IFOM-IEO Campus
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Istituto Nazionale Tumori

Mario Negri

Vita-Salute San Raffaele University
DiBiT-HSR Scientific Institute San Raffaele

4th Joint PhD Student Workshop

23rd-25th JANUARY 2006

Astoria Park Hotel
Riva del Garda (TN)

SCIENTIFIC AND ORGANIZING COMMITTEE

Isabella Alessandrini, Manuela Basso, Andrea Disanza, Sabrina Frata, Luca Libelli, Luigi Nezi, Silvia Masciarelli, Andrea Orsi, Claudio Storini

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JANUARY 23RD- SCIENTIFIC PROGRAM

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14:50 - 15:00	**Opening Remarks** **Andrea Musacchio**, IFOM-IEO Campus
15:00 - 15:20	**ROBERTO BOGGIO**, IFOM-IEO Campus "Gam1 and the Sumo pathway"
15:20 - 15:40	**ANDREA CROTTI**, Vita-SaluteS.Raffaele University "Understanding the role of STAT5 and STAT5ΔC in HIV infection/replication"
15:40 - 16:00	**CLAUDIO CIFERRI**, IFOM-IEO Campus "Molecular architecture of the human Ndc80-Hec1 complex, a critical constituent of the outer kinetochore"
16:00 - 16:20	**JILL MARTURANO**, Vita-Salute S.Raffaele University "Tumor antigen processing and presentation on MHC class II molecules for $CD4^+$ T cell recognition in health and disease"
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JANUARY 24TH- SCIENTIFIC PROGRAM

Morning session-MOLECULAR AND CELLULAR BIOLOGY

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09:20 - 09:40	**STEFANO BIRESSI**, Vita-Salute S.Raffaele University "The molecular basis of skeletal myoblast diversification. Gene expression analysis of purified embryonic and fetal myoblasts"
09:40 - 10:00	**ALESSIO MAIOLICA**, IFOM-IEO Campus "Protein-protein interaction by mass spectrometry"
10:00 - 10:20	**PIETRO VEGLIANESE**, Mario Negri Institute "Characterization of the AMPA receptors subunits and their associated trafficking proteins (ABP, NSF and PICK-1) in a mouse model of amyotrophic lateral sclerosis"
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10:50 - 11:10	**LAURA CARRASSA**, Mario Negri Institute "Molecular mechanisms regulating the G2 checkpoint focusing on chk1"
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15:30 - 15:50	**ERICA BUTTI**, Vita-Salute S.Raffaele University "IL-4 gene delivery induces clinical and functional recovery from experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis"
15:50 - 16:10	**CHIARA MARTINOLI**, IFOM-IEO Campus "Development and function of mucosal IgAs induced following oral infection with *Salmonella Typhimurium*"
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Afternoon session-CANCER BIOLOGY

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17:40 - 18:00	**FEDERICA POLATO**, Mario Negri Institute "Characterization of a gene important for p53-dependent, stress-induced apoptosis"
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JANUARY 25TH- SCIENTIFIC PROGRAM

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10:50 - 11:10	**VALERIA CAMBIAGHI**, IFOM-IEO Campus “Preleukemic phase analysis to understand the biological contribution of PML-RARα to the leukemogenic process”
11:10 - 11:30	**FRANCESCA DE FRANCO**, IFOM-IEO Campus “Role of quiescent stem cells in the maintenance of leukaemia cell populations”
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12:20 - 12:40	**SABINA SANGALETTI**, Istituto Nazionale Tumori “Stromal-derived SPARC regulates stroma cells-tumor cells interaction toward metastasis”
12:40 - 13:00	**LUIGI MADDALUNO**, IFOM-IEO Campus “L1CAM: a new regulator of dendritic cell trafficking”
13:00 – 14:30	Lunch

Afternoon session-CANCER BIOLOGY/SCIENCE AND SOCIETY

14:30 - 16:00	**Poster session**
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17:30 - 17:40	**Closing Remarks** …, Istituto Nazionale Tumori
17:40	End of the workshop

LECTURE

Linda Hendershot
Department of Genetics and Tumor Cell Biology,
St. Jude Children's Research Hospital
Memphis, US

SESSION A: CELLULAR AND MOLECULAR BIOLOGY

Roberto Boggio
Andrea Crotti
Claudio Ciferri
Jill Marturano
Cristina Sironi
Chris Madsen

JANUARY 23RD - ABSTRACTS

ORAL PRESENTATIONS

GAM1 AND THE SUMO PATHWAY

Roberto Boggio, Alfonso Passafaro, Paula Babarovic and Susanna Chiocca.
European Institute of Oncology, Department of Experimental Oncology, 20141 Milan, Italy

The Small Ubiquitin-related Modifier, SUMO-1, is a Ubiquitin like family member. The mechanism utilized to conjugate this molecule to its substrates includes three discrete enzymatic steps: the activation, which involves the activation enzyme E1; the conjugation step, which involves the E2 enzyme UBC9; the substrate modification step, which occurs through the cooperative association of UBC9 and the E3 protein ligases. We report here that the adenoviral protein Gam1 blocks the SUMO-1 pathway via its novel and unique interference with the E1 heterodimer SAE1/SAE2. Our results clearly indicate that Gam1 has a role in E1 enzymatic inactivation, both “in vitro” and “in vivo” followed by SAE1/SAE2 and UBC9 proteasomal degradation. Moreover, we have now characterized this mechanism by showing that Gam1 forms an ubiquitin E3 ligase complex containing Cullins, Elongins and the RING protein ROC-1 through its C-terminus degenerate SOCS domain. We demonstrate that Gam1 is necessary for the recruitment of SAE1/SAE2 in a Culs/EloBC/ROC-1 ligase complex and for their subsequently ubiquitinylation. Furthermore the proteasomal degradation of UBC9 seems to be a secondary effect of SUMOylation inhibition and is not tightly dependent on the Gam1 effect. These results show that Gam1, similarly to other viral proteins, is able to inactivate/degrade an essential cellular enzyme and to arrest a host basic pathway to assure a better environment for viral replication.

UNDERSTANDING THE ROLE OF STAT5 AND STAT5ΔC IN HIV INFECTION/REPLICATION

Andrea Crotti, Chiara Bovolenta , Elio Liboi, Elisa Vicenzi, and Guido Poli
AIDS Immunopathogenesis Unit, DiBiT-HSR, Via Olgettina 58, Milano, Italy + MolMed Spa + UNI Verona

The balance between the state of latency and productive replication in HIV infection is deeply influenced by multiple cytokines. Signal transduction pathways and transcriptional factors activated by several cytokines either transactivate or inhibit the HIV-LTR promoter sequence, thus influencing the state of viral latency. One of the most common signal transduction pathway shared by several cytokines is the JAK/STAT pathway. In this regard, we have previously shown that a constitutive activation of a C-terminal truncated STAT5 (STAT5ΔC) and of STAT1alpha occurs in CD4+ T cells of most individuals with progressive HIV disease. STAT5ΔC isoforms retain DNA binding, but maybe impaired or lack the transcriptional activity typical of full-length STAT5. We are attempting to define whether indeed STAT5ΔC isoforms of lengths comparable to those observed in HIV+ individuals could indeed act as dominant negative factor on STAT5-dependent gene transcription. In addition, we are investigating whether STAT5 activation can influence the state of HIV infection and replication. Up to date, constitutively activated STAT5ΔC is less potent than STAT5 FL but it does not seems to act as a dominant negative element on STAT5-dependent gene transcription, at least at the studied cotransfection ratios (1:1 and 2:1). Using an HIV-based reporter gene assay in transfected 293-derived cells, we observed that the expression of constitutively activated STAT5 FL activated the HIV-1 LTR driven GFP expression. Furthermore, GM-CSF stimulation of chronically-infected promonocytic U1 cells induces activation of STAT5 followed by viral expression. Constitutively activated STAT5 ΔC showed a reduced capacity of transactivating the HIV-1 LTR than STAT5 FL in the same model system. Of interest, U1 cells express a STAT5 ΔC of length comparable to that observed in HIV-infected individuals. These observations are consistent with a direct positive role of STAT5 in inducing HIV transcription.

MOLECULAR ARCHITECTURE OF THE HUMAN NDC80-HEC1 COMPLEX, A CRITICAL CONSTITUENT OF THE OUTER KINETOCHORE

Ciferri C, De Luca J, Monzani S, Ferrari KJ, Wyman C, Salmon ED, Musacchio A.

The Ndc80 complex is a constituent of the outer plate of the kinetochore and plays a critical role in establishing the stable kinetochore-microtubule interactions required for chromosome segregation in mitosis. The Ndc80 complex is evolutionarily conserved and contains the four subunits Spc24, Spc25, Nuf2, and Ndc80 (whose human homologue is called Hec1). All four subunits are predicted to contain globular domains and extensive coiled coil regions. To gain an insight into the organization of the human Ndc80 complex, we reconstituted it using recombinant methods. The hydrodynamic properties of the recombinant Ndc80 complex are identical to those of the endogenous HeLa cell complex and are consistent with a 1:1:1:1 stoichiometry of the four subunits and a very elongated shape. Two tight Hec1-Nuf2 and Spc24-Spc25 subcomplexes, each stabilized by a parallel heterodimeric coiled coil, maintain this organization. These subcomplexes tetramerize via an interaction of the C- and N-terminal portions of the Hec1-Nuf2 and Spc24-Spc25 coiled coils, respectively. The recombinant complex displays normal kinetochore localization upon injection in HeLa cells and is therefore a faithful copy of the endogenous Ndc80 complex.

TUMOR ANTIGEN PROCESSING AND PRESENTATION ON MHC CLASS II MOLECULES FOR $CD4^+$ T CELL RECOGNITION IN HEALTH AND DISEASE

Jill Marturano and Maria Pia Protti (CIGTP, Dibit, H San Raffaele, via Olgettina 58, 20132 Milan, Italy)

$CD4^+$ T cells play an important role in orchestrating the anti-tumor response but little is known about the intracellular events leading to the formation of tumor antigen CD4 epitopes and how these epitopes are recognized *in vivo* in patients during the progression of neoplastic disease.
We focused on MAGE-3, an ideal candidate for cancer immunotherapy because it is expressed in many types of solid tumor but not in healthy tissues, and for which we previoulsy identified after *in vitro* priming several naturally processed epitopes recognized by $CD4^+$ T cells from healthy donors.
To study of the pathways leading to the formation of CD4 epitopes of this protein, we focused on two MAGE-3 epitopes. MAGE-$3_{156\text{-}175}$, expressed by antigen presenting cells in association with DR alleles after processing of endocytosed MAGE-3 protein and MAGE-$3_{286\text{-}300}$, expressed also directly in association with DR alleles at the surface of MAGE-3 positive melanoma cells. We used selective inhibitors of lysosomal and cytosolic enzymes in different types of MAGE-3 expressing cells, and then evaluated as a readout their recognition by $CD4^+$ T cells specific for the peptides corresponding to the two selected MAGE-3 regions. Results obtained so far will be shown.
To evaluate the relevance of the spontaneous occurring anti-MAGE-3 specific $CD4^+$ T cells *in vivo* in neoplastic patients, we selected a population of melanoma patients with MAGE-3 expressing tumor. We are testing the *ex-vivo* qualitative and quantitative response to all the promiscuous MAGE-3 CD4 epitopes identified so far, using different techniques.

FUNCTIONAL AND BIOCHEMICAL CHARACTERIZATION OF EFA6, A KEY PLAYER IN CYTOSKELETAL REMODELLING

Cristina Sironi, Vittoria Matafora, Angela Bachi, Daniela Talarico
DIBIT, H.S.Raffaele, via Olgettina 58, 20132 MILANO

EFA6 is preferentially expressed in brain tissue and is believed to be involved in actin cytoskeleton remodelling and neurite outgrowth. Through its Sec7 domain, it is able to activate the small GTPase ARF6, known to play a role in endosomal-plasma membrane recycling and in cytoskeleton dynamics. Overexpression of EFA6 in neuronal cells induces neurite outgrowth, while in HeLa cells it induces membrane ruffling. The C-terminal of EFA6 appears to be required for the full biological activity of the protein ; the PH domain is important for its localization to the membrane; the presence of two putative coiled coil motifs might suggest a possible interaction with other proteins, likely involved in the cytoskeletal remodelling signalling pathway. To further characterize the COOH terminal of EFA6, we have prepared tagged constructs that encompass different portions of the PH domain and of the coiled coil motifs. Overexpression of an entire PH-coiled coil construct in neuronal cells induces neurite branching, while in HeLa cells it induces cell elongation and it colocalizes with F-actin on dorsal membrane ruffles. By expressing various mutants in both cell types we have identified the regions which are important for the biological activity, and we are trying to isolate possible interactors by immunoprecipitations followed by mass spectrometry analysis . Moreover we are investigating whether EFA6 undergoes any post-translational modification, as this might represent a mechanism to modulate interaction with effector proteins. With these approaches, we would like to elucidate the components of the EFA6 pathway, and to better understand its role in cytoskeleton dynamics.

COMPLETE ALANINE-SCAN OF HUMAN UPAR

Chris Madsen, Annapaola Andolfo, Nicolai Sidenius, IFOM

Urokinase-receptor (uPAR) is involved in cancer biology by numerous molecular mechanisms. Expression of uPAR in an epithelial cell line lacking endogenous uPAR and its natural ligands induces major morphology change and cell migration. Since uPAR lacks catalytic activity and has no transmembrane and cytosolic domains, the molecular changes rely entirely on intracellular signaling through other cell surface proteins e.g. integrins. It is the aim of this study to map the physical binding site(s) of uPAR responsible for this migratory transition and to identify the signaling receptor(s) conceivably interacting directly with uPAR. For this purpose we generated 255 stably expressing uPAR-mutants each containing a single amino acid substituted by an alanine residue. Residues responsible for the morphology change are being superimposed on the crystal structure of uPAR to reveal the critical interaction sites. The preliminary data has established the high-affinity binding site between uPAR (Arg92 and Tyr93) and vitronectin. Disruption of this interaction site completely obstructs cell adhesion, cytoskeleton rearrangement, and cell migration without affecting other functions of uPAR, thereby demonstrating uPAR/vitronectin binding to be required for uPAR-induced cell migration of human embryonic epithelial cells. At this stage we have not yet completed the screening of all mutants and therefore not characterized the binding site to the cell surface molecule transducing the intracellular signaling. However, the generation of a complete set of uPAR-mutants emphasizes the immense potential of this system to further dissect the molecular structure of uPAR implicated in cancer biology.

SESSION A- CELLULAR AND MOLECULAR BIOLOGY

JANUARY 23RD - ABSTRACTS

POSTER

A1

A PROTEOMIC APPROACH TO INVESTIGATE THE MONOUBIQUITIN PROTEOME IN MAMMALIAN CELLS

Elisabetta Argenzio, Barbara Oldrini, Pier Paolo Di Fiore and Simona Polo
IFOM, FIRC Institute for Molecular Oncology, Milan, Italy

Protein ubiqutitination is one of the most important post- translational modification.
The binding of polyubiquitin chains to proteins, as a signal for their degradation through 26S proteasome, has been studied since a long time but less is known about the attachment of a single ubiquitin molecule to target proteins. Currently, it is believed that protein monoubiquitination, as well as phosphorylation, is a mechanism to reversibly regulate several cellular pathways.
In order to understand the role of protein monoubiquitination, a proteomic approach has been used to identify the specific proteins modified by monoubiquitin after different treatments in mammalian cells expressing Flag-6xHis-Ubiquitin. We are setting up a purification protocol relying on a) in vitro proteasome digestion of cellular lysates, b) double affinity purification, c) reversed phase chromatography and LC-MS/MS analysis. In vitro 26S proteasome treatment was performed to enrich the cell lysate in monoubiquitinated proteins that were subsequently purified by denaturing nickel-affinity chromatography and anti-Flag immunoprecipitation.

A2

A ROLE FOR THE PRE-MRNA 3' END PROCESSING FACTOR CF I_M IN MRNA EXPORT AND TRANSLATION?

C. Aringhieri, S. Cardinale, S. Cugusi, P. Bonetti, and S.M.L. Barabino
Department of Biotechnology and Biosciences, University of Milano-Bicocca

The expression of eukaryotic genes is a process with many individual steps. These include transcription and pre-mRNA processing in the nucleus, mRNA export through the nuclear pore complex, translation in the cytoplasm, and finally decay of the mRNA and protein. Although these processes were traditionally thought of as entirely separate events, it has recently become apparent that *in vivo* they are all highly interconnected.
Pre-mRNA splicing enhances mRNA 3'end processing, nuclear export and translational utilization. Several members of the SR family of splicing factors are nucleo-cytoplasmic shuttling proteins that stimulate mRNA export through the recruitment of the export receptor NXF1/TAP. They have also a role in mRNA quality control (non-sense mediated decay, NMD). NMD is in turn associated with the exon junction complex (EJC), a set of splicing-dependent proteins that are deposited at a specific position upstream of exon–exon junctions.
CF I_m is multisubunit factor that is required for pre-mRNA 3'end processing. We previously showed that CF I_m 68 kDa subunit interacts with a subset of shuttling SR proteins. We now report that both CF I_m59 and CF I_m68 have nucleo-cytoplasmic shuttling properties. In contrast, CF I_m25 does not appear to shuttle and requires one of the larger subunits to be imported into the nucleus. Nuclear export of CF I_m 59 and 68 kDa subunits is dependent on mRNA traffic. We are currently investigating interactions between CF I_m68 and EJC/NMD proteins. Preliminary immunoprecipitation experiments show that CF I_m68 can be found associated with RNP particles containing EJC/NMD subunits suggesting a role for CF I_m in mRNA export and translation.

A3

THE ROLE OF SUMO IN THE STABILITY OF DIVERSE TRANSCRIPTIONAL REPRESSOR COMPLEXES

Paula Babarovic and Susanna Chiocca. Department of Experimental Oncology, European Institute of Oncology, Milan, Italy

Post-translational modifications of proteins have critical roles in many cellular processes. One of recently discovered post-translational modification system is sumoylation. This modification involves four steps and different enzymes: SUMO activating enzyme E1, SUMO conjugating enzyme E2 (UBC9), SUMO ligases E3s and SUMO specific proteases. Available data currently implicate SUMO in the regulation of protein-protein interaction, subcellular localization and protein-DNA interactions. One of the proteins known to be sumoylated is HDAC1; component of Sin3A/HDAC, REST/CoREST and NuRD complexes. We report here that sumoylation is important factor in stabilization and maintenance of protein complex Sin3A/HDAC. Immunobloting and Q-PCR results show that the protein levels of the subunits of this complex is lower in cells where the SUMO pathway has been silenced. with their mRNA level unaltered. Using the approach of fusion proteins, we obtained HDAC1 wt, HDAC1 mutated in target SUMO sites (K444, K476R) and HDAC1 mutated in phosphorylation sites (S421, 423A) fused in frame with SUMO-1. Our coIP indicate that HDAC1 mutated in two phosphorylation sites, known to be inactive and unable to bind the other components of the complex, once fused with SUMO-1 becomes once again enzymaticly active and able to bind the other proteins. These results leads to a model by which once SUMO pathway is blocked the proteins involved in this complex are impaired to bind to the other components of the complexes and more accessible to the degradation machinery.

A4

A YEAST-BASED GENETIC SCREEN TO UNCOVER A NOVEL ROLE OF EPSINS IN CELL VIABILITY

C. Bartocci, M. Foiani and P.P. Di Fiore
IFOM, FIRC Institute of Molecular Oncology, Milan, Italy.

Epsins play an important role in receptor-mediated endocytosis in mammalian cells. A series of evidence also supports a role for these proteins in linking the endocytic process to signalling networks. While single ablation of either *epsin1* or *epsin2* is viable, double KO mice are embrionically lethal. Similarly, *Saccharomyces cerevisiae* cells lacking either of the highly conserved epsins, Ent1p and Ent2p, are viable, while double deletion cells are unviable. The portion of either protein sufficient for viability is the one containing the ENTH domain at the N- terminus. Deletion of the UIM-containing C-terminal portion, while impairing endocytosis, does not affect viability. Thus it is possible to distinguish two different functions of yeast epsins, carried out by distinguishable portions of the protein, one involved in internalization, the other in cell viability, independent of endocytosis.
Based on the above, I have undertaken a genetic screen with a yeast centromeric cDNA library to identify genes that, once overexpressed, are able to rescue the lethality of the *ent1ent2* double mutant.
Using this approach I aim to identify genes involved in signalling and cell cycle regulation. Studies of these genes may contribute to understanding this yet unexplored role of epsins.

