in development and in cell response to stress. Despite the increasing number of cellular functions and target factors identified, the regulation of HIPK2 catalytic activity is poorly understood. To characterize the mechanism of HIPK2 activation, we focused on HIPK2 autophosphorylation. Mass-spec analysis of HIPK2 identified 22 S/T sites of autophosphorylation and phosphorylation by other kinases, plus one Y site of autophosphorylation, the Y354. This site belongs to the putative activation loop of HIPK2 and is constitutively phosphorylated. This Y354 phosphorylation is an autocatalytic event, as shown by the lack of Y354 phosphoryaltion in HIPK2 kinase dead mutant and by the ability of HIPK2 kinase domain to autophosphorylate after dephosphorylation. A non-phosphorylatable Y354F mutant has a reduced ability to autophosphorylate and to phosphorylate substrates on S/T. These data demonstrate that HIPK2 is a dual specificity Y-regulated kinase (DYRK). DYRKs are characterized by two subsequent, mutually exclusive features: a transient ability to autophosphorylate on Y, catalyzed by ribosome-bound intermediate, and a loss of this ability with the acquisition of S/T kinase activity, catalyzed by the mature kinase. Interestingly, the Y354F mutant retains a Y autophosphorylation activity mimicking the DYRK ribosome-bound intermediates. In contrast to the nuclear localization of wild-type HIPK2, Y354F mutant has a cytoplasmic, juxtanuclear aggregated distribution. Aggregates are partially insoluble, ubiquitylated and colocalize with MTOC suggesting that they consists of unfolded proteins recruited into the so called "aggresomes" to be cleared by the autophagic pathway. Based on these observation, we are testing whether the activation-loop Y non-phosphorylatable Y354F mutant is unable to acquire the mature conformation after disengagement from ribosome or, alternatively, if Y dephosphorylation signals for degradation pathway/s.

P4.41

Shedding light on the mitochondrial permeability transition

J. Sileikyte[1], V. Petronilli[1], A. Zulian[1], F. Dabbeni-Sala[2], P. Nikolov[3], P. Bernardi[1], F.Ricchelli[4]
[1]C.N.R. Institute of Neuroscience and Dept of Biomedical Sciences, Univ. of Padova, Padova, Italy
[2]Dept of Pharmacology and Anesthesiology, Univ. of Padova, Padova, Italy
[3]Bulgarian Academy of Sciences, Institute of Organic Chemistry, Sofia, Bulgaria
[4]C.N.R. Institute of Biomedical Technologies at the Dept of Biology, Univ. of Padova, Padova, Italy

The mitochondrial permeability transition (PT) is a sudden increase in the permeability of the inner mitochondrial membrane (IMM) to solutes with molecular masses up to 1,500 Da. This process is due to opening of a voltage- and Ca^{2+}-dependent, cyclosporin A-sensitive, high-conductance channel called the PT pore (PTP). Its involvement in pathological states and in the loss of cell viability is widely recognized, but its molecular identity remains elusive. The long standing idea that the PTP may form at inner-outer membrane contact sites, and that it may be constituted by the adenine nucleotide translocator in the IMM and VDAC in the outer mitochondrial membrane (OMM) has not been confirmed by genetic ablation of these proteins. Yet, the PT can be regulated by proteins that interact with the OMM such as hexokinase, and by ligands of the OMM translocator protein of 18 kDa (TSPO, formerly known as peripheral benzodiazepine receptor). As of today, however, it is not clear whether the OMM is necessary for the PT to occur; and what regulatory properties, if any, it may contribute to the PTP. We have studied the properties of the PTP in rat liver mitochondria and in mitoplasts retaining inner membrane ultrastructure and energy-linked functions. Like mitochondria, mitoplasts readily underwent a PT following Ca^{2+} uptake in a process that maintained sensitivity to cyclosporin A. On the other hand, major differences between mitochondria and mitoplasts emerged in PTP regulation by ligands of the TSPO. Indeed (i) in mitoplasts the PTP could not be activated by photooxidation after treatment with dicarboxylic porphyrins endowed with protoporphyrin (PP) IX configuration, which in intact mitochondria bind TSPO; and (ii) mitoplasts became resistant to the PTP-inducing effects of N,N-dihexyl-2-(4-fluorophenyl)indole-3 acetamide and of other selective ligands of TSPO. Thus, the PT is an IMM event that is regulated by the OMM through specific interactions with TSPO.

P4.42

Activation of Hypoxia Inducible Factor(s) as a protective stratagem against various cell injuries

R. Spagnuolo[1], L. Tacchini[1], C. Rumio[1], B. La Ferla[2], E. Gammella[1], G. Cairo[1]
[1]Dept of Human Morphology and Biomedical Sciences- Città Studi, Univ. Milano, Milan, Italy
[2]Dept of Biotechnology and Bioscience & CINMPIS, Univ. Milano Bicocca, Milan, Italy