A5

PROTEOMIC ANALYSIS OF ASTROCYTIC SECRETION IN A MOUSE MODEL OF FAMILIAL AMYOTROPHIC LATERAL SCLEROSIS

Basso M., Tortarolo M., Massignan T., Salmona M., Bendotti C., Bonetto V. Dulbecco telethon Institute, Mario Negri Institute, via Eritrea 62, 20157 Milan–Italy.

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by selective degeneration of motor neurons. About 10% of ALS cases show familial inheritance, 20% of which are caused by mutations in the gene encoding copper, zinc superoxide dismutase (SOD1). The role of these mutations in the pathogenesis and the peculiar selective motor neuron loss remain to be explained. Evidences from experimental models supports the view that a functional cross-talk between neurons and non-neuronal cells is crucial for the induction of motoneuronal death in ALS. The aim of this work is to investigate the role of astrocytes in the pathogenesis of familial ALS. For this purpose we analyzed the secretome from culture of astrocytes expressing the pathogenic mutated SOD1 or wild-type SOD1. Secreted proteins were separated by two-dimensional electrophoresis, and the gel maps were compared by computerized image analysis. The most abundant proteins secreted were identified by MALDI mass spectrometry. About thirty proteins are differentially secreted by astrocytes expressing the mutated SOD1 in comparison with controls. We are currently identifying these proteins. These preliminary results suggest that the only expression of mutated SOD1 has an impact on astrocyte secretion pathways. We are now planning to compare astrocyte secretome with the secretome of motor neuron/astrocytes co-cultures carrying or not the mutation.

A6

ROLE OF TIP60 IN CELLULAR DNA DAMAGE RESPONSE MECHANISMS

Samantha Bennett and Bruno Amati
Department of Experimental Oncology, European Institute of Oncology, Milan, Italy

Tip60, an HIV-1-Tat interactive protein, is a nuclear histone acetyltransferase (HAT) with the ability to acetylate both histones and several non-histone protein substrates. It has been shown that Tip60, and members of the Tip60 HAT complex, are recruited by transcription factors like MYC, E2F and p53 to target promoters and co-operate in transcriptional activation. Through these interactions Tip60 has been found to have a role in many diverse cellular pathways including the response to DNA double strand break repair and apoptosis.
However direct and mechanistic evidence, particularly for a function of Tip60 as a p53 co-activator and its role in the DNA damage response (DDR), are missing. We have begun to address this issue by inducing DDR in cell lines infected with a lentivirus RNAi against Tip60. We are also using MEFs, Pre-B and B cells derived from Tip60 wild type and Tip60 heterozygous mice in the same experiments to further elucidate Tip60's function in these pathways.

A7

UNRAVELLING THE PATHOGENESIS OF CMT4B1 NEUROPATHY: ROLE OF THE MTMR2/DLG1 COMPLEX IN SCHWANN CELLS

Annalisa Bolis, Silvia Coviello, Peter J. Brophy, Alessandra Bolino
Dulbecco Telethon Institute, San Raffaele Scientific Institute, Milano,

Inactivation of *Mtmr2* in Schwann cells is sufficient and necessary to provoke the CMT4B1 neuropathy with myelin outfolding .We demonstrated that the Mtmr2, a phosphatase that preferentially dephosphorilates PtdIns(3,5P)$_2$ and PtdIns(3)P, interacts with Dlg1/SAP97, a scaffolding molecule belonging to the MAGUK family of proteins. Dlg1 homologues have been located in several types of cellular junctions and play role in cell polarity and membrane addition. We proposed that Mtmr2/Dlg1 regulates membrane homeostasis at paranodal loops of Schwann cells -a region flanking the node of Ranvier- where also myelin outfoldings preferentially arise. We first demonstrated Dlg1 expression in Schwann cells, at paranodal myelin and at Schmidt-Lantermann incisures. In *Mtmr2*-null mouse fibers Dlg1 staining is almost lost. To understand which is the function of the Mtmr2/Dlg1 complex in Schwann cells we are identifying molecular partners of Dlg1 in Schwann cells using the yeast two-hybrid approach. The candidate interactors will be tested for their expression in myelin-forming Schwann cells and the interaction with Dlg1 will be confirmed by co-immunoprecipitation and co-localisation at the endogenous level. Isolated Schwann cells or Schwann cells/DRG co-cultures from wild type and mutant mouse will be used to demonstrate the Mtmr2/Dlg1 complex function in nerve.

A8

ALGORITHMS FOR SCORING DATA OF CROSS-LINKED PEPTIDES OBTAINED ON A FT-LTQ MASS SPECTROMETER

Dario Borsotti, Alessio Maiolica, Juri Rappsilber.
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Cross-linking combined with mass spectrometry can be a powerful tool in order to discover protein-protein interactions. The major problems are to observe the cross-linked peptides against the background of the non-linked peptides, and to interpret the relative fragmentation spectra.
The standard tools for protein identification match the MS data to single peptides. They are not able to associate one MS/MS to a pair of cross-linked peptides during search. We are working on software able to match the fragmentation dataset versus a list of potential cross-linked peptides, and sort them on the base of a new scoring system.
We performed an experiment that first chemically cross-linked the members of a small protein complex, which were then subjected to standard LC-MS/MS analysis. Here we focus on the algorithmic solutions that allow automatically to spot and interpret the MS/MS spectra of the cross-linked peptides. The developed method first calculates the *b*- and *y*- ion series for all the in-silico digested proteins, and then matches every peptide against all the MS/MS spectra using a score that reward the most complete ions series. In the second step, the pair of high score individual peptides is found that together adds up to the mass of the precursor of the cross-linked peptide. Finally, we take advantage of the MS3 spectra, in order to increase confidence in our identification. Manual validation shows that our software tool succeeds in identifying cross-linked peptides on the bases of fragmentation spectra.

A9

ROLE OF COMPLEMENT IN TUBULAR CELL ACTIVATION AND INJURY

Buelli S., Moioli D., Cassis P., Zanchi C., Morigi M., Zoja C., Noris M. and Remuzzi G.
Mario Negri Institute for Pharmacological Research, Bergamo, Italy

Abnormal filtered plasma proteins during proteinuric nephropathies have an intrinsic renal toxicity promoting interstitial inflammation and fibrosis. Ultrafiltered and/or locally synthesized third part of complement (C3) play a pivotal role in the progression of tubulointerstitial damage, but its role as promoter of renal injury upon protein challenge has not been established.
Here the effect of protein overload on complement activating properties of cultured proximal tubular cells (PTEC) was investigated. Cells exposed to human serum -source of complement- showed strong C3 and MAC deposition in a dose-dependent manner and the exposure to albumin, transferrin and IgG –mimicking a proteinuric condition- before serum addition resulted in an increase of such deposits. These events were accompanied by a decrease of exogenous factor H binding -the principal regulator of the complement alternative pathway- with a concomitant reduction of heparan sulfate, the major binding site of factor H, on cell surface in response to protein challenge. These findings indicate that proteinuric condition causes loss of PTEC capability to inhibit complement system activation. Protein overload also induced the activation of the transcription factors C/EBP and NF-kB, known regulator of C3 gene expression. This transcription activity was associated with enhanced C3 mRNA levels. These findings indicate protein overload as a promoter of complement activation and C3 gene induction in PTEC, events that might contribute to the progression of renal disease.

A10

ANALYSIS OF Chk2, ATM AND Nbs1, THREE PROTEINS INVOLVED IN THE CELLULAR RESPONSE TO DNA DAMAGE

Giacomo Buscemi, L. Zannini, L. Chessa and D. Delia. Department of Experimental Oncology, Istituto Nazionale Tumori, Milan.

Chk2 protein, a kinase with a key role in the response to DNA damage, is activated by ATM, the protein mutated in ataxia telangiectasia (AT) and targets several substrates causing cell cycle arrest, DNA repair or apoptosis.

We demonstrated that Chk2 activation by γ-radiation requires, besides ATM, Nbs1, the gene product involved in the Nijmegen Breakage Syndrome (NBS), a disorder that shares with AT chromosome fragility, radiosensitivity and radioresistant DNA synthesis. While in normal cells Chk2 undergoes a time-dependent increased phosphorylation and activation, in NBS cells these events are impaired. The same defects can be complemented by the reintroduction of wild type Nbs1, but not by a deletion mutant unable to form a complex with Mre11 and Rad50 in the nucleus. These results underline the main role of Nbs1 complex in ATM-Chk2 pathway and suggest that some defects in NBS and AT cells may result from defective Chk2 activation.

Since Nbs1 and ATM are involved in DNA breaks resolution, we investigated the lesion specificity of Chk2 response. Normal lymphoblastoid cells were exposed to different DNA damaging agents and proteins modifications and activation were evaluated. We demonstrated that ATM and Chk2 respond respectively to a low (<3) and a higher (>19) amount of double strand breaks per cell, but not to other lesions, underlining the specificity and the different sensitivity of DNA damage response components.

A11

MOLECULAR BASIS OF ENDOTHELIAL CELL DIFFERENTIATION: A POSSIBLE ROLE FOR SOX18 AND SOX7 TRANSCRIPTION FACTORS

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In order to characterize the molecular events occurring during endothelial differentiation, we used murine embryonic stem cells (ES) induced to differentiate to endothelial cells (EC) *in vitro*. Gene expression profile was evaluated at different stages of differentiation and compared to undifferentiated ES cells, to EC isolated from whole embryo and from adult mice. We found that Sox18 and Sox17 transcription factors are transiently expressed during differentiation, while they are barely detectable in undifferentiated or fully differentiated EC. Sox18 is up-regulated in embryonic derived EC as compared to EC from adult mice. Sox7 was high during ES differentiation and did not decrease in mature endothelial cells. Silencing Sox18 and Sox7 by siRNA in differentiating ES cells modified the expression level of classic endothelial markers. Furthermore, double knockdown Sox18+Sox7 in *Danio Rerio* embryos led to alterations in the vascular system characterized by edema in the cardiac region and reduced or absent circulation in the trunk. Single morphants lacking either Sox18 or Sox7 expression showed mild circulation defects or normal vascular phenotype respectively, suggesting a redundancy between the two proteins.
These data support the concept that Sox18 and Sox7 play a role in endothelial cell differentiation and are consistent with the vascular alterations observed in mice and humans carrying inherited mutations of the Sox 18 gene.

A12

ROLES OF SGS1 AND SRS2 DNA HELICASES AND THEIR ASSOCIATED FACTORS IN PREVENTING DNA RECOMBINATION AT DAMAGED REPLICATION FORKS.

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S.cerevisiae SGS1 belongs to the highly conserved family of RecQ DNA helicase genes that also includes human BLM, WRN and RECQ4, respectively altered in Bloom's, Werner's and Rothmund-Thomson genome-instability syndromes. Several studies indicate that deregulation of DNA recombination is a hallmark of these genetic disorders at molecular level.
Accordingly, recent evidences obtained in our lab using 2D gel electrophoresis technique indicate that Sgs1, together with the UvrD helicase Srs2, prevents unscheduled recombination events at damaged replication forks. Moreover, Sgs1 and Srs2 physically interact and form a large protein complex together with the evolutionally conserved endonuclease Mre11. This protein complex containing Sgs1, Srs2 and Mre11 reorganizes in response to checkpoint activation in different DNA damage-specific sub-complexes. Our data also indicate that other factors physically interact with both Srs2 and Sgs1 ; intriguingly, some of them are required for genome stability maintenance in yeast and have human orthologues which are inactivated in cancer prone diseases.
We are currently investigating whether these proteins might play a role in Sgs1 and Srs2-dependent pathway that regulate DNA recombination at damaged replication forks.

A13

EFFECT OF DIOXIN ON BONE CELL PROTEOME

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2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), the most toxic and biologically active member of the dioxins, is the prototypical compound of this class . Recently, it has been suggested that the antiestrogenic/antiandrogenic effects of TCDD observed in reproductive organs may also be observed in "non-reproductive endocrine organs" such as bone. As many hormones influence bone homeostasis, the bone status is an interesting target to study the role of TCDD as an endocrine disrupter. A few studies have shown that TCDD impairs bone tissue composition and function. Given that osteoporosis incidence is increasing in the Western world and experimental data shows that organochlorines have adverse effects on bone homeostasis, one may hypothesise that exposure to endocrine disrupting chemical, such as dioxin, may be a risk factor for osteoporosis. The aim of this project is to investigate the possible disturbance of bone homeostasis evoked by TCDD, using a proteomic approach on *in vitro* models of bone cells. The proteomic platform will include protein separation by bi-dimensional electrophoresis and protein identification by MALDI-TOF-MS and LC-MS/MS.
The main objectives are: (i) to characterise the effects of TCDD on bone cells, by identifying proteins that are specifically up- or down regulated in response to TCDD, (ii) to identify and define an integrated set of specifically responsive proteins within the pathway(s) of interest, (iii) to identify biomarkers of early events in dioxin toxicity for risk assessment. This PhD project lays in the framework of the EU project BONETOX (QLRT-2001-02528).

A14

FUNCTIONAL ROLE OF PARAPLEGIN/AFG3L2 COMPLEX IN MITOCHONDRIAL METABOLISM

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Paraplegin is a ubiquitous nuclear-encoded mitochondrial protein and it forms a high molecular weight complex with its homologous protein, AFG3L2, in the mitochondrial inner membrane. Paraplegin was identified because its inactivation leads to a neurodegenerative disease, Hereditary Spastic Paraplegia (HSP). Our studies on HSP patients' primary fibroblasts revealed a complex I deficiency and increased sensitivity to oxidative stress. This impairment of the respiratory chain activity could result in increasing radical oxygen species (ROS) production and oxidative stress damage.
What is the role of paraplegin complex in mitochondria of neuronal cells? We hypothesise that a selective effect of paraplegin deficiency in neurons could be due to high ATP consumption and high susceptibility to oxidative damage in neuronal cells. Furthermore, since neurons position their mitochondria in regions of intense ATP consumption we will assess whether mitochondrial trafficking at the periphery of the cell is affected.
Therefore, we are generating a paraplegin-deficient human neuroblastoma cell line, SH-SY5Y, either by homologous recombination strategy or by RNA inteference. This cellular model will allow the study of the role of paraplegin/AFG3L2 complex in mitochondrial metabolism, ATP production and mitochondrial transport throughout the cell body in differentiated versus undifferentiated neuronal cells.

A15

INCREASED ETHANOL CONSUMPTION AND RESISTANCE IN EPS8 NULL MICE CORRELATES WITH ALTERED ACTIN DYNAMICS AND NMDA RECEPTOR CURRENTS

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Actin dynamics in dendritic spines is an important component of synaptic plasticity. We found that KO mice for Eps8, an adaptor protein that regulates actin dynamics downstream of growth factor signaling, display increased ethanol consumption and tolerance. The fact that their ethanol metabolism is normal, indicates that Eps8 null mice have an increased resistance to ethanol due to alterations in the central nervous system.
Eps8 is highly and homogeneously expressed in cerebellar glomeruli, where it colocalise with F-actin. Since we found that also in vitro Eps8 and F-Actin are both present in synaptic-puncta, we used cultured cerebellar granule neurons as model system. Exposure of neurons to ethanol leads to relocalisation of Eps8 away from prominent clusters along neurites concomitant with the depolymerisation of F-Actin. Neurons derived from KO mice in contrast were resistant to ethanol induced actin depolymerisation. We found tha Eps8 is present in the post-synaptic compartment, in particular it is part of the NMDA receptor complex. Consistent with the model that actin remodeling is involved in regulating NMDA receptor activity, NMDA receptor currents are increased in eps8-/- mice and remain sustained even after ethanol exposure. Thus, Eps8 plays an important role in normal brain function and in the processes that lead to alcohol addiction by regulating actin remodeling and synaptic transmission.

A16

EFFECTS OF VECTOR DESIGN ON INTEGRATION SITE SELECTION BY MLV-BASED RETROVIRAL VECTORS.

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Retroviral integration follows non-random patterns in mammalian genomes, with a peculiar preference for active chromatin regions. In particular, MLV-based retroviral vectors preferentially target regions immediately flanking transcriptional start sites. This may result in gene activation and/or deregulation, accounting for a risk of insertional mutagenesis. The molecular basis of the preference of MoMLV pre-integration complexes for promoter-proximal regions is unknown. Here we investigate the effect of vector design on the integration pattern of an MLV-derived retroviral vector in the context of human CD34+ hematopoietic cells. We have designed three distinct vectors sharing an identical backbone (GFP gene under the control of viral LTRs and internal SV40 promoter driving the expression of ΔNGFR reporter gene) but differing in their LTR elements. One construct maintains wild type LTRs; a second vector contains a partially deleted U3 region and in the third construct the U3 deleted region is replaced with two erythroid-specific, autoregulatory elements (HS1 and HS2) of the GATA-1 gene. A linker-mediated PCR method has been optimized to evaluate the integration pattern of the three different constructs in hematopoietic cells. About 200 integration events have been collected for each vector and mapped onto the human genome. Our data indicate that LTR modification affects the normal MLV integration pattern, suggesting that vector design may have an impact on the safety characteristics of retroviral vectors.

A17

CONTRIBUTING TO BUILD AN UNDERSTANDING OF KINETOCHORE ARCHITECTURE

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Inaccurate separation of sister chromatids causes aneuploidy, one of the common features of cancerous cells. Kinetochores are multiprotein complexes that play an essential function in sister chromatid segregation by serving as scaffolds for spindle-microtubule binding. Kinetochores assemble dynamically onto centromeric DNA from many subcomplexes. Even the simplest kinetochore, that of *Saccharomyces cerevisiae*, is estimated to consist of around 60 different proteins. Little is known at present with concern to the architecture of kinetochores. In my PhD project, I would like to contribute to the understanding of how the kinetochore assembles from different subcomplexes, how individual subcomplexes form and interact with each other and how these interactions contribute to different functions of the kinetochore.
I started my analysis from the ROD-ZW10-Zwilch (abbreviated as RZZ) complex, which is essential for the mitotic checkpoint and the recruitment of the microtubule motor Dynein at the kinetochore, and its interaction with the core kinetochore component Zwint-1. In one line of investigation, I am using recombinantly expressed proteins and subcomplexes in crystallization trials in the hope to be able to solve their structure using X-ray crystallography. The recombinant proteins and subcomplexes will also be used in different protein interaction assays to study their interaction with other kinetochore components already available in the laboratory, including the Ndc80/Hec1 complex and the Mtw1/Mis12 complex. Furthermore, I will use a library of purified mitotic kinases available in the laboratory to identify sites of phosphorylation that might be expected to play a role in kinetochore assembly.

A18

ENLARGEOSOME TRAFFICK: FROM EXOCYTOSIS TO ENDOCYTOSIS AND MEMBRANE SHEDDING

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We are studying a new cytoplasmic vesicle, the enlargeosome (E), competent for a form of exocytosis $[Ca^{2+}]_i$-dependent and tetanus toxin-insensitive. Es are widely expressed and differ from all classical organelles (e.g. early and recycling endosomes, lysosomes, ER, Golgi and TGN). Their marker is a huge protein, which is retained in their lumen, named desmoyokin/Ahnak (d/A).
Following exocytosis, which accounts for an increase from 10 to 20% of the cell surface, two different retrieval pathways have been identified: in the first case d/A positive membranes are internalised in non-acidic vesicles, which differ from the various type of known endosomes. In the second vesicles around 1μm in diameter are shedded from the PM: immuno-EM analysis shows the presence of d/A on the surface of these structures. The two processes are modulated by $[Ca^{2+}]_i$ and PI3K activity.
The ongoing characterization of additional properties of Es such as their protein composition and the machinery and pathways of their trafficking to and from the plasma membrane, is expected to expand our understanding about the function of these vesicles, opening the way to the study of their role in pathology, concerning in particular processes such as cell migration and survival.

A19

UNRAVELLING THE PATHOGENIC MECHANISM OF ARX MUTATIONS LEADING TO SEVERAL FORMS OF MENTAL RETARDATION, EPILEPSY AND XLAG.

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Arx (aristaless-related homeobox gene) was originally identified as the gene responsible of an inherited form of West syndrome, a disease characterized by early onset epilepsy, infantile spasms and mental retardation (Stromme et al., 2002). However, recent findings have revealed that Arx mutations are the causes of some forms of inherited pure mental retardation and epilepsy diseases as well as of a lissencephaly phenotype (Kitamura et al., 2002). How this heterogeneous spectrum of malformations is induced by Arx mutations is still not understood and Arx functions during brain formation and function are still largely unknown. Arx is a gene highly conserved in animal evolution and its orthologs are isolated in mouse, chick, Xenopus and Drosophila. We showed that during murine development Arx expression was detected both in the GABAergic neurons of the basal ganglia and in the tangential migrating ones (Colombo et al., 2003), in the proliferative cortical regions, in the eminentia thalami and in the ventral thalamus. We are currently investigating the brain malformations occurring in Arx deficient mice. These mice exhibited hypoblastic olfactory bulbs and an extremely reduced olfactory cortex pointing to a general failure in olfactory system organization. Moreover, medial cortical structures as the choroid plexus, the dentate gyrus and hippocampus were either absent or strongly reduced. GABAergic differentiated neurons were decreased and failed to migrate properly into the cerebral cortex. These findings revealed specific functions played by Arx in different neural structures.

A20

ENDOTHELIAL CELL-CELL JUNCTIONS: CONTACT INHIBITION OF CELL GROWTH AND PROTECTION FROM APOPTOSIS

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Endothelial cell-cell junctions play a critical role in signaling and in mantaining endothelium homeostasis. In particular, they control cell proliferation and protection from apoptosis. Vascular-Endothelial-cadherin (VE-cad) is a key component of the adherens junctions. We observed that wt endothelial cells (ECs) in sparse conditions as well as VE-cad ko ECs in confluent conditions are not contact inhibited and are more susceptible to apoptotic stimuli in comparison to confluent wt ECs. A microarray experiment (Affymetrix platform) was designed to investigate the role of VE-cad in the regulation of EC phenotype and behavior through signal transduction and gene expression changes. We compared VE-cad positive and null murine ECs, in sparse and confluent culture conditions. Raw data were analyzed using different statistical approaches. We identified 2 subsets of genes under the control of VE-cad expression alone and 8 under the control of VE-cad expression and clustering. Each subset will be functionally classified using different algorithms allows in order to define regulated signaling pathways or gene families. A comparison of the promoter regions of co-regulated genes, searching for transcription factor binding sites, could suggest a common transcription factor, or a combination of transcription factors, responsible for the observed expression profiles.

A21

THE ROLE OF αSG IN THE PROLIFERATION OF SATELLITE CELLS

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Sarcoglycans are dystrophin-associated proteins (DAPs), forming a tight complex necessary for the physiological function of skeletal and cardiac muscles. Mutations in genes coding for sarcoglycans and sarcoglycan null mice produce cardiomyopathy and/or muscular dystrophy in animal models and humans. Satellite cells are a population of muscle progenitors able to activate, divide and differentiate in myocytes in response to the cell damage.
In this study we analyzed the number of clones and the number of cells in each clone of satellite cells, isolated from wt and αSG null mice. Preliminary results showed that satellite cells from αSG null mice produced a greater number of clones with a smaller number of cells in each clone. Moreover, we have shown, that in contrast to C2C12, satellite cells express αSG also during proliferation. In order to understand the role of αSG in the proliferation phase we isolated and cloned satellite cells from dystrophic and control muscle and tested them for responses to growth factors. Preliminary results show a possible involvement of αSG with FGF signaling. By Immunoflorescence analysis we have observed a partial colocalization of FGFR1 and αSG only during proliferation.
Our future studying will concern some in vivo proliferation assays by directly injection into tibialis of wt and dystrophic satellite cells different labeled. Finally we will compare the adhesion capacity of wild type and dystrophic satellite cells through a cell adhesion assay in order to understand whether the loss of αSG could affects the cell adhesion capacity.

A22

A PREP-1 NULL MUTATION IS EMBRYONIC LETHAL AT E7.5.

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The Prep-1 Meinox protein is able to interact with all Pbx proteins, to prevent their export from the nucleus and to cooperate in DNA binding site selection. We have generated a Prep-1 null mouse by deleting its homeodomain by homologous recombination. The phenotype of the
Prep1$^{-/-}$ mutation is embryonic lethality at E7.5.
Importantly, we have analysed in detail the mutation to exclude a dominant negative expressing the amino terminal region of the protein. First, by immunoblotting analysis we have not found any other form than wild type Prep-1 protein in heterozygous mice. Moreover, we have cloned and sequenced the mRNA at the site of the insertion of the vector and have found a series of stop codons preceding the LacZ part of the vector. Finally, we have analyzed the size of the LacZ protein and found that it was indistinguishable from the same protein expressed in a cell line. We therefore conclude that the mutation is indeed a null mutation and that Prep-1 is required for early (gastrulation) developmental processes. We hypothesize that the complete absence of Prep-1 protein causes the total absence of all Pbx proteins.
Initial analysis of gastrulation markers, like brachyury, suggest a defect in early gastrulation. A detailed study of the pattern of expression of Prep1 between E6.0 and E7.5 together with the expression of other markers of gastrulation should help us to understand better the Prep1 Knock-out phenotype.

A23

EPS8-IRSP53 COMPLEX CONTROLS ACTIN REMODELING DOWNSTREAM CDC42

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A large repertoire of actin-binding proteins regulates the dynamic assembly and spatial organization of actin filaments. Coordination and integration of their activities is essential to control site-directed actin polymerization *in vivo.* These proteins are, in turn, the targets of various signaling pathways emanating from diverse extracellular stimuli. Within these pathways, Rho GTPase family members, play a key role, acting as molecular switches on which signaling inputs converge and are transduced into coordinated output-events regulating site-directed actin dynamic. Eps8, the prototype of the Eps8-family proteins, participates in the transduction of signal from Ras to Rac leading to actin remodeling. Moreover Eps8-family proteins display barbed end capping activity, which resides in the C-terminal "effector" region. The full length Eps8 is auto-inhibited *in vitro* and the association with its interactor Abi1 relieves this inhibition, unveiling novel modalities of regulation of capping through protein:protein interactions. This led us to search for novel interactors of Eps8 capable of regulating its activity, and to link Eps8 to upstream signaling events. The scaffolding protein insulin receptor tyrosine kinase substrate p53 (IRSp53), a ubiquitous regulator of the actin cytoskeleton, mediates filopodia formation downstreamo the smal GTPase Cdc42.
Here we show that Eps8 interacts with Irsp53 *in vitro* and *in vivo* and show that the Eps8-IRSp53 complex is involved in actin citoskeleton remodeling leading to filopodia formation, by bundling actin filaments, downstream the small GTPase Cdc42.

A24

MECHANISMS RESPONDING TO DNA DOUBLE STRAND BREAKS DURING S-PHASE

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In the recent years, a lot of work has been concentrated in trying to understand how cells deal with DNA double strand breaks. The response to these lesions is a coordinated action of specialized repair and checkpoint proteins, which act together to prevent pathological transitions at the break and to promote repair. The key players and repair mechanisms adopted in the G1 and G2 phases of the cell cycle are now becoming clear but still, little is known on how cells deal with these chromosomal lesions during S-phase and, importantly, what happens when the DNA replication machinery encounters a DSB.
Using yeast as a model system I'm studying the cellular response to a site specific DSB during S-phase. In particular, by using two-dimensional agarose gel electrophoresis, I'm analyzing the replication fork transitions occurring at a DSB site in wt cells or in mutants altered in the DNA damage response.

A25

REPLICATION FORKS AND FRAGILE SITES: YEAST AS A MODEL SYSTEM TO STUDY THE FATE OF REPLICATION INTERMEDIATES AT RECOMBINATION HOT SPOTS

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Eukaryotic chromosomal DNA replication initiates at multiple sites called origin elements and replication forks move bidirectional through the genome from these sites. Replication fork progression may slow down at specific genomic locations known as replication slow zones and at repetitive sequences. These chromosomal loci represent hot spots for recombination and, in human cells, are known as fragile sites. The checkpoint response contributes to maintain the integrity of replication forks encountering fragile sites.
Using yeast as a model system I aim to investigate the physiological and pathological transitions of replication forks encountering specific genomic locations that are known to cause the slowing down of the forks. I will use the 2D gel approach that will allow me to analyse the quality and the fate of the replication intermediates in wild type cells and in mutant defective in the DNA damage response. The analysis will be carried out in genetic backgrounds in which the fragile sites have been positioned at convenient genomic locations.

A26

SPECIFIC CHROMATIN STRUCTURES, DEFINED BY MNASE DIGESTION OF CHIP-READY CHROMATIN, REVEAL THE PROXIMITY BETWEEN ENHANCER AND MINIMAL PROMOTER OF UPA GENE.

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We have studied the interaction of the enhancer (E) with the minimal promoter (MP) of the uPA gene, located at less than 2Kb from each other, in PC3 cells, that contain multiple copies of the gene itself and that constitutively express it. By using ChIP we show that antibodies against Sp1, that specifically bind theMP of uPA gene, and p300 enriched the immunoprecipitate material in both enhancer and MP sequences. The average size of the chromatin fragments, after crosslinking and sonication, is around 600 bp. However, the bulk of the sonicated material also contains fragments of 2 kb or more, raising the possibility that enhancer and MP may be present on the same genomic fragment after sonication. In order to exclude this and to confirm the proximity between the two regulatory elements, we set-up a newapproach by coupling MNase digestion to ChIP. MNase digestion of X-linked chromatin from PC3 cells allowed us to identify a number of large, cleavage-resistant fragments in the region of the minimal promoter, but not in the enhancer region. Furthemore, we show that they posses specific chromatin compositions, which could be associated with different transcription states of the uPA gene. In particolar, a specific MNase resistant fragment contains the elongating form of RNA Polymerase II (RNAP II),phosphorylated in serine 2 of the CTD. Interestingly only a nucleosome core-size fragment (145 bp) that spans the enhancer shares the same exact chromatin composition, HMGN protein and RNAP II content of the structure on the MP. All together this results provide evidences on the proximity of the enhancer and the MP of the uPA gene and that this specifically occurs through structures actively engaged in transcription. Moreover this method allows to fine map the DNA involved in this interaction. MNase-coupled ChIP represents a powerful tool to study long-range chromatin interactions in transcriptional regulation.

A27

THE KINETOCHORE-BINDING DOMAIN OF MAD1 AND ITS BINDING PARTNERS

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The spindle checkpoint delays sister chromatid separation until all chromosomes have undergone bipolar spindle attachment. Mad1 and Mad2 are key effectors for the establishment of the checkpoint. Mad1 is a 718-aa coiled-coil protein associated with nuclear pores during interphase. During prometaphase, Mad1 is localized at the unattached kinetochores where it acts as Mad2 receptor. The N-terminal region of Mad1 is required for its localization at the kinetochore. We are interested in identifying Mad1-binding proteins that may mediate kinetochore recruitment and release of Mad1 by means of mass spectrometry analysis on the immunopurified endogenous Mad1. On the other hand, to characterize the structure of the Mad1 N-terminal domain, two different N-terminal fragments have been successfully expressed and purified. The two constructs have been further characterized using biochemical approaches. The combination of hydrodynamic experiments and the samples behaviour on size exclusion chromatography leads to the extrapolation of their native molecular weight. This value confirms the predicted dimeric state of both constructs. Furthermore, we would like to confirm whether the constructs are the minimum fragment localizable at the kinetochore by transfecting HeLa cells with myc-tagged versions of both samples. If the constructs contain the kinetochore-binding domain, we should observe the inability of these cells to arrest in mitosis in the presence of nocodazole.

A28

REGULATORY POLARITY ACROSS FIRST EXON OF HUMAN GENES: FIRST INTRON AS HOTSPOT OF ANTISENSE TRANSCRIPTION

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Recent experiments employing genomic tiling arrays have highlighted serious gaps in our understanding of genome function. Binding of transcription factors far away from annotated promoters and large amounts of unknown transcripts inside and outside of the boundaries of known genes have been reported.
We analyzed the regulatory potential of the whole amount of genomic sequences of protein coding genes available in RefSeq database. Different methods were used: CpG islands analysis, transcription factor binding sites detection and promoter prediction. The analysis revealed a strong accumulation of regulatory sequence in a region proximal to the first exon - first intron junction.
We took advantage of different types of transcriptional data to evaluate the presence of antisense transcripts starting from this region. Cap-analysis gene expression (CAGE) data generated by FANTOM consortium and cDNA and 5'ESTs available in GenBank database were mapped on genomic sequences to identify the genomic location of 5' end of antisense transcripts. We detected a preferential localization of antisense transcription start sites in the first intron and first exon of genomic sequences. Even in this case a strong accumulation was detected in first exon - first intron junction, indicating a critical role for this region.
Some of identified cases were tested with wet lab experiments that confirmed promoter activity in the first intron and the presence of antisense transcripts.

A29

CELL-CYCLE KINASES REGULATE CELLULAR RESPONSES TO DOUBLE STRAND BREAK

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Double Strand Breaks (DSBs) lesions may occur frequently as a result of metabolic reactions, faulty chromosome replication or exposure to mutagenic agents. One single DSB in yeast can lead to ATR/Mec1-mediated checkpoint activation when the break cannot be repaired or when certain repair events generate extensive regions of ssDNA.
We recently showed that a single HO-induced DSB is not sufficient per se to activate a checkpoint response in G1 and S phase. Furthermore the activity of CDK1 is required for the correct processing of a chromosomal lesion occurred in G2. Theese observations lead to the question of how the cells deal with a DSB in different cell-cycle phases. To address this issue, I took advantages by using specific genetic backgrounds to analyze different mechanisms of DSB repair and checkpoint activation. In particular, I'm addressing the roles of CDK1 and other cell-cycle kinases in linking the appropriate pathway of DSB repair to the checkpoint response throughout the cell cycle.
I'm also setting up a genetic systhem to visualize the events of homologous recombination and non homologous end-to-end joining at the DSB locus, thus elucidating how CDK1 partecipate to the processes.

A30

TELOMERE ASSOCIATION, LENGTH REGULATION AND TELOMERASE ACTIVITY MODULATION BY THE RAD9/RAD1/HUS1 DNA-DAMAGE CHECKPOINT COMPLEX

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Telomeres cap the ends of chromosomes and are maintained via a specialized mechanism involving the telomerase reverse transcriptase enzyme. Here we report that the mammalian Rad9/Rad1/Hus1 cell cycle checkpoint complex (911) is a novel constituent of the mammalian telomere and is required for telomere homeostasis. Significantly, Hus1-deficient primary mouse embryonic fibroblasts, as well as thymocytes from conditional Hus1 knockout mice, demonstrated severe telomeric shortening, as well as chromosomal fusions. 911 was found in association with telomerase enzyme activity and it was required for efficient telomerase activity in vitro. Taken together, these findings identify an unanticipated function for 911 at telomeres in mammals and provide a mechanistic link between the activity of DNA-damage checkpoint proteins and the telomere maintenance machinery.

A31

CHARACTERIZATION OF THE DIFFERENT GLUTAMATE TRANSPORTERS: FOCUS ON THE PRESYNAPTIC SUBTYPES AND THEIR ROLE IN NEURODEGENERATIVE DISEASES.

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Glutamate homeostasis in the CNS is guaranteed by specific transporters (EAATs) that mediate glutamate re-uptake. Five EAATs have been described; the glial transporter EAAT2/GLT1 seems to play the major role in glutamate removal, although some evidence suggest a role played by a presynaptic transporter located in nerve terminals. This EAAT will be of great relevance in pathological conditions where a reversal mode of operation of transporters could result in glutamate accumulation in the extracellular space. Neurons seem to be the principal source of glutamate in this condition, thus the nerve terminal transporter is an interesting tool to investigate. The demonstration of the presence of a presynaptic transporter is still problematic, in particular because crude synaptosomal preparations are contaminated by glial elements. The preparation of purified neuronal fractions can be useful in clarifying this matter. We evaluated the abundance of neuronal and glial elements and their glutamate uptake kinetics (Km and Vmax) in purified fractions obtained by two different methods. Results indicated that purified preparations were enriched in specific elements and that the crude synaptosomes are mainly constituted by neuronal elements. The different preparations also showed different capacities of glutamate uptake, with higher Vmax in the purified neuronal fractions. The distribution of the different EAAT in these fractions is currently under investigation.

A32

CHROMATIN ASSOCIATED FUNCTIONS OF p27

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Cyclin E, CDK2 and the CDK inhibitor p27 have been proposed to associate with pre-replication complexes in Xenopus, where p27 degradation might coordinate the loading of mcm proteins (Furstenthal et al. 2001a; 2001b). No such interaction was reported in mammalian cells. In the mouse, deletion of either CDK2 (Berthet et al. 2003; Ortega et al. 2003) or cyclins E1 and E2 (Geng et al. 2003; Parisi et al. 2003) do not affect cell cycle progression, and hence are likely to be compensated during embryonic development. Interestingly, however, cyclin E double-KO cells cultured in vitro were defective in cell cycle re-entry from quiescence, and showed a defect of mcm loading on chromatin (Geng et al. 2003). For these reasons, we hypothesized that both loading of p27 on origins and its degradation, controlled by cyclin E/CDK2, might underlie the defect seen in cyclin E-null cells. For example, these cells might load p27 on origins but might be unable to degrade it, hence preventing mcm loading. Loss of CDK2, on the other hand, may either be compensated by another CDK, or may lead to a failure to load p27 on origins. In order to address these hypotheses, we have initiated a series of experiments aimed at exploring whether p27 is indeed loaded on replication origins in human and mouse cells. Preliminary cell fractionation data show that part of p27 is recovered with a chromatin fraction in quiescent cells and, later, at the G1-S transition. We are using Chromatin Immunoprecipitation to address the association of p27 with specific genomic sequences. Further characterization of these interactions will be presented.

A33

SON OF SEVENLESS SIGNALING

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Sos1 (Son of sevenless) plays a well-established role in the signal transduction from receptor tyrosine kinases (RTK) to the Ras-Mapk pathway. Upon activation RTKs, Sos1 is recruited to the plasma membane via an interaction with the adapter protein Grb2. This re-localization allows Sos1 to act as Guanine nucleotide exchange factor (GEF) for the small G protein Ras. The activation of Ras by Sos1 is tightly controlled by a negative feedback loop that implies the phosphorylation of Grb2 by Erk, which in turn results in reduced binding affinity to Sos1.
Surprisingly and contrary to the to the current dogma, we recently found that in some cell lines the association of Grb2 and Sos1 is growth factor dependent. This raises questions about the mechanisms preventing the SH3 domain of Grb2 from binding to the proline rich region of Sos1.
Interestingly, Sos1 can also acts as a Gef for the small RhoGTPase Rac via its DH domain near the N-terminal portion. This activity seems to be regulated by post-translational modification and cellular localization of Sos-1. However this function is much less understood and is one major topic of our research. Two different events can trigger the activation of Sos1 as a Rac Gef. One is its engagement in a complex with Eps8, Abi1 and PI3K and the other implies the phosphorylation of one or possibly more tyrosine residues by Abl. In each case the structure of Sos1 has to change dramatically, since the DH domain is sterically blocked by the PH domain. The first attempt to solve this question will be to map the tyrosines that are phosphorylated by Abl.