Hypoxia Inducible Factor(s) (HIFs) transactivate a variety of genes that help the cell to survive under harsh environmental conditions. In addition to genes that reprogram cellular metabolism, anti-apoptotic genes are HIF-1 targets. However, HIFs also activate pro-apoptotic BNIP3. We have recently demonstrated that the iron chelator Dexrazoxane protects cardiomyocytes from doxorubicin-induced toxicity by increasing the expression of the HIF-regulated anti-apoptotic proteins survivin, MCL1 and heme oxygenase (Spagnuolo et al., Br. J. Pharmacol 2011: 163, 299-312). Therefore, to explore the possibility that a pharmacological strategy based on small molecular mimics of hypoxia could be exploited in an attempt to limit anthracycline cardiotoxicity, we examined HIFs levels and activity, as well as protection from doxorubicin damage, in H9c2 cardiomyocytes pre-exposed to DMOG, an antagonist of α-ketoglutarate which activates HIFs under normoxic conditions.
Moreover, we investigated the role of the modulation of pro-apoptotic and anti-apoptotic HIF-1 target genes in a model of lipopolysaccaride (LPS)-mediated inflammation. It has been shown that D-glucose-induced activation of the sodium-dependent glucose transporter-1 (SGLT-1) down-regulates the NF-kB-dependent pro-inflammatory response (Palazzo et al., J. Immunol. 2008:181, 3126-3136). Given the interplay between HIF-1 and NF-kB we previously found (Tacchini et al., J. Biol. Chem 2008:283, 20674-20686), we investigated the possible role of HIF-1 expression in LPS-exposed HT29 and A549 cell lines, in the absence or presence of D-glucose, the SGLT-1 agonist BLF50 (La Ferla et al., ChemMedChem. 2010:5,1677-80), and the glucose analog 3-OMG, that induces transporter activity, but is not metabolized.
These studies may provide insights into the protective role of HIFs under pathological conditions.

P4.43

Cytotoxic versus cytostatic responses in oncogene-addicted cells following oncogene inhibition

D. Torti, A Bertotti, L Trusolino
Laboratory of Molecular Pharmacology, Institute for Cancer Research and Treatment (IRCC) & Dept of Oncological Sciences, University of Torino Medical School, Candiolo (TO), Italy

The oncogene addiction principle states that the malignant traits of cancer cells may be halted through the suppression of one single oncogene. Both *in vitro* and *in vivo* models suggest that different biological phenotypes follow the blockade of the addictive oncogene, including either growth arrest/senescence or apoptosis. We have observed that upon pharmacological inhibition of the Met receptor the Met-addicted cancer cell lines GTL16 and EBC1 feature a cytostatic as opposite to cytotoxic response, respectively. Indeed, using multiparametric measure of cell cycle/viability and apoptosis, we showed that Met-inhibited GTL16 cells undergo G_0/G_1 cell-cycle arrest whereas EBC1 suffer overt apoptosis. Moreover, these two biological responses were accompanied by different activation profiles of a number of pro-apoptotic or anti-apoptotic signaling effectors, including p38, JNK, and NF-kB. In order to identify other potential molecules responsible for these biologic outcomes we performed a target-focused gene expression profile interrogating 44 genes with a definite function either in cell-cycle or apoptotis regulation. We observed a comparable downmodulation of cell-cycle genes in both GTL16 and EBC1 cell lines; on the contrary, we found that pro-apoptotic transducers including the BH3-only family genes *BMF* and *HRK* were potently induced only in EBC1 cells. At present, gain/loss of function experiments are planned to determine the mechanistic role of these proteins. In a translational perspective, we foresee that the acknowledgment of the molecular mechanisms governing the type of biological response to targeted drugs will greatly benefit therapeutic strategies in addicted tumors.

P4.44

Anti-apoptotic effects of phenolic extrcts from *theombra cacao l.*, clovamide and epichatechin on rat cardiomyoblasts undergoing oxydative stress

A. Zamperone[1], S. Pietronave[1], J.D. Coïsson[2], F. Travaglia[2], M. Locatelli[2], A. Martelli[2], M. Arlorio[2], M. Prat[1]
[1]Dept of Medical Sciences, Eastern Piedmont A.Avogadro Univ., Italy
[2]Dept of Chemical, Alimentary, Pharmaceutical and Pharmacology Sciences & DFB Center, Eastern Piedmont A.Avogadro Univ, Italy

Cacao and its derivatives display antioxidant and antiradical properties, which can have a role in the maintenance of human health. On the basis of many studies of the in vivo bioavailability of polyphenols contained in the cacao, it can be inferred that some of them (phenolic acids, epicatechin and catechin, clovamide, dimeric procyanine, minor flavonoids) may be largely bioaccessible and bioavailable. Cardiovascular diseases are the first cause of morbidity and mortality in western countries. Phenolic compounds contained in the cacao have been reported to be beneficial in pathologies linked to hypertension, dyslipidemies and other inflammatory diseases related to cardiovascular diseases [1,2]. The anti-radical properties of clovamide, a minor cacao component[3], potential anti-platelet aggregant[4], could help in reducing the consequences of cardiac ischemic damage, which is followed by the oxidative stress in the reperfusion phase, at the end responsible for the loss of myocytes[5]. Herein we have studied the radical scavenging properties of clovamide, epicatechin and rosmarinic acid in a model of rat cardiomyoblasts (H9c2 cell line), evaluating their inhibition on ROS (reactive oxygen species) release induced by hydrogen peroxide. Also polyphenols extracted from cacao (fractioned by Solid Phase Extraction in low, medium and high molecular weight and characterized in HPLC-DAD), as well as theobromine, were studied in this cell model. The anti-apoptotic activity of these compounds in cells treated with H2O2 was evaluated as well. At micro-nanomolar concentrations, clovamide, epicatechin and rosmarinic acid dramatically inhibited ROS release and protect H9c2 cells from H2O2–induced apoptosis, evaluated both in a TUNEL assay and cytofluorimetrically. These data are a further support of the bioactive beneficial role of the cacao in the context of cardiovascular pathologies and in particular in the protection towards ischemic injury.

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