A34

EPSIN FUNCTION IN ENDOCYTOSIS AND CELL SIGNALING

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Endocytosis is a mechanism present in all eukaryotic cells. Epsins are proteins highly conserved from yeast to man which are selectively enriched at sites of endocytosis. In the mammalian genome, there are three epsin genes. These proteins are cytosolic with a characteristic three domain structure which permits it to interact with the other proteins of the endocytic mechanism. By dominant negative interference and other studies, epsins were demonstrated to have an important role in the endocytosis. In addition, yeast studies have shown an epsin function in actin remodeling. In the meantime it was shown that epsin in Drosophila, liquid facet, is involved in the activation of the Notch signaling pathway. In fact epsin is an essential gene that is required for the activation of Notch signaling pathway by potentiating the endocytosis of its ligand.
In our lab we have generated epsin1 and epsin2 knock- out mice. Surprisingly, epsin single mutants do not show major phenotypical defects. Since the two epsins have similar domain structure, interactors and overlapping tissue distribution, we hypothesize that the expression of one is compensating for the lack of the other.
Based on the recent observations, in invertebrates, that epsin is required for full Notch activation and the epsin knock- out show severe embryonic phenotype, we decided to generate the epsin double knock-outs (DBKOs). We could observe a mendelian distribution of epsin heterozygous matings that indicates embryonic lethality of our DBKOs. In fact our DBKOs show severe defects in the cardiovascular system, the first organ system that is functional at this stage, with altered vasculogenesis in extraembryonic tissues and angiogenetic blockage in the primary vascular plexus remodeling. Real- Time PCR analyses on a variety of transcripts involved in early organogenesis show a dramatic alteration of most Notch reads-out expression. These data represent direct genetic proof of epsin espistaticity in Notch signaling.

A35

MOLECULAR MECHANISMS UNDERLYING THE ROLE OF SYNAPSIN I IN EPILEPSY

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The synapsins (syn) are a family of synaptic vesicle (SV)-associated phosphoproteins implicated in the control of neurotransmitter release and neuronal development. The recent identification of a non-sense mutation in the *synI* gene in a family displaying X-linked recessive partial epilepsy and/or mental retardation points to a role for syn I in human epilepsy. Null mutant mice for syn I have been generated and have been shown to undergo partial seizures, thus representing a good model for idiopathic partial epilepsy of genetic origin.
The goal of this project is the elucidation of the molecular mechanisms by which the reported mutation in the syn I gene leads to the development of an epileptic phenotype.
A mutated version of human syn I bearing the non-sense mutation described in epileptic patients has been introduced in embryonic hippocampal neurons derived from syn I KO mice. The targeting of mutated syn I has been tested by co-localization with endogenous synaptic markers. Neuronal cells trasfected with either wild type or mutated human syn I have been treated with specific stimuli in order to analyze their phosphorylation pattern. siRNA technology will be exploited to discriminate whether the epileptic phenotype of the KO mice is due to altered neuronal development or to impaired SV recycling at mature synapses.

A36

INDUCING NEURAL COMMITMENT IN BLASTOCYST OUTGROWTHS AND MORPHOLOGICAL CHARACTERIZATION OF NEURAL ROSETTES.

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SCRI, DIBIT, San Raffaele, Milan Italy

Neural progenitor commitment may be obtained using specific in vitro conditions starting from embryonic stem cells. ES derived neural cells can be maintained in vitro for long time and exhibit stem cells properties as self-renewal ability and multipotency. We optimised an in vitro culture system to derive neural progenitors directly from freshly isolated bovine blastocysts. Embryos were seeded on STO-feeder layers and the derived outgrowths were expanded in a serum-free medium. Under these conditions, embryonic cells grew in colonies exhibiting multiple rosettes morphology. Neural rosettes are fascinating structures constituted by neural progenitors that assemble together in an orderly array. Intriguingly, these structures exhibit similar features described for the neural tube. We propose that in vitro formation of neural rosettes correspond to a bidimensional recapitulation of neural tube formation during embryonic development.Moreover, in our model neural rosettes were further cultured giving rise to stable cell lines of neural progenitors that exhibited features of neural stem cells. Detailed characterization of these lines is under way.

A37

NPM ISOFORMS†: CONDITIONAL KNOCKOUT MOUSE

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NPM (also known as Nucleophosmin, B23, NO38) is a ubiquitously expressed nucleolar phosphoprotein which acts as a molecular chaperone and shuttles between nucleus and cytoplasm. It is also implicated in ribogenesis, cell proliferation and it is supposed to have a role in DNA damage response. Furthermore, the NPM gene is mutated and rearranged in a number of haematological disorders. NPM exists in two phosphorylated variants, NPM1 and NPM2 (B23.1 and B23.2, respectively). NPM1 is mostly nucleolar, while NPM2 is present in cells in low levels and is detected both in cytoplasm and nucleoplasm. These two isoforms differ from each other in short specific regions at their carboxyl-termini. The functions of each isoform are not well understood. To investigate their specific roles, we decided to generate a conditional knockout mouse for NPM1 and NPM2. We isolated the NPM locus using a Bac Library and then we cloned the gene by means of a new technique called ìrecombineeringî (recombination-mediated genetic engineering): a method based on homologous recombination in E. Coli using recombination proteins provided from (phage. After the generation of the targeting vectors for the two isoforms, we started the homologous recombination in ES cells.

A38

RIBOSOMES HOST PKC ACTIVITY ABLE TO REGULATE PROTEIN SYNTHESIS.

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Protein synthesis is a very energy expensive process; translation is highly regulated at multiple steps. The initiation phase is the rate limiting step and it is mediated by several proteins known as eukaryotic Initiation Factors (eIFs). eIFs are target of complicated signal transduction pathways.
Protein Kinase C (PKC) is a well known kinase involved in fundamental cellular processes such as growth and differentiation. There are twelve different isoenzymes with different cellular localization and molecular targets. Receptor of Activated C Kinase 1 (RACK1) is a scaffold protein for active PKCβ2 and we recently showed to be bound to cytosolic ribosomes.
To further explore the role of PKC in protein synthesis, we defined which PKC isoform can translocate onto cytoplasmatic ribosomes. We show that RACK1 docks activated PKCβ2 on cytoplasmic ribosomes. In condition of PKC stimulation, ribosomes exhibit a specific PKC activity.
In several cell models, PKC activation leds to an increase of translation, mostly through undefined pathways. Peptides able to specifically block the binding of RACK1 to PKCβ2 isoform greatly impair PKC stimulated translation.
In conclusion, we speculate that ribosomes host a kinase platform able to directly transduce signals to the ribosomal machinery.

A39

CHARACTERIZATION OF THE ROLE OF RAC3 IN NEURONAL FUNCTION

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Rac proteins are members of the Rho family of GTPases involved in the regulation of actin dynamics. The three highly homologous Rac proteins in mammals are the ubiquitous Rac1, the haematopoiesis-specific Rac2, and the least characterized Rac3. Rac3 mRNA is widely and specifically expressed in the developing nervous system, with highest concentration at embryonic day 13 in the dorsal root ganglia and ventral spinal cord. At P7 Rac3 is particularly abundant in populations of projecting neurons in several regions of the brain, including the cortex and the hippocampus. To examine the function of Rac3 *in vivo* we generate mice with a targeted deletion of the coding region of the gene. Rac3 knockout animals survive embryogenesis and show no obvious developmental defects. Interestingly, specific behavioural differences were detected in the Rac3-deficient animals, since motor coordination and motor learning on the rotarod were superior to their wild type littermates. No obvious histological or immunohistochemical differences were observed at major sites of Rac3 expression. This results indicate that in vivo Rac3 activity is not strictly required for normal development in utero, but may be relevant to later events in the development of a functional nervous system. I'm performing detailed analysis of neurons in culture to identify cellular correlates of the behavioral phenotype observed.

A40

CHARACTERIZATION OF THE PTB DOMAIN IN THE Eps8 PROTEIN FAMILY

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Eps8, the prototype of the Eps8-family proteins, is involved in signalling leading to actin remodelling by mediating Rac activation. Recently, Eps8 has been show to possess barbed end capping activity, thus increasing the complexity of its role in actin-dynamics. In mammals, three different Eps8-related genes (Eps8L1, Eps8L2, Eps8L3) share a collinear topology with a putative PTB domain at the N-terminus, a central SH3 domain and a C-terminal "effector region". The "effector region" is responsible for capping activity, whereas the SH3 domain forms macromolecular complexes with the Abi1 scaffold and the Rac-specific GEF, Sos1. In contrast, the function of the PTB domain remains unknown. PTBs display binding specificity for peptides containing the NPXY motif, with different requirement with respect to the phosphorylation status of the tyrosine residue. Furthermore, they are also involved in a variety of signal transduction pathways, often by binding to tyrosine-kinases or cytokine receptors, and endocytic/exocytic processes. To characterize the PTB domain of the Eps8 family, the structure and interactions of this domain will be characterized. Cell membrane receptors binding to the Eps8PTB domain, identified from a screening of GST-peptides, will form the basis for characterizing domain interactions. Since the PTB domain of Eps8-related proteins is not highly conserved, variability in interacting partners is expected, thus suggesting putative specific functions for each different member of this protein family.

A41

MESENCHYMAL STEM CELLS ARE RENOTROPIC, HELP REPAIRING THE KIDNEY AND IMPROVE FUNCTION IN ACUTE RENAL FAILURE

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Injury to a target organ can be sensed by bone marrow stem cells that migrate to the site of damage, undergo differentiation and promote structural and functional repair. This remarkable stem cell capacity prompted us to investigate the potential of mesenchymal and hematopoietic stem cells to cure acute renal failure. We elected to use the model of renal injury induced in mice by the anticancer agent cisplatin. Injection of mesenchymal stem cells of male bone marrow origin remarkably protected cisplatin-treated syngeneic female mice from renal function impairment and severe tubular injury. Y-chromosome containing cells localized in the context of the tubular epithelial lining and displayed binding sites for *Lens culinaris* lectin, indicating that mesenchymal stem cells engraft the damaged kidney and differentiate into tubular epithelial cells, thereby restoring renal structure and function. Mesenchymal stem cells markedly accelerated tubular proliferation in response to cisplatin-induced damage, as revealed by higher numbers of Ki67 positive cells within the tubuli in respect to cisplatin mice given saline. Hematopoietic stem cells failed to exert beneficial effects. These results offer a strong case for exploring the possibility that mesenchymal stem cells by virtue of their renotropic property and tubular regenerative potential may have a role in the treatment of acute renal failure in humans.

A42

GENETIC SCREENS TO IDENTIFY CHECKPOINT-DEPENDENT PATHWAYS CONTROLLING THE STABILITY OF REPLICATION FORKS

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Chromosome integrity during DNA replication is essential for preventing genome rearrangements and cancer. When replication pauses the stability of stalled forks is controlled by the checkpoint response, which in the yeast *Saccharomyces cerevisiae* is mediated by the Mec1/Rad53 kinases. Results obtained in our laboratory indicate that Rad53 prevents the accumulation of abnormal replication intermediates thus allowing replication forks to restart DNA synthesis following pausing of the replication machinery. In fact, hydroxyurea (HU)-treated *rad53* cells accumulate DNA structures resembling recombination intermediates that impede replication resumption when the inhibitor is removed.
The Rad53 kinase phosphorylates several factors implicated in chromosome replication and recombination. However, it is still unclear whether these phosphorylation events are required to prevent the collapse of replication forks in response to replication stress or intra-S DNA damage. Further, it is reasonable to think that not all of the targets of Rad53 kinase play a crucial role in protecting stalled forks. Hence, in order to identify those relevant factors implicated in facilitating replication restart in response to replication stress, I will use the yeast *S.cerevisiae* to screen for mutants able to rescue the HU sensitivity of *rad53* cells.

A43

SEARCHING FOR MASTER GENE(S) GOVERNING NEUROSECRETORY COMPETENCE

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Our work is based on the hypothesis that competence for regulated neurosecretion results from a specific gene programme governing expression of neurosecretory organelles and of their function.. Our task is the identification of the master gene(s) of the programme. As the main tools we use PC12–TrkA and PC12-27, two defective clones that mantain the general phenotype of rat PC12 cells, but lack all components of the neurosecretory machinery . Previous studies had shown that fusion with other wt cells can yield revertants expressing all PC12 properties, possibly due to master gene(s) activity. In order to reveal in the revertants the acquired chromosomes and/or chromosomal fragments new fusions have been performed between PC12-TrkA cells and human lymphocytes and hybrid cells have been studied by cytogenetics.. The presence of one (or more) of these chromosome/fragment(s) in all neurosecretion-positive hybrid clones, as well as their absence in those incompetent for neurosecretion, has opened the search for the mapping and identification of master gene(s) and for the study of its (their) mechanism(s) for the coordinate expression of neurosecretion.

SESSION A: CELLULAR AND MOLECULAR BIOLOGY

Angelo Lombardo
Stefano Biressi
Alessio Maiolica
Pietro Veglianese
Laura Carrassa
Vera Magistroni

SESSION B: IMMUNOLOGY

Paramita Baruah
Veronica Huber
Barbara Cassani
Erica Butti
Chiara Martinoli
Paola Pittoni

SESSION C: CANCER BIOLOGY

Raffaella Di Micco
Paola Bonetti
Federica Polato
Cinzia Brambilla

JANUARY 24TH - ABSTRACTS

ORAL PRESENTATIONS

DEVELOPMENT OF NOVEL LENTIVIRAL VECTORS FOR GENE CORRECTION OF INHERITED MUTATIONS

Angelo Lombardo, Christian M. Beausejour, Lucia Sergi Sergi, Philip D. Gregory, Michael C. Holmes, Luigi Naldini. HSR-TIGET, San Raffaele Scientific Institute, Milan, Italy

X-linked severe combined immunodeficiency is a fatal monogenic disorder caused by mutations in the IL2RG gene. Recently, the disease was successfully treated by retroviral vector (RV) mediated gene replacement in hematopoietic stem cells (HSC). Unfortunately, leukemia developed in a fraction of treated patients, and its origin was linked to RV insertional mutagenesis. Thus, although stable genetic modification is required for HSC gene therapy, the random distribution of retroviral integration poses a challenge for the safe use of RV in HSC. An alternative approach to gene replacement is the correction of the endogenous gene using engineered zinc finger protein-based nucleases (ZFNs) to specifically target a DNA double stranded break at the site of mutation. One mechanism the cell uses to repair these breaks is homologous recombination (HR), and specific nucleotide substitution can be made in the genome by delivering an extrachromosomal donor molecule to serve as repair template during HR. By this approach mutations may be corrected and normal gene function restored. Here we developed a new delivery platform based on integrase-defective lentiviral vectors, which takes advantage of the viral proficiency both to introduce template DNA and transiently express ZFNs in the same cell. Using this approach, we reached unprecedented levels of gene correction at the IL2RG locus in cell lines, and we are currently testing correction of IL2RG gene mutation in X-SCID patient cells. Our results provide proof of principle of a new gene therapy strategy with a unique combination of safety and potential efficacy.

THE MOLECULAR BASIS OF SKELETAL MYOBLAST DIVERSIFICATION. GENE EXPRESSION ANALYSIS OF PURIFIED EMBRYONIC AND FETAL MYOBLASTS.

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A cell-sorting method for isolating myoblasts at different stages was developed using a mouse strain with the GFP gene targeted into the Myf-5 locus. Isolated embryonic and fetal myoblasts show an intrinsically different behaviour *in vitro* (i.e. differentiation/fusion ability, response to myogenesis inhibitors). With the aim to understand the molecular mechanisms responsible of these phenotypic differences the changes in gene expression between the two populations has been evaluated by microarray. In particular we decided to investigate the role of the homeobox gene ARX, which appears to be more expressed in embryonic myoblasts. In situ hybridization and real-time PCR approaches have confirmed that ARX expression is predominant in the myogenic areas during the embryonic stage, decreases during the late prenatal life and after birth in the mouse. Studies on fibroblasts converted to a myogenic fate by the expression of different MRFs and analyses on KO embryos for different muscular determination genes reveal a certain degree of redundancy in the mechanisms necessary for activating ARX expression. When over-expressed in the myogenic cell line C2C12 ARX is able to accelerate the process of differentiation (interestingly embryonic myoblasts differentiate more rapidly than their fetal counterpart). Moreover when co-expressed with MyoD in 10T1/2 fibroblast ARX seems to significantly enhance the expression of markers of muscular differentiation by CAT assay. These and other evidences let us conclude that ARX has the role of promoting embryonic myogenesis.

PROTEIN-PROTEIN INTERACTION BY MASS SPECTROMETRY

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Large protein complexes play a key role in many biological processes. An emerging and relatively new approach that combines chemical cross-link with mass spectrometry can efficiently complement techniques such as NMR and X-ray. Cross-linking combines physiological working conditions with the ability to investigate large structures. Detecting those tryptic peptides that contain the cross-link site allows establishing protein complex topology and gives an estimation of neighboring protein regions and thereby candidate areas for interaction surfaces. The common way to identify cross-linked peptides is by measuring their exact mass. The number of cross-linked peptide matching an observed mass increase as the complexity of the system increases. For large protein complexes hundreds of possible peptide combination may match any observed mass. Here we show an approach that uses a type of cross-linking reagent present in a heavy and light form (deuterated and not deuterated) together with additional information from peptide fragmentation (MS^2 and MS^3) leading to an unambiguous identification of the cross-linked peptides. If we use a 1:1 mix of the two forms of the reagent all peptides that have incorporated the reagent are present has doublet in the MS. It is possible to "enrich" for the crosslinked species, directing the search to use only fragmentation spectra obtained from precursors that are present as doublet, spaced by a certain mass value in the MS. Here we present the overall experimental strategy of cross-linking and automatic identification of the linked peptides by LC-MS and automatic data interpretation using a novel algorithm and applications on protein complexes.

CHARACTERIZATION OF THE AMPA RECEPTORS SUBUNITS AND THEIR ASSOCIATED TRAFFICKING PROTEINS (ABP, NSF AND PICK-1) IN A MOUSE MODEL OF AMYOTROPHIC LATERAL SCLEROSIS.

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Amyotrophic Lateral Sclerosis (ALS) is a fatal motor neuron degenerative disease characterized by a progressive loss of the upper and lower motor neurons. The excitotoxicity is considered to play a major role in its pathogenesis and the dysfunction of the glutamate AMPA receptors appears to be highly involved in the selective motor neuron death. AMPA receptors are made by four different subunits (GluR1-4) whose stoichiometry is crucial to alter the motor neuron calcium permeability, which contribute to the excitotoxicity. Different proteins have been characterized to modulate the trafficking of the AMPA receptors subunits between cytoplasm and membrane such as: N-ethylmaleimide-sensitive fusion protein (NSF), AMPA binding protein (ABP) and protein interacting C-Kinase1 (PICK-1). In this study, we aimed to analyse the distribution of the AMPA receptor subunits, and their associated trafficking protein in the spinal cord of a transgenic mouse model of ALS (TgSOD-1G93A) during the disease progression. In particular, we examine the subcellular distribution (dendrites and perykaria) of these proteins in the motor neurons. Preliminary results obtained by confocal microscopy and Western blot analysis showed a selective increase expression of the PICK-1 and NSF in the spinal cord ventral horn of transgenic mice involved respectively in the endocytosis and in the membrane fusion of the AMPA receptor subunits. Alterations of these proteins could be able to delocalize the AMPA receptors from the membrane thus influencing the calcium permeability responsible of the excitotoxity.

MOLECULAR MECHANISMS REGULATING THE G2 CHECKPOINT FOCUSING ON CHK1

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The checkpoint kinase (chk) 1 is an essential component of the DNA damage checkpoint acting as the effector of the DNA damage to block the cell cycle at the G2-M transition. We structurally and functionally characterized the 5' flanking region of the human Chk1 gene starting from a PAC clone containing the 5' regulatory region of the human Chk1 gene and it was possible to demonstrate that the isolated region with promoter activity is down-regulated by p53 confirming previous data showing that Chk1 is transcriptionally down-regulated in a p53 dependent manner after DNA damage. To further understand the interplay between p53 and Chk1 in the G2 checkpoint, we studied the cellular response to anticancer treatments in isogenic cellular systems differing in the expression of Chk1 and p53. The small interfering RNA technique (siRNA) was used to efficiently down-regulate the expression of Chk1 in the HCT-116 colon carcinoma cell line and in its isogenic systems in which p53 and p21 have been inactivated by homologous recombination. We also knocked down by siRNA the kinase protein Chk2, another important component of the G2 checkpoint finding out that the inhibition of Chk1 and not of Chk2 in p21-/- and p53-/- cell lines caused a greater abrogation of the G2 block and a greater sensitization to IR and cis-DDP treatments than in the parental cell line with an intact G1 checkpoint. These data emphasize the role of chk1 as a possible molecular target to inhibit in tumors with a defect in the G1 checkpoint to increase the selectivity and specificity of anticancer treatments.

BIM PROMOTER IS HIGHLY METHYLATED IN MALIGNANT LYMPHOID CELL LINES, LEADING TO DOWNREGULATION OF BIM EXPRESSION AND PROTECTION FROM APOPTOSIS

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Bim, a proapoptotic, BH3-only, Bcl-2 family member, is the major physiological antagonist of the antiapoptotic Bcl-2 proteins in B and T lymphocytes. We performed a CpG Islands prediction analysis on Bim promoter, identifying a putative CpG Island. Using a Bisulfite Modification-Clonal Sequencing Analysis (BMCSA), we investigated the methylation status of 19 CpG sites in the Bim promoter in 12 malignant hematological cell lines. An homogeneous, very high level of methylation was present in all the lymphoid cell lines and a variable level of methylation in the myeloid cell lines. The lowest Average Level of Methylation (ALM) was found in lymphocytes from healthy donors. With Real Time PCR analysis we have found that there is a significative correlation between the level of methylation and Bim expression (p=0,0091). We treated the cell lines with the demethylating agent 5-azacytidine. The changes in the methylation status of Bim promoter were evaluated by BMCSA and the corresponding induction of Bim by Real-Time PCR and by Western Blot. The demethylation of Bim promoter led to a potent induction of Bim at the mRNA and protein level. To address the biological role for the methylation of Bim promoter, we generated a TET-ON inducible system for BimS (the most potent proapoptotic isoform of Bim) in the highly methylated NPM/ALK+ Karpas-299 cell line, showing that, following an induction of Bim expression, the cells are potently induced to apoptosis. We conclude that Bim promoter is actively methylated in several leukemias/lymphomas of T and B origin and that its methylation is associated with the downregulation of Bim expression and with protection from apoptosis.

INNATE OPSONINS: MODULATING PERCEPTION OF CELL DEATH AND INFLAMMATION

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Pentraxin 3 and C1q are members of humoral innate immune response that can selectively bind to dying cells and modulate phagocytic clearance. We are investigating the effect of PTX3 and C1q on the interaction between apoptotic cells and dendritic cells (DCs) in an *in vitro* system. PTX3 can inhibit phagocytosis of apoptotic cells by DCs while C1q enhances it. In addition PTX3 inhibits the cross presentation of cell associated antigens from apoptotic cells. Moreover, DCs produce PTX3 and C1q in response to various microbial components and we have found that the presence of apoptotic cells modifies the PTX3 production from dendritic cells. We also found that PTX3 and C1q can directly influence the function of human monocyte derived DCs in response to endotoxin. In addition C1q increases T cell activation and IFN-γ polarisation following stimulation of naïve CD4+ T cells with LPS treated DCs. Moreover PTX3 modulates many functional effects of C1q, including binding to apoptotic cells and subsequent C3 activation. PTX3 and C1q act as fine modulators of dendritic cell function and their local availability could modify the outcome of the interaction between dendritic cells, apoptotic cells and microbial agents and thus influence the initiation/maintenance of autoimmune responses.

HUMAN CRC CELLS INDUCE T-CELL DEATH THROUGH RELEASE OF PROAPOPTOTIC MICROVESICLES: ROLE IN IMMUNE ESCAPE

Huber V, Fais S, Iero M, Lugini L, Canese P, Squarcina P, Zaccheddu A, Colone M, Arancia G, Gentile M, Seregni E, Valenti R, Ballabio G, Belli F, Leo E, Parmiani G, Rivoltini L.

Background & Aims: Normal and neoplastic cells release MV, whose effects on the immune system still need to be elucidated. Because human CRC cells are hypothesized to escape immune recognition by expressing proapoptotic molecules, we investigated whether MV bearing FasL and TRAIL and inducing apoptosis of activated T cells are secreted by CRC cells both in vitro and in affected patients. Methods: FasL and TRAIL expression were analyzed in CRC cells and purified MV by flow cytometry, WB, and immunoelectron microscopy. MV tumor origin was assessed through simultaneous detection of lysosomal (CD63) and adenocarcinoma (CEA) markers. Proapoptotic activity of MV was evaluated by annexin V/propidium iodide staining and caspase activation in T cells, including CD8+ T cells from CRC patients. Results: CRC cells showed a granular pattern of TRAIL and FasL expression, suggesting a secretory behavior. These proapoptotic molecules were detected on isolated MV, together with class I HLA, CD63, and CEA. MV induced FasL- and TRAIL-mediated apoptosis of activated CD8+ T cells generated from CRC patients. MV with comparable phenotypes and functions were found in plasma from patients with advanced disease, whereas vesicular structures expressing FasL and TRAIL were also detected in CRC specimens. Conclusions: CRC induces T-cell apoptosis through the release of FasL- and TRAIL-bearing MV both in vitro and in vivo. This mechanism of immune escape has potential implications as a prognostic factor and could be targeted for the development of new antitumor therapies in CRC patients.

INVESTIGATING THE MOLECULAR MECHANISMS OF IMMUNE DEFICIENCY IN ADA-SCID: IMPLICATIONS FOR GENE THERAPY APPROACH

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Accumulation of adenosine (Ado) and deoxyadenosine (dAdo) due to the absence of adenosine deaminase (ADA) results in a form of Severe Combined Immunodeficiency (ADA-SCID), recently successfully treated with gene therapy. Using polyclonal T cell lines established from ADA-SCID patients before and after gene therapy, we investigated the biochemical pathways responsible for the pathogenesis of the disease and the efficacy of gene therapy in restoring normal metabolic and immunological functions.
We found that the expression of functional ADA in transduced T cells resulted in normalization of the apoptosis induced by Ado/dAdo in defective cells, as well as correction of in vitro proliferative responses and cytokine production. The impairment in ADA-/- cells was due to an intrinsically reduced ERK1/2 MAPK activation, which is completely abolished in presence of toxic metabolite.
Normalization of immunological functions was also observed at the clonal level in CD4+ T cell clones generated from two gene therapy treated patients.
Interestingly, such clones showed different level of ADA expression suggesting a position dependent expression of the transgene. Based on mapped retroviral vector integration site by inverse-PCR, we are currently evaluating the correlation between gene expression and cell functions with vector integration.

IL-4 GENE DELIVERY INDUCES CLINICAL AND FUNCTIONAL RECOVERY FROM EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS, AN ANIMAL MODEL OF MULTIPLE SCLEROSIS.

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Widespread demyelination, axonal loss and reactive astroglial scar formation are typical hallmarks of inflammatory CNS demyelinating syndromes, such as multiple sclerosis (MS). The multifocal and recurrent nature of MS makes experimental cellular and gene therapy approaches aiming at the restoration of CNS tissue integrity, very much complicated. We established a reliable gene therapy-based system for delivering anti-inflammatory cytokines directly within the CNS. Helper-dependent adenoviral vectors (HD-Ad) engineered with the IL-4 gene (AdIL-4) and the green fluorescence protein (GFP) as reporter (AdGFP) were injected into the celebrospinal fluid (CSF) circulation of immunocompetent mice and monkeys trough the cisterna magna (i.c.). The i.c. injection of AdIL-4 vectors allowed persistent transduction of ependimal and leptomeningeal cells, without toxicity, and long-term production of IL-4 into the CSF. After injection into mice affected experimental MS, IL-4 treatment induced progressive clinical recovery. Indeed, the therapeutic effect was accompanied by increased CNS production of pro-inflammatory chemokines (e.g.CCL17) and increased numbers of CNS-infiltrating regulatory T cells with known suppressant function. CNS delivery of HD-Ad engineered with genes coding for soluble anti-inflammatory molecules might then represent an promising therapeutic option for chronic and multifocal inflammatory CNS disorders.

DEVELOPMENT AND FUNCTION OF MUCOSAL IgAs INDUCED FOLLOWING ORAL INFECTION WITH *Salmonella typhimurium*

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In the gastrointestinal tract potentially dangerous agents such as viruses, pathogen bacteria and toxins, are mixed together with commensal bacteria and dietary protein. A number of molecular and cellular mechanisms evolved to discriminate between harmful and harmless antigens, maintain the integrity of mucosal surfaces and fight infections. A key component of the intestinal immune response is represented by secretory IgAs (SIgAa). This class of antibodies is produced by B cells activated in the gut-associated lymphoid tissue (GALT) and is delivered in the lumen by intestinal epithelial cells. SIgAs have been traditionally attributed a neutralizing function, exerted by blocking antigens and bacteria in the lumen, neutralizing intracellular viruses and excreting antigens.
We show that following oral infection with invasive strains of *Salmonella typhimurium*, mice effectively secrete high levels of anti-*Salmonella* IgAs in the intestine. Nevertheless, these antibodies seem not to be able to completely block further access of bacteria. A possibility we are investigating is that SIgAs allow IgA-coated bacteria access to GALT, but somehow block their pathogenicity. In this way, beside the described mechanisms, we propose that SIgAs exert their neutralizing activity also by providing a reservoir of "safe" antigens for the immune system in secondary lymphoid organs.

THYMIC SELECTION OF NKTi CELLS

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Invariant NKT(i) cells are innate T lymphocytes expressing a conserved semi-invariant TCR consisting, in mice, of the invariant V□14-J□18 TCR-□ chain paired mostly with V□8.2 and V□7. Unlike conventional T cells, which recognise peptides presented by MHC molecules, NKTi cells recognise glycolipids presented by CD1d molecules, which are also remarkably conserved in mice and humans. The cellular requirements for thymic positive and negative selection of NKTi cells are only partially understood. To get insight into NKTi cell selection mechanisms, we generated transgenic (tg) mice expressing human CD1d (hCD1d) either on immature thymocytes or on thymic Dendritic Cells (DCs), which are the cells suspected to play a role in the selection of NKTi cells. The hCD1d tg mice were crossed with mice genetically deficient in the endogenous mouse CD1d ($CD1d^{0/0}$ mice), to generate mice that express either only tg hCD1d on thymocytes or on DCs, or hCD1d on either thymocyets or DCs plus the endogeneous mouse (m)CD1d, or only the endoneous mCD1d. The analysis of these mice reveal three unexpected features of innate T cells, namely that: *i*. selective expression of hCD1d on thymocytes is sufficient for positive selection of NKTi cells; *ii*. both thymocytes and APCs may independently mediate NKTi cells negative selection; and *iii*. additional interactions of NKTi cells with CD1d expressed on cells other than thymocytes modulate their functional status.

RAS-INDUCED SENESCENCE OCCURS VIA A DNA-DAMAGE RESPONSE THAT IS DEPENDENT ON REACTIVE OXYGEN SPECIES-MEDIATED HYPERPROLIFERATION AND ON DNA REPLICATION

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Expression of oncogenic Ras in primary mammalian cells results in a permanent cell cycle arrest similar to replicative cellular senescence. We demonstrate that oncogenic Ras-induced senescence (RIS) is the consequence of the activation of a robust cellular DNA-damage checkpoint response (DDR) and that DDR-inactivated cells do not undergo RIS. Reactive oxygen species (ROS) generated by oncogenic Ras are mitogenic and trigger an initial hyperproliferative phase that precedes senescence. RIS cells arrest with a partially re-replicated DNA with replication origins having re-fired multiple times and their DNA shows preferential loss of heterozygosity (LOH) at fragile sites. If DNA replication is prevented, oncogenic Ras expression does not trigger a DDR. *In vivo*, activated Ras is associated with DDR activation in benign mouse hyperproliferative skin lesions. Therefore, RIS is the consequence of a DDR caused by ROS-mediated hyperproliferation and DNA re-replication.

NUCLEOPHOSMIN IS REQUIRED FOR DNA INTEGRITY AND p19ARF PROTEIN STABILITY

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Nucleophosmin (NPM) is a nucleolar phosphoprotein that binds the tumor suppressors p53 and p19ARF (Arf), and is thought to be indispensable for ribogenesis, cell proliferation and survival after DNA-damage. The NPM gene is the most frequent target of genetic alterations in leukemias and lymphomas, though its role in tumorigenesis is unknown.
At this purpose we decided to study the phenotype of the mouse null for the expression of NPM. Lack of NPM expression results into accumulation of DNA damage, activation of p53, widespread apoptosis and mid-stage embryonic lethality. Fibroblasts explanted from null embryos fail to grow and acquire rapidly a senescent phenotype.
Transfer of the NPM mutation into a p53-null background rescued apoptosis in vivo and fibroblast proliferation in vitro. Cells null for both p53 and NPM grow faster than control cells and are more susceptible to transformation by activated oncogenes, such as mutated Ras or overexpressed Myc.
In the absence of NPM, Arf protein is excluded from nucleoli and is markedly less stable. Our data demonstrate that NPM regulates DNA integrity and, through Arf, inhibits cell proliferation, and are consistent with a putative tumor suppressive function of NPM.

CHARACTERIZATION OF A GENE IMPORTANT FOR P53-DEPENDENT, STRESS-INDUCED APOPTOSIS

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The present thesis regards the characterisation of an unknown gene named DRAGO (DRugs Activated Gene Overexpressed), previously isolated in our group as a p53-inducible gene by differential expression analysis in cells following DNA damage. The molecular structure of the gene was characterized revealing the presence of a coding region constituted by six exons, a large 3'UTR and two long introns. The length of those regions suggested a strong gene regulation at transcriptional/post–transcriptional levels. The activity of a putative promoter region was increased by p53. An even stronger promoter activity in response to p73 and its isoforms was observed. The presence of DNA mutations in the gene has been investigate by SSCP analysis and DNA sequencing. Among the human cancer cell lines and tumors tested so far, no mutations were detected. The functional role of DRAGO has been studied by ectopically overexpressing it in eukaryotic cells. An inhibitory effect on cell growth and the formation of a large number of vacuoles leading to the disruption of the plasmatic membrane could be observed upon its overexpression. The phenotypic changes induced by DRAGO overexpression were therefore evaluated using specific deletion mutants showing that cells can tolerate the expression of DRAGO mutants with deletion of a few aminoacids in the C–terminus. The theoretical prediction was supported by immunofluorescence experiments in DRAGO transiently expressing cells, showing that the fluorescence was localised in the membrane. DRAGO–null mice have been generated: they are viable and do not present any macroscopic alterations. DRAGO –/– MEFs were more resistant to drug treatments than wild–type MEF due to the inability of the cells to activate apoptosis.

INTERFERENCE WITH MECHANISMS OF TELOMERE MAINTENANCE BY TARGETING hPOT1 IN HUMAN CANCER CELLS.

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The maintenance of human telomeres, repetitive G-rich sequences at the end of chromosomes, is an essential feature that guarantee the survival of cancer cells. Thus far, two mechanisms of telomeres maintenance (TMMs) have been identified: the *de novo* synthesis of telomeric DNA catalyzed by telomerase and the Alternative Lengthening of Telomeres (ALT) mechanism. Since genes involved in ALT are presently unknown, specific ALT inhibitors are still lacking. However, the structure/function of telomeres could be disrupted in both telomerase- and ALT-positive tumor cells by targeting telomere-associated proteins. In the present study, we exploited a siRNA-mediated approach to target the human protection of telomere 1 (hPOT1) protein, whose role in the regulation of the telomerase activity is not yet ascertained. Specifically, two siRNAs, targeting different consesus sequences within the hPOT1 mRNA, have been exogenously delivered to telomerase-positive MCF-7 breast cancer cells. Results obtained at different time points after transfection showed a significant inhibition of hPOT1 mRNA expression levels. Such inhibition was paralleled by a modest impairment of cell proliferation and significant changes in cell morphology. Furthermore, the exposure of cells to siRNAs did not affect the activity of telomerase. This evidence corroborate previous data supporting the role of hPOT1 as a negative regulator of telomerase functions.

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JANUARY 24TH - ABSTRACTS

POSTERS

A44

LIGAND-INDUCED MONOMER/DIMER DYNAMICS OF uPAR IN LIVING CELLS BY 2-PHOTON FLUORESCENCE FLUCTUATION SPECTROSCOPY

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The urokinase plasminogen receptor (uPAR) is a gpi-anchored protein, which, by interacting with ligands and transmembrane proteins regulates cell adhesion, migration and proliferation in normal and pathological situations. However, it is currently unknown how activated uPAR selects different signaling pathways. Our working hypothesis is that uPAR-uPAR interactions and/or partitioning in membrane microdomains might be required for selecting the activation pathway. We have applied 2-photon excitation fluorescence correlation spectroscopy (FCS) and photon counting histogram (PCH) approaches to follow membrane dynamics and oligomerization of uPAR in living cells. FCS measures diffusion coefficients while PCH distinguishes the diffusing molecular forms by molecular brightness. Stable clones of HEK293/uPAR-EGFP-gpi were obtained, in which the fluorescent chimera of uPAR retains all functions of the wt receptor. We show that mobile uPAR is either monomer or dimer, and a fraction of receptors are immobilized in the bottom membrane. Monomers and dimers are heterogeneously distributed in the cell surface and diffuse in two distinct domains. The internalization induced by uPA-PAI-1 depletes the dimers from the membrane, but does not eliminate any of the two diffusive components, suggesting that the segregation of uPAR in membrane microdomains is independent on dimerization.

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A45

GENERATION OF FUNCTIONAL NUCLEAR EXPORT SIGNALS (NES) IN AML-ASSOCIATED NPM MUTANTS

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Nucleophosmin (NPM) is an abundant nucleus-cytoplasmic shuttling protein that has been implicated in several cellular processes, including ribogenesis, DNA repair, centrosome duplication, cell cycle progression and stress response. Both a NLS and a NES have been described to be present in its sequence, but, in physiological conditions, the vast majority of NPM protein resides in the nucleus. However, in about 35% of Acute Myeloid Leukemia patients, mutations occurring within the NPM exon coding for a putative nucleolar localization signal (exon 12th), in the C-terminal domain, induce cytoplasmic localization of the protein. It has been shown that the most common NPM mutant (Mut-1) re-localizes to the nucleus when cells are treated with leptomycin B, a drug that inhibits CRM1-dependent nuclear export.
In this study we address the molecular mechanism that drives the aberrant cytoplasmic localization of NPM mutants. We show that, despite genetic heterogeneity, all the mutations isolated so far in NPM exon 12th lead to the formation of new C-terminal sequences that always contain a nuclear export signal (NES) consensus, which is absent in the wt protein. Furthermore, we report a new case in which NPM carries a mutation in the splicing donor site of exon 9, that again introduces a NES consensus sequence in the C-terminal portion of the translate. We show that peptides corresponding to NES of MutA and of MutExon9 are both *per se* sufficient to drive the nuclear export of a reporter nuclear protein.

A46

MOLECULAR PHYSIOLOGY OF PLASMA CELL DIFFERENTIATION.

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Plasma cells are terminally differentiated secretory cells that produce huge amounts of antibody. Emerging evidences indicate that the unfolded protein response (UPR), a response triggered to cope with accumulation of misfolded proteins in the ER, may play a key role in B lymphocyte to plasma cell differentiation during which proteins accumulate in the ER because of the very high rate of protein synthesis. In mammals the UPR is mediated by three transducers in the ER membrane: Ire1, which activates the transcription factor XBP1, ATF6, a bZIP transcription factor and PERK which phosphorilates eIF2-α inhibiting most cap-dependent protein synthesis. ATF6 and XBP1 induce expression of UPR genes.

Our study aims to understand the involvement of the UPR in B cell differentiation.

The data we obtained in a murine model of B cell differentiation, I.29μ[+] cell line, show induction of a transcription factor downstream of eIF2-α phosphorylation, CHOP. This suggests that PERK is activated during B cell differentiation, although we cannot rule out that the expression of CHOP could be induced by different eIF2-α kinases. As previous evidences in different models indicate that CHOP has a pro-apoptotic role and affects the redox environment during the UPR we reasoned that it could affect plasma cells viability and Igs polymerization. Thus we further investigated the role of CHOP in B cell differentiation studying B lymphocytes and plasma cells from CHOP[-/-] mice. Results will be shown here.

A47

FROM EGFR ACTIVATION TO EPS15 MONOUBIQUITINATION

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Eps15 (EGF receptor Pathway Substrate 15), a protein originally identified as EGFR substrate, is monoubiquitinated upon EGF treatment. Our previous experiments suggested that Nedd4 is the E3 ligase involved. In our work we want to identify the signalling events that induce monoubiquitination using an integrated approach based on specific pharmacological inhibitors and molecular genetics tools. Treatment with specific inhibitor of receptor kinase activity (AG1478 and GEINISTEIN) and Src kinase activity (PP1) abolish eps15 monoUb, indicating that this event depends on proteins acting at the first steps of RTK signaling cascade. Our initial results show that the kinase activity of the EGFR, but not its phosphorylation sites are required, suggesting a possible downstream regulation exerted by one of the kinases, probably Src, activated by this RTK. Nedd4 belongs to an E3 family that comprise 8 members with similar architecture. A second part of the project aims to clearly establish the E3 ligase involved in esp15 monoUb using siRNA oligos, antibodies as well as GFP-tagged constructs already available in the lab. Results will be presented.

A48

ROLES OF EBF2 TRANSCRIPTION FACTOR IN CEREBELLAR DEVELOPMENT

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EBF2 is one of four mammalian members of an atypical helix-loop-helix transcription factor family. We have generated a mouse carrying a null mutation of *Ebf2*, a gene previously characterized in the context of *Xenopus laevis* primary neurogenesis and neuronal differentiation (Pozzoli et al., 2001). In addition to deficits in neuroendocrine and olfactory development, and peripheral nerve maturation (Corradi et al., 2003; Wang et al., 2004), *Ebf2* null mice feature an ataxic gait and obvious motor deficits associated with striking abnormalities of cerebellar development. The number of Purkinje cells (PCs) in the *Ebf2* null is markedly decreased resulting in a small cerebellum with notable foliation defects. This stems from the defective migration of a specific PC subpopulation that subsequently dies by apoptosis. The surviving PCs in the mutant cerebellar cortex display disorganized and defasciculated axons, and exhibit redundant branches. In the adult cerebellum, the normal striped cerebellar topography is severely disrupted, and the zebrin II-negative PC subclass is lost. Detailed analyses are underway to identify the molecular targets of EBF2 that mediate these remarkable effects.

A49

THE PRDM GENE FAMILY AND CANCER: A HIGH-THROUGHPUT APPROACH

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The human PRDM gene family consists of 17 known members characterized by the presence, generally at the N-terminus, of the PR domain, a 130 amino-acid region that shares 20-30% sequence homology with the SET domain of histone methyltransferases. A variable number of Zn finger domains are also present at the C-terminus of the protein. These proteins are expressed in two forms that differ by the presence or absence of the PR domain, referred as PR-plus and PR-minus, respectively. The protein product that retains the PR domain is anti-tumorigenic, while the product that lacks the PR domain is oncogenic and over-expressed in tumor cells.
Preliminary experiments revealed the existence of other unidentified variants, besides the PR-plus and PR-minus isoforms. The PRDM5 protein exists in two different isoforms that differ for the presence or absence of the exon6 that contains the third of sixteen Zn fingers. The functional role of these splicing variants is under study.
We are currently investigating the PRDM loci using tiling arrays from NibleGen to systematically analyze the structure and expression pattern of human PRDM genes in normal and neoplastic cells. We designed a tiling array representative of the PRDM genes, including the 17 loci and the flanking regions. Preliminary results showed that the PRDM genes are expressed at very low levels, hampering the fine mapping of the coding regions. We are currently setting the best conditions of probe synthesis to detect the PRDM transcripts.

A50

ROLE OF THE TRANSCRIPTION FACTOR PREP1 IN APOPTOSIS

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TALE proteins, including the MEINOX and PBC subfamilies, are homeodomain transcription factors. Prep1, belonging to the MEINOX family, heterodimerizes with Pbx proteins, in a DNA-independent manner. The complex is translocated to the cell nucleus, where it binds the DNA through the homeodomains of Prep1 and of the Pbx partner, and acts as a transcriptional regulator. Through the use of different interaction surfaces, the Pbx and Prep1 complex could interact with Hox proteins forming trimeric complexes and expanding DNA target selectivity . In a previous work , the functional role of Prep1 during early zebrafish development was investigated using a morpholino antisense oligonucleotides strategy. In particular, Prep1 morphants showed the massive apoptotic cell death, particularly in the hindbrain and spinal chord. In an other work was reported that Meis1 (a member of Meinox subfamily) overexpression strongly induces apoptosis in a variety of cell types in vitro through a caspase-dependent process. In this study, we have investigated the role played by Prep1 in the apoptotic behaviour of mammalian cells, using MEFs (Mouse Embryo Fibroblasts) cells obtained from WT and Prep1 hypomorphic mice. By flow cytometric analysis, using Annexin V staining (which marks apoptotic cells), we observed that MEFs, extracted from Prep1 hypomorphic embryos were more sensitive to apoptosis after UV induction. The apoptotic phenotype was supported by a strong increase of active Caspase 9 and active Caspase 3, by a decrease of the level of Bcl-X_L protein, an antiapoptotic member, and an increase of Bax protein, a proapoptotic member. We investigate also the effect on cell survival after overexpression of Prep1, using different cell lines. Interestingly, we observed that the overexpression of Prep1 induces cells to be more sensitive to apoptosis after UV irradiation. These results could contribute to the understanding of biological role of Prep1 and its involvment in programmed cell death.

A51

TRANSLATIONAL REGULATION OF BACE-1 EXPRESSION

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Alzheimer's disease (AD) is a neurodegenerative disorder characterized by deposition of beta-amyloid in the brain of Alzheimer's disease patients.
BACE-1, the β-site amyloid precursor protein (APP) cleaving enzyme 1, is the secretase responsible for production of beta-amyloid.
Published data indicate a complex and strong regulation of BACE-1 expression on the transcriptional and post-transcriptional level, implying that misregulation of BACE-1 expression could contribute to Alzheimer's disease development.
Interestingly, the BACE-1 promoter region lacks characteristic "CAAT" and "TATA" boxes upstream of the transcription start site (TSS). The BACE-1 mRNAs synthesized are heterogeneous in length, which is considered to be due to different possible polyadenylation sites being processed. In addition, alternative splicing leads to the synthesis of at least 3 different isoforms.
The 5'-untranslated region (5'-UTR) of BACE-1 mRNA is relatively long (446 bp), GC rich, and contains 4 upstream open reading frames (uORFs).
We have found that the 5'-UTR of BACE-1 mRNA is strongly inhibitory for translation *in-vivo* and *in-vitro*. This inhibition of translation can only be partially explained by the presence of the uORFs; we have found that other sequence elements within the 5'-UTR also contribute to the inhibition.
We intend to study the molecular mechanism underlying this process in more detail by characterization of 5'-UTR sequence elements that are important for the translational inhibition of BACE-1 *in-vivo* and *in-vitro*.
The results of these studies will be presented.

A52

IDENTIFICATION AND CHARACTERIZATION OF NEW JUNCTIONAL MARKERS SPECIFIC FOR THE LYMPHATIC ENDOTHELIUM

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The lymphatic vasculature complements the blood vasculature by maintaining tissue fluid homeostasis. Due to the common lineage of the lymphatic and blood endothelium, lymphatic endothelial cells (LEC) have been poorly characterized, mainly because of the lack of specific markers and technical limitations in isolating pure LEC cultures.
We have focused our work on the poorly characterized cell-cell lymphatic junctions. Previously performed gene profiling analysis, comparing blood and lymphatic endothelial cells, showed that these two cell types differ in the expression of various genes encoding for junctional protein families. These differences relate to the already known morphological differences. Following a subtractive immunization protocol, we have isolated a panel of 22 murine monoclonal antibodies and tested them by immunofluorescence on a cell line expressing other known lymphatic markers such as podoplanin, LYVE-1 and Prox-1. Of these supernatants, we selected 3 subclones which displayed a junctional-like staining in immunoflurescence on LEC and not on HUVEC cells. These same subclones recognized a single band of approximately 65-70 KDa in Western Blot under reducing conditions. We are now attempting to identify this putatively novel LEC-specific junctional marker and determine whether it interacts with other known junctional proteins. This can provide new insights in the understanding of lymphatic vascular functions in homeostasis and disease and in the differences between blood and lymphatic vasculature development.

A53

GLYCYRRHIZIC ACID, A NATURAL INHIBITOR OF THE PROINFLAMMATORY ACTIVITY OF HIGH MOBILITY GROUP BOX 1 PROTEIN (HMGB1), FUNCTIONAL AND STRUCTURAL STUDIES,

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HMGB1 is a 22 kDa protein composed of two similar DNA binding domains (HMG box A and B) with an acidic C-terminal tail of 30 residues. It has also a pivotal role as an extracellular mediator, acting as a cytokine for lethal systemic inflammation, arthritis and local inflammation: recent studies have shown that HMGB1 induces diffuse inflammation in skeletal muscles and is highly expressed in dystrophic muscles. The understanding of the mechanisms underlying its inflammatory activity are therefore of enormous clinical interest and recent research has focused on the identification of effective HMGB1 inhibitors. We have identified glycyrrhizic acid (GL) as a new modulator of the cytokine activity of HMGB1: GL is a natural triterpene exhibiting a wide range of known pharmacological properties (e.g. anti-inflammatory activity). By NMR chemical shift mapping and fluorescence we have shown that GL and its derivative carbenoxolone interact directly with HMGB1 full length with micromolar affinity, and that interaction sites are mainly clustered on the first helix of the two HMG boxes, involving highly conserved residues in the HMG family. From the functional point of view, GL inhibits the HMGB1 induced migratory response and proliferation of endothelial cells and mesoangioblasts. These results suggest that GL and derivatives are potential anti-inflammatory drugs which will be also tested as potential therapeutic agents in the treatment of muscular dystrophy.

A54

THE "MAD2 TEMPLATE" MODEL OF SPINDLE CHECKPOINT ACTIVATION AND PROPAGATION IN BUDDING YEAST.

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The inheritance by daughter cells of complete copies of their genome is central to the process of cell proliferation. The spindle assembly checkpoint delays anaphase until all sister chromatid pairs have attained bipolar attachment to spindle microtubules. Defects in this cell cycle surveillance mechanism contribute to chromosome instability and aneuplody, which are both hallmarks of cancers. Anaphase is triggered by the proteolysis of one of the subunits of Cohesin by Separase, which is normally kept inactive by Securin. Securin ubiquitination by APC/C leads to anaphase onset. The spindle assembly checkpoint targets the APC/C activator Cdc20 with Mad2 and BubR1. Mad2 adopts a closed conformation when bound to its kinetochore receptor Mad1 or its target in the checkpoint Cdc20, and an open conformation when unbound to these ligands. Recently, we showed that a closed conformer of Mad2 constitutively bound to Mad1, rather than Mad1 itself, is the kinetochore receptor for cytosolic open Mad2, and that the interaction of open and closed Mad2 conformers is required to sustain the spindle checkpoint in humans. The spindle assembly checkpoint is conserved between higher eukaryotes and budding yeast *S. cerevisiae*. Here we show that the biochemical properties of yeast protein are comparable to those of the human protein and we use complementetion in yeast to validate the "Mad2 template" model.

A55

RAC1 IS NECESSARY FOR AXONAL SORTING IN THE PERIPHERAL NERVOUS SYSTEM

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Schwann cell interaction with extracellular matrix components is required for sorting of large diameter axons and their myelination Mice with specific inactivation of β1 integrin in Schwann cells present a dysmyelinating neuropathy with a block in axonal sorting similar to laminin-2 deficient mice. We postulate that β1 integrin null Schwann cells lack the signals responsible for cytoskeleton rearrangement during ensheathment of axons. β1 integrin null Schwann cells have a reduced surface due to an impairment in ruffle formation, when plated on laminin. Rho small GTPase Rac1 promotes the formation of lamellipodia, and it is expressed and present in an active form in myelinating Schwann cells. By Rac1 activity assays on sciatic nerve lysates, we found that β1 integrin null Schwann cells have a reduced Rac1 activity. Cdc42 activity, instead, is unchanged in β1 integrin null nerves, suggesting that sorting is mediated through Rac1 signalling. To confirm this hypothesys, we generated Rac1 conditional null mice in the Schwann cell lineage. Axonal sorting is defective in the absence of Rac1, demonstrating that β1 integrin-Rac1 signalling pathway controls axonal segregation in the peripheral nervous system.

A56

CROSSTALK BETWEEN ENDOTHELIAL ADHERENCE JUNCTIONS AND TGFβ SIGNALING

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Endothelial adherence junctions (AJ) contain vascular endothelial (VE)-cadherin, mediating endothelial cell-cell (EC) homophilic adhesion, and the *armadillo* proteins β-catenin, γ-catenin and p120 which anchor VE-cadherin to the actin cytoskeleton and regulate target gene expression. VE-cadherin transfers intracellular signals controlling vascular homeostasis through the association with both catenins, preventing their nuclear localization, and growth factor receptors.
The notion that TGFβ-mediated endothelial-to-mesenchymal transformation is impaired in β-catenin null EC and that the knock-out of VE-cadherin strikingly resembles that of the TGFβ endothelial co-receptor (CD105) prompted us to investigate a possible crosstalk between TGFβ and AJ pathways in EC. TGFβ mediates its cellular effects through serine threonine receptors (TβRI, TβRII) coupled to the transcriptional regulators Smads. To assess whether VE-cadherin/β-catenin signaling regulates TGFβ pathway, we performed Smad-dependent reporter gene assays in β-catenin null EC and in VE-cadherin-expressing CHO cells in which cytoplasmic β-catenin pool is reduced. The results show enhanced TGFβ/Smad reporter activity in β-catenin null EC and in VE-cadherin/CHO cells, suggesting that β-catenin function inhibits TGFβ transcriptional activity. Since AJ signaling impacts TGFβ pathway, we tested whether VE-cadherin physically interacts with TGFβ receptors. Briefly, VE-cadherin, CD105 and TβRII have been transiently expressed into recipient cells and co-immunoprecipitated complexes analysed. The results show that both CD105 and TβRII associate with VE-cadherin in TGFβ-dependent cells. These findings demonstrate a functional crosstalk between TGFβ and AJ pathways providing prospects for better understanding the role of AJ proteins in TGFβ-mediated effects in endothelial cells.

A57

ORSI

A58

FUNCTIONAL ROLE OF HETEROTRIMERIC G PROTEINS ON INSULIN GRANULES

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Heterotrimeric G proteins have been identified not only at the plasma membrane, but also on intracellular organelles, suggesting that G protein-coupled receptor (GPCR)-mediated signal transduction systems might also operate at the internal membranes in mammalian cells. In particular, Gαi proteins have been found on the membrane of insulin granules in β cells, where they seem to be involved in insulin secretion. In order to study the role of Gαi on insulin granules a chimeric protein was generated, consisting of IA2, an integral membrane protein of insulin granules, fused to a constitutively active form of Gαi. Expression of this fusion protein in β cells led to the accumulation and constitutive activation of Gαi specifically on the enriched organelles, resulting in the formation of insulin granule clusters nearby the centrosome. These findings suggest that a heterotrimeric G protein - and perhaps GPCR – mediated mechanism might be involved in regulating the distribution and the transport of secretory organelles along the microtubule cytoskeleton.

A59

AN UNCONVENTIONAL DILEUCINE AND A NOVEL CYTOSOLIC MOTIF ARE REQUIRED FOR THE LYSOSOMAL/MELANOSOMAL TARGETING OF OA1

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Ocular albinism type 1 is an X-linked disorder characterized by severe visual abnormalities and the presence of giant melanosomes in the skin and eyes. The protein product of the *OA1* gene is a pigment cell-specific membrane glycoprotein, displaying features of G protein-coupled receptors and exclusively localized to lysosomes and melanosomes. To dissect the signals responsible for the intracellular localization of OA1 we generated LAMP1/OA1 chimeras and OA1 mutants at the cytosolic domains of the protein. By this approach we identified two separate sorting signals that are both sufficient and necessary for intracellular retention and lysosomal/melanosomal localization in melanocytic and non-melanocytic cells: an unconventional dileucine motif within the third cytosolic loop and a novel motif, characterized by a tryptophan-glutamic acid doublet, within the C-terminal tail. Both motifs must be mutated to promote the plasma membrane localization of OA1, suggesting that they can independently drive its intracellular targeting. In addition, both motifs act similarly as lysosomal sorting signals in non-melanocytic cells, but appear to carry different specificities in melanocytic cells. Our findings indicate that OA1 contains multiple unconventional signals responsible for its lysosomal/melanosomal localization, and reveal a remarkable and unforeseen complexity in the regulation of polytopic protein sorting to specialized secretory organelles.

A60

RLP-A AND RLP-B : TWO NOVEL RAS LIKE PROTEIN

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The c-Myc gene product is a transcription factor that can both activate and repress gene expression. A micro array screen previously performed in our group identified a novel Myc repressed gene. This gene has been provisionally named RLP-A (for Ras-like protein A) and a second gene RLP-B was identified by its homology to RLP-A. RLP-A and RLP-B are members of the Ras family. All Ras-related GTPases contain five highly conserved domains (G1-G5), function as guanine nucleotide-dependent molecular switches and regulate a diverse array of cellular functions. By aligning RLP-A and RLP-B with other small G proteins we notice the presence of these five conserved domains (G1-G5) and the absence of consensus sequences for lipid modification and posttranslational modification for targeting to membranes. We are addressing whether these proteins are functionally similar to the other small G proteins.
We used immunofluorescence to address the localization of RLP-A. We observed that the endogenous protein was present in the nucleolus of NIH3T3 cells and co-localised with nucleolar markers, such as nucleolin, fibrillarin, NPM and UBF (RNA polymerase I upstream binding transcription factor) in interphase. RLP-A stained the centre of the nucleolus, co-staining with fibrillarin (FCs) and UBF. In mitotic cells RLP-A appears to remain associated with NORs (Nucleolar Organizing Region) and is still co-localized with UBF. We performed ChIP (Chromatin Immunoprecipitation) in NIH3T3 cells on rDNA promoter sequences using the termination site after the 28S sequence as a negative control. Surprisingly, we found RLP-A on this termination site, and less on the promoter. ChIP on rDNA gene, in order to map the entire rDNA cluster, will confirm RLP-A as a nucleolar protein that associates with rDNA chromatin in cells.
Knock-down experiments will be undertaken to address the cellular function of these proteins.

A61

IN SILICO TOOLS FOR THE SCREENING OF OESTROGEN RECEPTOR BINDING AFFINITY

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Oestrogen Receptor (ER) is a ligand-dependent transcription factor which plays a critical role in the growth, development and maintenance of a diverse range of tissues. ER is present in two isoforms (ER-α and ER-β) that exhibit overlapping but distinct tissue distribution patterns and differ in their ligand-binding ability and transactivational properties. There is an increasing concern related to xenobiotics that can mimic oestrogens and interfere with ER functions. At the same time, there is a general lack of adequate information about possible estrogenic effects for a large amount of industrial chemicals.
In silico tools are valuable instruments to provide a virtual screening of large amount of compounds allowing the identification of those with a major need of deeper *in vitro* and *in vivo* investigations permitting a more rational use of the resources. The ability of *in silico* tools to discriminate active versus inactive compounds in relation to their potential disruption effects on the oestrogen receptor will be here discussed. Several algorithms and techniques capable to describe the activity pattern of chemicals are nowadays available to solve this problem by modelling approach. We will propose here an overview of different approaches adopted to study datasets of compounds for which Relative Binding Affinity (RBA) values for ER were given. Different techniques have been adopted to describe molecules, along with the interacting macromolecules. Advantages and disadvantages of different approaches will be discussed. Acknowledgments to EASYRING project.

A62

FUNCTIONAL CHARACTERIZATION OF MAMMALIAN COP9 SIGNALOSOME AND ITS DOWNSTREAM EFFECTOR huCOP1 E3 LIGASE.

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COP9 signalosome (CSN) is a multimolecular complex conserved throughout evolution from yeast to mammals. Functionally CSN has been linked to the control of ubiquitin mediated proteolysis pathway through the regulation of multiple E3 ligases that in turn drive the degradation of many proteins involved in cell cyle control. In our work we focused on two parallel issues concerning CSN function and regulation. On the one hand we characterized COP9 signalosome subcellular distribution, By immunofluorescence and biochemical analysis, we found that in contact inhibited, non proliferating epithelial cell lines, CSN is mainly cytosolic, whereas disruption of epithelial integrity by *wound healing* assay induces rapid CSN nuclear re-localization. The molecular mechanism underlying the differential distribution of CSN under these conditions is under investigation. On the other hand, we focused on the effectors of mammalian CSN. In our lab we cloned the human orthologue of *Arbidopsis Thaliana* COP1, which is one of the first CSN effectors identified. We showed that huCOP1 displays the features of an E3 ligase, which functionally acts downstream the CSN. Moreover we found that huCOP1 binds and induces the degradation of the transcription factor c-Jun. We are now investigating which are the physiological stimuli inducing COP1 dependent c-Jun degradation. Moreover understanding whether the signalosome subcellular distribution can affect huCOP1 function, would shed some light on the still controversial issue of mammalian CSN functional regulation.

A63

PROTEASOME RESTRICTS HIV-DERIVED VECTOR GENE TRANSFER IN HEMATOPOIETIC STEM CELLS

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The therapeutic potential of hematopoietic stem cell (HSC) gene therapy can be fully exploited only if we reach efficient gene transfer into HSCs without compromising their biological properties. Although HSCs can be transduced by HIV-derived lentiviral vectors (LVs) in short *ex vivo* culture, they display low permissivity to the vector, requiring cytokine stimulation to reach high-frequency transduction. Using stringent assays of competitive xenograft repopulation, we show that early-acting cytokines synergistically enhanced human HSC gene transfer by LV without impairing engraftment and repopulation capacity. Using S-phase suicide assays, we show that transduction enhancement by cytokines was not dependent on cell cycle progression and that LVs can transduce quiescent HSCs. Pharmacological inhibition of the proteasome during transduction dramatically enhanced HSC gene transfer, allowing reaching very high levels of vector integration in their progeny *in vivo*. Thus, LVs are effectively restricted at a post-entry step by the activity of this proteolytic complex. Unexpectedly, cytokine stimulation substantially down-regulated proteasome activity in hematopoietic progenitors, providing a possible mechanism by which cytokines enhance permissiveness to LV gene transfer. These findings demonstrate that antiviral responses ultimately mediated by the proteasome strongly limit the efficiency of HSC transduction by LVs, and establish improved conditions for HSC-based gene therapy.

A64

CHARACTERIZATION OF PUTATIVE HUMAN ORTHOLOGUES OF *S.cerevisiae* SRS2 DNA HELICASE.

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Several human cancer prone diseases, including Bloom's (BS) and Werner's (WS) genome instability syndromes, is due to mutations in DNA helicase genes. Particularly, the genes altered in BS and WS disorders belong to a unique and evolutionarily conserved family of RecQ helicases that also includes Sgs1 in *S.cerevisiae*. The RecQ helicases play an essential role in the control of the homologous recombination repair pathway of which deregulation is thought to be one major cause of genome instability. In yeast, Sgs1 recombination function is strictly related to the one of Srs2, which belongs to a different family of UvrD of DNA helicases.
Previous work done in our lab wishes to identify putative human orthologues of Srs2 that have not been found yet. By database searching, we have identified at least two human genes as putative Srs2 orthologues, which are under investigation. We found that one of them, namely hFbh1, is indeed able to complement some defects of *srs2* mutants in yeast; intriguingly it has been shown that hFbh1 is not only a DNA helicase, but also contains an F-box domain which functions as E3 ubiquitin ligase.
We are currently investigating the role(s) of these putative Srs2 orthologues in the maintenance of genome stability in human cells.

A65

LENTIVIRAL VECTOR MEDIATED GENE THERAPY FOR WISKOTT-ALDRICH SYNDROME : EVIDENCE FOR EFFICACY AND SAFETY

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Wiskott-Alsrich Syndrome (WAS) is a severe X-linked primary immunodeficiency. Transplantation of hematopoietic stem cells (HSC) from HLA-identical sibling donors is a resolutive and safe treatment but it is available to a minority of patients. A treatment based on the transfer of genetically corrected autologous HSC could be applicable to all patients. We investigated the efficacy and the safety of WAS gene transfer with lentiviral vector using untransformed T cells and HSC from WAS patients. We tested 3 vectors encoding the WAS protein (WASP) under the control of different promoters : the ubiquitous PGK promoter and the WASP full-length (1,6 kb) and minimal (0,5 kb) promoters. Transduction levels in WAS T cells were found to be comparable for each vector.The fraction of WASP+ T cells increased during long-term *in vitro* culture indicating that transduced cells have a selective growth advantage. WASP expression reached normal levels and no over-expression was observed. After transduction with all 3 vectors, correction of functional defects was achieved. Based on these results we selected the vector with the 1,6 kb promoter for further preclinical development. Clones of transduced CD4+ T cells were generated to understand how many vector copies are necessary to correct WAS T cells and to perform safety studies. CD34+ HSC were isolated from mobilized peripheral blood or bone marrow of donors and of a WAS patient. These cells were transduced with WASP-encoding lentiviral vectors. Preliminary results indicate that WAS and normal CD34+ cells were efficiently transduced and that *in vitro* differentation (number of CFC) occurred normally.

A66

MECHANISM OF AURORA B ACTIVATION BY INCENP AND INHIBITION BY HESPERADIN

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Aurora family serine/threonine kinases control mitotic progression, and their deregulation is implicated in tumorigenesis. Aurora A and Aurora B, the best-characterized members of mammalian Aurora kinases, are 60% identical but bind to unrelated activating subunits. The structure of the complex of Aurora A with the TPX2 activator has been reported previously. Here, we report the crystal structure of Aurora B in complex with the IN-box segment of the inner centromere protein (INCENP) activator and with the small molecule inhibitor Hesperadin. The Aurora B:INCENP complex is remarkably different from the Aurora A:TPX2 complex. INCENP forms a crown around the small lobe of Aurora B and induces the active conformation of the T loop allosterically. The structure represents an intermediate state of activation of Aurora B in which the Aurora B C-terminal segment stabilizes an open conformation of the catalytic cleft, and a critical ion pair in the kinase active site is impaired. Phosphorylation of two serines in the carboxyl terminus of INCENP generates the fully active kinase.

A67

CHARACTERIZATION OF SPINDLE ASSEMBLY CHECKPOINT PROTEIN DYNAMICS BY *IN VITRO* RECONSTITUTION

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The spindle assembly checkpoint (SAC) imparts fidelity to chromosome segregation by delaying anaphase until all sister chromatid pairs have become bipolarly attached. The components of the SAC reside at kinetochores (KTs), proteins scaffolds required to capture microtubules. Certain components of the SAC, including Bub1, Mad1, and a portion of Mad2 form a catalytic platform at KTs that recruits, activates, and releases a diffusible wait signal containing a rapidly exchanging portion of Mad2. We reconstituted a model of KT recruitment of the SAC protein Mad2 using purified components. Fluorescent molecules were immobilized on a flat glass surface inside a laminar flow chamber and imaged using a confocal microscope. The kinetic parameters of the reconstituted biochemical interactions were determined with fluorescence recovery after photobleaching (FRAP) and found them to be remarkably consistent with those measured in living cells for the recruitment of Mad2 to KTs. Furthermore, the application of a hydrodynamic flow will allow us to detect rapid changes in fluorescence upon addition of new SAC components under continued microscopy observation. The emerging data show the strong predictive value of our *in vitro* solid-phase assay based on accurately defined biochemical systems.

A68

VE-CADHERIN CLUSTERING IS DETERMINANT FOR TIGHT JUNCTION ORGANIZATION.

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The establishment of adherens junctions (AJs) between endothelial cells is thought to be necessary for the correct organization of tight junctions (TJs). Gene expression patterns, by Affymetrix analysis, of VE-cadherin null endothelial cells derived from *in vitro* differentiated murine embryonic stem (ES) cells was compared to the profile of the same cells transduced with the cDNA coding for VE-cadherin, the main constituent of AJs in endothelial cells. VE-cadherin clustering in confluent cultures dramatically upregulated claudin-5, one of the main constituents of TJs in endothelial cells. Claudin-5 seems to be the only TJ marker influenced by VE-cadherin clustering, since the lack of this molecule alters neither the level of expression nor the junctional localization of the other TJ constituents.
β-Catenin was shown to play an important role in the regulation of claudin-5 expression by VE-cadherin clustering. Several "gain of function" approaches inducing an increase in the amount of nuclear β-catenin caused a downregulation of claudin-5. Based on these observations, we hypothesize that β-catenin could be a negative regulator of claudin-5 expression.
The putative involvement of the PI3K-AKT-FKHR (FOXO1) pathway an its crosstalk with the β-catenin pathway is being studied.
The demonstrated relationship between claudin-5 and VE-cadherin suggests that the correct organization of TJs is dependent on AJ formation and stabilization.

A69

CHROMATIN MODIFICATIONS AND RETENTION OF HMGB1 BY APOPTOTIC CELLS

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When a cell dies due to apoptosis, HMGB1 is retained into the nucleus firmly bound to chromatin, thus preventing its release and so the generation of an inappropriate signal for neighbouring cells.
To date, we know that HMGB1 does not appear to be modified in apoptotic cells while chromatin undergoes chemical or structural transitions, including histone hypoacetylation , that allow irreversible HMGB1 binding. In mammalian cells, the only core histone modification unique to apoptosis known so far is histone H2B phosphorylation at Serine 14 (S14).
I have now demonstrated that TSA treatment of apoptotic cells is able to inhibit both HMGB1 binding to apoptotic chromatin and histone H2B (S14) phosphorylation but it is not able to revert the phosphorylation of H2B when it is already present. Moreover, I demonstrated that the treatment with TSA does not affect Mst1 kinase activity but the inhibition of H2B (S14) phosphorylation correlates with its acetylation at Lys12 and Lys15.
Using photobleaching techniques, we also demonstrated the lack or the inhibition of caspase 3 inhibits the H2B (S14) phosphorylation and allows HMGB1 to move into the nucleus with kinetics similar to those measured in living cells.
We also demonstrated that HMGB1 co-immunoprecipitates with nucleosomes enriched in (S14)Ph-H2B, but *in vitro* HMGB1 has similar binding affinity for the phosphorylated or unphosphorylated H2B tail.

A70

THE ROLE OF Eps15 HOMOLOGY (EH) DOMAIN : YEAST TWO-HYBRID SCREENINGS IN *C.ELEGANS*

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Eps15 homology (EH) domain is an evolutionarily conserved protein-protein interaction domain that was first identified as a repeated motif in Eps15, a substrate of EGF receptor. Although endocytosis has been the main process in which the EH domain containing proteins have been implicated, some interactors of EH domain-containing proteins have suggested that the EH domain is involved also in diverse processes such as vesicle recycling, intracellular signal transduction, and nuclear-cytosolic shuttling.
Caenorhaobditis elegans was chosen as the model system as all of the four conserved families of EH proteins are represented by single orthologues in their genome; INT-1(Intersectin family), EHS-1(Eps15 family), RME-1(EHD family), and Y39B6A.38(Reps/POB family). The EH domains of each proteins were cloned and used as baits for four independent yeast two-hybrid screenings against cDNA library. The map of interactions mediated by the EH domains in four different protein families could allow us to have a look at the extent of EH network that may not be well-portrayed by studies on the interactions of individual EH proteins.
Currently, Y2H screens have found 10 putative interactors of INT-1, 14 for EHS-1 and 14 for Y39B6A.38, some of which are under validation. The screening of RME-1 is under way to complete the map of EH interactions found by Y2H.

A71

CHARACTERIZATION OF DNA-DAMAGE RESPONSE IN CAENORHABDITIS ELEGANS

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Cells respond to the generation of DNA damage by mounting a coordinate set of actions known as the DNA-damage response (DDR). Studied manly in isolated cells, DDR is still poorly characterized in living organisms. We chose the nematode Caenorhabditis elegans as a multicellular model system to approach this problem. In the worm's germline, DNA damage can induce both cell cycle arrest and apoptosis, two responses that are spatially separated. Firstly, we set up two techniques that allowed us to study the DDR in worms. We discovered that living worms exposed to X-rays accumulate DNA double-strand breaks in all cells, as detected by TUNEL assay, but only germline cells are able to mount a DDR as demonstrated by immunofluorescence experiments. Therefore, the DDR signaling cascade can be triggered only in proliferating cells of the germline. In addition we discovered small number of cells with a low but detectable DDR in untreated worms. These are meiotic cells undergoing extensive DNA recombination, most likely responsible for the DDR signaling detected. Presently we are determining the genetic requirements for the generation of the DDR signal, employing mutants for several checkpoints activated in the ATM/ATR cascade. Our ultimate goal will be to study the potential engagement of the DDR signaling cascade in a variety of fundamental processes such as aging at the organismal level.

A72

EX-VIVO GENE THERAPY FOR GLOBOID CELL LEUKODYSTROPHY

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Globoid cell leukodystrophies (GLD) is a rare disease due to the deficiency of the lysosomal enzyme Galactocerebrosidase (GALC). The enzymatic deficiency results in intracellular storage of undegraded metabolites in central and peripheral nervous systems, leading to progressive demyelination. We are testing the effectiveness of a gene therapy strategy for GLD based on hematopoietic stem cells (HSC) and lentiviral vectors (LV) in the murine models of the disease. Homozygous defective neonates were transplanted with genetically corrected -/- HSC, supplemented with accessory wild type cells to reduce short-term toxicity of lethal conditioning. We observed a significant improvement in the phenotype of treated mice and prevention of mayor histopathological and neuroradiological signs of the disease. When moving to the more amenable approach of transduction of both HSC and accessory cells, we observed failure of transduced cells to rescue transplanted animals from lethal conditioning. These data, together with preliminary *in vitro* data showing apoptosis in GALC over-expressing HSC, suggest that GALC over-expression might be detrimental in HSC. *In vitro* experiments are currently on going to rule out the actual mechanisms responsible for this toxic effect. Moreover, we are developing alternative strategies to achieve controlled and safe GALC expression in HSC upon LV gene transfer, such as the use of the human GALC promoter.

A73

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In the last years, an increasing number of reports showed that HSCs participate in muscle regeneration. Although, the property of HSCs to incorporate into muscle fibers has been an area of intensive study, the fine characterization of the sub-population within the hematopoietic compartment and the ability of these cells to be reprogrammed remain elusive. This study aims to investigate the existence of a myogenic molecular program that BM cells follow prior to their fusion and the identification of myogenic factors involved in this program. In order to address the above question we set up a BM-myoblasts co-culture assay, which allows to score the contribution to myotube formation *in vitro*. BM cells participated to myotubes formation and expressed Myosin Light Chain in co-culture with primary myoblasts. Interestingly, the satellite specific myogenic marker Myf5 was expressed in mononucleated cells before their incorporation to myotubes. Employing the same co-culture protocol, it was also observed that BM cells derived from MyoD-/- or Pax7-/- mice have a reduced myogenic potential. Our results suggest that the transcription factors Myf5, Pax7 and MyoD are implicated in the BM to muscle transition.

A74

THE ROLE OF GIT1 IN MEMBRANE TRAFFICKING, CYTOSKELETON REMODELLING AND ADHESION

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A fine coordination between cytoskeleton remodelling and membrane trafficking is required in several cellular processes, including cell motility. GIT1 is a member of a family of GTPase-activating proteins for ARF GTPases that affect endocytosis, adhesion and migration. GIT1 associates to paxillin, and to a complex including the Rac/Cdc42 exchanging factors PIX/Cool and the kinase PAK. We found that overexpression of βPIX induces the accumulation of endogenous and overexpressed GIT1 at large intracellular structures similar to those induced by an ArfGAP-defective mutant of GIT1. Immuno-histochemical analysis and immuno-electron microscopy revealed that these structures include the endogenous transferrin receptor, suggesting that alteration of the endogenous GIT1-PIX complex could affect the endocytic compartment. The analysis of the endogenous GIT1 revealed that the protein is localized at cell periphery, both at actin-rich protrusions and focal adhesions, as well as at a perinuclear compartment. A better characterization of the GIT1 perinuclear staining is in progress. Furthermore, we are analysing the effects of GIT1/βPIX knock-down by siRNA on actin organization, adhesion, membrane trafficking and cell motility.

A75

ANALYSIS OF THE FUNCTIONAL DOMAINS OF THE CELL CYCLE CHECKPOINT KINASE Chk2

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DNA lesions induced by environmental agents or internal metabolism activate multiple checkpoint pathways that coordinate DNA repair and cell cycle progression.
Chk2 protein is a kinase that, upon activation by DNA damage in an ATM- and NBS1-dependent manner, phosphorylates several substrates causing checkpoint activation and cell cycle arrest in G1, S and G2/M transitions.
To get an insight on Chk2 function and regulation we decided to find out its interacting proteins.
For this purpose we have undertaken yeast two hybrid screens using the full lenght or Chk2 deleted of the kinase domain as baits and two different cDNA libraries. Several putative interactors have been identified and the relation between Chk2 and some of these proteins has been analyzed, but no association was confirmed *in vivo*.
A third yeast screen, using Chk2 deleted of the FHA domain as bait, enabled us the identification of karyopherin-α2 (KPNA-2) as a putative interactor. KPNA-2 is a protein involved in nuclear import of nuclear localization signal (NLS) containing proteins. We confirmed Chk2/KPNA-2 association by pull-down and co-immunoprecipitation assays, and we mapped, by the use of Chk2 and KPNA-2 deletion or missense mutants, the aminoacidic regions that mediate the interaction. Regarding Chk2, in particular, we have identified the NLS motif that is crucial for binding to KPNA-2 and Chk2 nuclear translocation.

B1

ALESSANDRINI

B2

GENE EXPRESSION PROFILING OF MURINE EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS AND HUMAN MULTIPLE SCLEROSIS PATIENTS.

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Multiple sclerosis (MS) is one of the most common autoimmune diseases of the central nervous system (CNS) which causes are still unknown. It is believed to be a polygenic disease characterized by both inflammation and neurodegeneration hallmarks. For these reasons, animal models for the disease have been developed and studied recently. The murine experimental autoimmune encephalomyelitis (EAE) is a well-established model that shows clinical and physiopathological aspects of MS and can reflect in part the disease in humans. In order to understand better the on-set and progression of the disease,
we have used microarray-based technology. We have compared gene expression profiling from CNS of EAE mice and from MS human patients peripheral blood mononuclear cells (PBMC). We have analyzed spinal cord and brain of two different strains of mice obtained at various time points of the disease and three different clinical courses of human MS: Relapsing-remitting (MSRR), Primary-progressive(MSPP) and Secondary-Progressive(MSSP). Statistics and data-mining software were used to identify differentially expressed genes that were unique or in common among all the disease stages, either in humans or in mice. Moreover, a comparison between human and mouse shows evidences that specific genes could be good biological markers for the disease and that some genes could be interesting target for therapeutics.

B3

DEFINITION OF THE ANTIGENIC REPERTOIRE IN LUNG CANCER.

Glenda Canderan, Paola Gruarin, Paolo Dellabona and Giulia Casorati

Non-small cell lung carcinoma (NSCLC) is the first cause of cancer death, and responds poorly to conventional therapies prompting the search of innovative therapeutic approaches. Immunotherapy is a promising new strategy to treat cancer. The prerequisite for its application in NSCLC is the definition of antigens selectively expressed by this tumour. To this aim, we attempted a classic «expression cloning» strategy, based on the screening of transfected tumour-derived cDNA libraries by specific $CD8^+$ T cell clones. Patient-derived DCs were used to cross-present tumour-associated antigens (TAA) from a NSCLC cell line (CaPo13) to autologous T cells. We obtained 52 tumor-specific $CD8^+$ T cell clones, displaying a limited TCR diversity. We focused on five T cell clones, three of which were identified at a molecular level in the tumor infiltrating lymphocytes (TILs). One of these clones recognised an unkonwn antigen shared by different NSCLC cell lines. Since the tumour-specific T cells clones grow very slowly, their TCR α and β chains were transduced into a mouse T cell line engineered with the human CD8 coreceptor. These transduced T cell lines express the grafted TCR on the membrane, and are able to produce IL2 upon the engagement of the TCR and the CD3 molecule, but till now they showed no ability to recognize Capo13 cells. This is probably due to a lower sensitivity to antigen stimulation by the transfected T cell lines in comparison with T cell clones. We are currently trying to improve the sytem.

B4

DYNAMICS OF HIV-1 RNA DECAY IN CD14+CD16+ MONOCYTES FOLLOWING COMBINED NUCLEOSIDE/NON-NUCLEOSIDE THERAPY OF SOUTH AFRICAN PATIENTS WITH ADVANCED SUBTYPE C INFECTION.

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Aims*:** To assess the relative contribution of CD14+CD16+ monocytes to HIV-1 persistence following treatment with nucleoside/non-nucleoside analogue therapy. ***Design*:** Intensive longitudinal monitoring (during the first year of therapy) of twelve African patients treated generic antiretroviral therapy. All 12 patients were dually-infected with HIV-1 subtype C and HHV-8. ***Methods*:** Immunophenotyping and flow-based *in situ* hybridization of HIV-1 *gag/pol* RNA levels and correlation with CD4+ T-cell restoration and plasma viral load clearance. ***Results: All patients had detectable *gag/pol* RNA in both cellular compartments. Patients with relatively intact immune systems (CD4+ >300 cells/μl) had the lowest levels of viral RNA in both reservoirs. Patients with intermediate CD4+ cell counts showed significantly increased HIV-1 RNA in the T-cell reservoir only, in association with decreased CD8+ T-cell counts. Patients with severe CD4+ T-cell depletion (<100 cells/μl) had the highest frequency (%) of HIV-1 RNA positive monocytes. Post-treatment clearance of viral RNA in monocytes was multi-phasic and paralleled decay in plasma and CD4+CD45RO+ T-cells. At the end of 6 months, the net reduction in intracellular RNA was 82.9% in monocytes and 52.2% in CD4+CD45RO+ T-cells. Sustained, post-treatment suppression of full-length viral RNA in monocytes was independent of CD4+ T-cell restoration, and related to high, sustained CD8+ T-cell numbers. ***Conclusions/Discussion*:** CD14+CD16+ monocytes are a major *in vivo* reservoir of HIV-1 RNA in African patients infected with subtype C viruses. Triple combination therapy with RT inhibitors is highly effective in eliminating HIV-1 RNA from the monocyte compartment

B5

JAM-A DEFICIENT POLYMORPHONUCLEAR CELLS SHOW REDUCED DIAPEDESIS IN PERITONITIS AND HEART ISCHEMIA-REPERFUSION INJURY IN MICE

Corada M, Chimenti S, Cera MR, Vinci M, Salio M, Fiordaliso F, De Angelis N, Villa A, Bossi M, Staszewsky L, Vecchi A, Motoike T, Latini R and Dejana E
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JAM-A (Junctional Adhesion Molecule-A) is a transmembrane adhesive protein expressed at endothelial junctions and in leukocytes. In this work we report that JAM-A is required for the correct infiltration of polymorphonuclear leukocytes (PMN) into an inflamed peritoneum or in the heart upon ischemia-reperfusion injury. The defect was not observed in mice with an endothelium- restricted deficiency of the protein, but was still detectable in mice transplanted with bone marrow from *JAM-A*$^{-/-}$ donors. Microscopic examination of mesenteric and heart microvasculature of *JAM-A*$^{-/-}$ mice showed high numbers of PMN adherent on the endothelium or entrapped between endothelial cells and the basement membrane. In vitro, in the absence of *JAM-A,* PMN adhered more efficiently to endothelial cells and basement membrane proteins and their polarized movement was strongly reduced (chemotaxis, Dunn chamber assay…). This work describes a novel role of JAM-A in controlling PMN diapedesis through the vessel wall.

B6

RECEPTOR-DEPENDENCY AND IMMUNOLOGICAL PROPERTIES OF HEAT SHOCK PROTEIN-PEPTIDE COMPLEXES IN HUMAN TUMOR

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The specificity of the immune response generated by Heat Shock Protein vaccinations is a result of the peptides chaperoned by HSPs. The HSP gp96 and HSP70 functions as chaperone for a number of peptides derived from tumor antigens. *In-vitro* progresses toward defining the nature of the peptides outlining the HSP antigenic repertoire and their pathway of HSP-mediated presentation are described here. We show that, in human setting, autologous monocytes pulsed with melanoma derived gp96 and HSP70 induce *in-vitro* anti-tumor T cells that include T cell clones recognizing a unique melanoma antigen. We study how activation of CTL changes according to the sequence context of the antigenic peptide bond to gp96. We designed Mart1-derived peptides containing the T cell epitope extended at the C- or N-terminus. The capacity of all synthesized peptides to bind purified gp96 is evaluated together with their ability to induce CTL activation in representation assays. Furthermore, the peptide showing the best *in-vitro* binding capacity is selected to study the uptake and processing pathway involved in the presentation of gp96-associated tumor antigens. Fluorescence microscopy analysis indicates a colocalization between labeled gp96 and its receptor CD91, although not of 100%. Other receptors are clearly involved in the gp96-mediated antigen uptake, eventually leading to a different cellular processing. Confocal analysis will address specifically this issue.

B7

ROLE OF INHIBITORY RECEPTORS ON T CELLS FOR TOLERANCE INDUCTION IN PATIENTS WITH PERSISTENT MIXED CHIMERISM AFTER ALLOGENEIC HEMATOLOGIC STEM CELL TRANSPLANTATION FOR β-THALASSEMIA

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Around 10% of patients with β-thalassemia develop a state of persistent mixed chimerism (PMC) between host and donor cells after allogeneic hematologic stem cell transplantation (HSCT), suggesting the achievement of a state of natural tolerance *in vivo*. Accumulating evidence suggests that expression of inhibitory receptors on T cells may be one of the mechanisms involved in peripheral tolerance. We have characterized the repertoire of polymorphic killer cell immunoglobulin-like receptor (KIR) genes carried by donor-recipient pairs. In a cohort of 3 and 6 patients with or without PMC, respectively, no apparent correlation was found between the presence of specific KIR haplotypes and PMC. As shown by immunofluorescence staining, a considerable percentage (>50%) of CD3+CD56+ T cells circulating several years after transplantation in four different patients with PMC, expressed inhibitory receptors whose HLA ligands were present in the patient. In order to investigate if these cells are involved in the mechanisms underlying PMC, we plan to purify them by cell sorting and/or cloning, and to characterize their origin (patient or donor) as well as their functional and phenotypical characteristics.

B8

SECRETED HMGB1 REGULATES THE MATURATION AND FUNCTION OF HUMAN DENDRITIC CELLS

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High mobility group box 1 (HMGB1) is a nuclear protein that is released by necrotic cells and acts in the extracellular environment as an inflammatory signal. Active secretion of HMGB1 has been documented for monocytes and macrophages. The activity of HMGB1 is mediated by the receptor for advanced glycation end-products (RAGE). We show that human dendritic cells (DCs) HMGB1 upon maturation. HMGB1/RAGE blockade leads to: decreased upregulation of surface markers and IL-12 production; decreased migration to MIP-3β and SDF-1 chemokines; impaired priming and polarization of naïve CD4 T cells by allogeneic DCs. The effects of HMGB1 on DCs were mediated *via* RAGE and involved the activation of p38 and ERK1/2 MAP kinases and of NF-κB. Stimulation of DCs derived from RAGE knock-out mice evidenced a significant decrease in the up-regulation of surface markers, in the production of IL-12 and in the activation of p38 and NF-κB, when compared to DCs derived from wild type animals. Our data reveal HMGB1/RAGE as an autocrine loop involved in the regulation of DC maturation. These results suggest that antagonists of HMGB1/RAGE might have therapeutic potential for the treatment of systemic human diseases.

B9

IDENTIFICATION OF GENES ASSOCIATED TO METASTATIC PHENOTYPE IN COLORECTAL CANCER: DISCOVERY OF NOVEL TARGETS FOR IMMUNOLOGICAL INTERVENTION.

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The aim of this study is the identification of genes involved in colorectal metastasis, in order to discover novel tumour associated antigens and, in general, novel immunological targets for an immune-based therapeutic approach. Gene expression profiling analysis was performed on two pairs of autologous colorectal cell lines derived from the primary tumour and from the corresponding metastasis. One pair is represented by two commercial cell lines, SW480 (primary tumour) and SW620 (lymph node metastasis), purchased from the ATCC. The other pair consists of two short-term cultures, Micol23m1 (primary) and Micol24m2 (liver metastasis), established at INT (Istituto Nazionale Tumori, Milan). RNA samples from each cell line were hybridized to Affymetrix GeneChips HG-U133A Plus 2.0 containing ~ 47,000 human transcripts. Comparative analysis revealed lists of genes differentially expressed in metastasis respect to the primary tumour cell lines. Only a few genes resulted commonly regulated in both metastatic cell lines compared to their primary counterparts: 26 genes are over-expressed and 75 down-regulated in both metastases. Some candidate genes with functional and immunological relevance were identified. Genes of interest were validated in terms of RNA expression using Real-Time PCR, both on the same samples used for the chip hybridization and on an independent set of cell lines.

B10

INVESTIGATING ASSEMBLY AND DYNAMICS OF HUMAN MEMBRANE IgE

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Human membrane IgE (mIgE) constitutes the B cell receptor (εBCR) of B cells responsible for IgE production. Regulated by complex genetic and environmental factors, B cells exposing εBCRs ultimately determine IgE-related immune-disregulations, such as allergic diseases and hyper IgE syndromes. Studying the BCR of IgE-producing B cells is therefore very important to understand IgE homeostasis. Due to their exceedingly low number, human εBCR^+ plasmacell precursors and memory B cells have been highly elusive to date. No human εBCR^+ cell line has been established and the differentiation stages leading to plasmacells and production of secretory IgE are yet debated and unclear. Given the scarce knowledge on mIgE biology and the urge to deepen the understanding on this issue, we investigated human mIgE isoforms transfected in mouse B cell lines corresponding to distinct B cell differentiation stages. Human mIgE was characterized in terms of its intracellular intermediates, propensity to assemble with Igα-Igβ heterodimers and capacity to translocate into rafts. Assembly of mIgE is being characterized in terms of endoplasmic reticulum-resident glycosylation and heavy-light chain association intermediates. Interestingly, when expressed in plasmacytoid cells (hence in absence of the accessory Igα-Igβ heterodimer) mIgE isoform long is exported to the cell surface as a folded and functional protein. We are presently observing the degree of cell activation, upon mIgE induced aggregation, depending on B cell type and presence of Igα-Igβ heterodimers. In addition, the localization of mIgE into rafts has been studied in resting state and after induced aggregation. As reported for γBCR, the εBCR localizes into rafs upon its clustering. The rate and dynamics of mIgE association with rafts reveals a complex aggregation-dependent pattern. Noteworthily, a detectable portion of mIgE (as well as mIgG) associates with rafts also in resting state. Indeed, BCRs exist as small oligomers prior to induced aggregation. We are now trying to understand if resting state γBCR and εBCR oligomers are raft-associated. In conclusion, mIgE is seemingly acting as a fully-equipped BCR.

B11

ROLE OF NEPHRIN IN THE GLOMERULAR PERMSELECTIVITY

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Nephrotic syndrome, a clinical disorder associated with primary and secondary glomerulonephritis of different etiology, is characterized by increased urinary protein excretion and profound changes in glomerular podocyte morphology resulting in massive remodeling of podocyte intercellular junction (slit diaphragm). This is the essential component of the filtration barrier. Recent disclosure of podocyte proteins has unravelled previous rather mysterious mechanisms that govern glomerular perm-selectivity in health and disease. We explored the role of nephrin and other podocyte proteins, including CD2AP and podocin, together with the integrity of the slit diaphragm in the pathogenesis of proteinuria in patients with acquired glomerular diseases. We observed that nephrin mRNA and protein were markedly reduced in glomeruli of proteinuric patients with IgA nephropathy (AmJNephrol 2003) and type II diabetes (KidneyInt 2004). Abnormalities were confined to nephrin to the extent that CD2AP and podocin were comparable in all subjects. Finally, in the same patients ultrastructural analysis revealed a remarkable reduction in the percentage of electron dense slit diaphragms as a consequence of targeted down-regulation of nephrin. Our results suggest that nephrin may represent the therapeutic target of treatments aimed at preventing proteinuria, in the hope of extinguishing renal disease. Experimental studies aimed at identifying the most effective pharmacological approach are ongoing.

B12

BONE MARROW TRANSPLANTATION AND IMMUNOTHERAPY FOR THE TREATMENT OF PROSTATE CANCER

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Spontaneous immune responses to tumors often develop but fail to eradicate the tumor. We have investigated anti-tumor immune responses in the Transgenic adenocarcinoma mouse prostate (TRAMP) model. TRAMP mice express the large T antigen (Tag) under the control of the probasin promoter, and develop prostate cancer after puberty. After 10 weeks of age the mice become tolerant to the tumor and fail to respond to a tumor specific vaccine (Degl'Innocenti et al., 2005). Allogeneic bone marrow transplantation (BMT) has been used to treat hematological malignancies, as in addition to a graft-versus-host-disease (GvHD) it elicits a graft-versus-tumor (GvT) immune response. We thus evaluated the possibility to break tolerance in TRAMP mice by performing BMT in a minor histocompability antigen (HY) mismatch setting (female bone marrow into male recipients). The HY antigen is expressed by male tissues and it is recognized by donor derived female T cells, able to mediate a GvH reaction. Male TRAMP mice were transplanted with female BM cells and vaccinated with Tag and HY specific vaccines, in the attempt to promote a tumor specific response in the context of a controllable GvHD. The immune response was evaluated in lymphoid organs by flow cytometry and the clinical outcome by immunohistochemistry of prostate-derived tissues. Our results indicate that BMT and vaccination restore the anti-tumor response in TRAMP mice, which correlates with a favorable clinical outcome.

B13

EPITHELIAL CELLS-DENDRITIC CELLS CROSSTALK IN THE PROCESSES OF TOLERANCE IN THE GUT.

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Tolerance is a central regulatory mechanism in the immunity. To achieve a balanced control between tolerance and immune responses, the gut immune system combines a number of mechanisms at different levels. These mechanisms regulate the response to pathogens and tolerance to commensal bacteria. In order to understand how the tolerance to commensal bacteria is achieved, we have developed a two partner co-culture system, that mimics the in vivo spatial distribution of two important players that regulate mucosal immune responses: polarized monolayer epithelial cells (ECs) and Dendritic cells (DCs). We sought if the ECs released factors are able to promote tolerogenic immune response. Thus, myeloid DCs were conditioned with polarized Caco2 supernatants and then co-cultured with naïve CD4+ T-cells in allogeneic mixed leukocyte reaction (MLR). Interestingly, such conditioned DCs did not only promote Th2 type responses, but also facilitated development of tolerogenic CD4+CD25+ T cells in MLR. Which are the factors involved in this process remains to be further elucidated.
It has been shown that flagellin, a ligand of TLR5, is sensed by ECs when introduced basolaterally, but not aplically. In our experimental system, virulent *Salmonellae typhimurium*, which is flagellin positive and is able to penetrate the ECs, increased the production of macrophage inflammatory protein 3α (MIP3α) and IL-8 by polarized Caco2 monolayers. In contrast, chemokine production was not observed in response to commensal bacteria and CpG DNA, which are not able to penetrate the ECs and therefore to approach the basolaterall side of the polarized monolayer. We assume, that the spatial distribution of the TLRs could be one of the cellular mechanisms of keeping the tolerance to commensial bacteria. In support to this hypothesis, our preliminary data show that CpG DNA, as a ligand of TLR9, was able to effectively evoke a response in polarized Caco2 cells only when it was introduced basolaterally.

B14

DEFECTIVE REGULATORY AND EFFECTOR T CELL FUNCTIONS IN PATIENTS WITH FOXP3 MUTATIONS

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Mutations in the FOXP3 gene are responsible for the autoimmune disease IPEX (Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked). In the mouse model of FOXP3 deficiency, $CD4^+CD25^+$ regulatory T (Tr) cells do not differentiate, thus causing lethal autoimmunity. In humans, a correlation between mutations of FOXP3, lack of $CD4^+CD25^+$ Tr cells and the development of IPEX has not yet been demonstrated. Aim of this project is the characterization of T cells isolated from IPEX patients. In the four patients included in this study, we observed that the number and cell surface phenotype of $CD4^+CD25^+$ Tr cells in peripheral blood is normal. Furthermore, purified $CD4^+CD25^+$ Tr cells from IPEX patients suppress the in vitro proliferation of effector T cells from normal donors. In contrast, IPEX $CD4^+CD25^+$ Tr cells are not able to suppress in the presence of a strong activation signal or of autologous effector T cells, unless they have been previously activated and expanded in vitro in the presence of exogenous IL-2. Interestingly, TCR-mediated stimulation of IL-2 and IFN-γproduction by PBMC from IPEX patients is significantly decreased compared to controls. These results indicate that in patients with FOXP3 mutations $CD4^+CD25^+$ Tr cells are present although they are impaired in their suppressive activity. Whether this is related to impaired responsiveness to different strength of TCR activation or due to lack of production of IL-2, remains to be clarified.

B15

CONVERSION OF $CD4^+$ $CD25^-$ LYMPHOCYTES INTO REGULATORY T CELLS IS THYMUS AND PROLIFERATION INDEPENDENT AND CONTRIBUTES TO TUMOR INDUCED T REG EXPANSION

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$CD4^+CD25^+$ regulatory T cells (T reg) play an essential role in controlling the immune responses to self antigens. In tumors, T reg depletion prior vaccination increases the chance to induce tumor rejection, but is insufficient to obtain a therapeutic response. We show that the presence of tumor breaks the tight control of T reg homeostasis and induces an increase of T reg number in draining lymph node and spleen but not in contro-lateral lymph node. In tumor bearing mice, recovery of T reg after depletion with the mAb anti-CD25 (PC61) is faster and double in number than in normal mice and occurs independently from the thymus, as shown in mice thymectomized prior depletion and tumor inoculation. The newly generated $CD4^+CD25^+$ T cells show the same features of naïve T reg since they express Foxp3, the costimulatory molecules CTLA-4, GITR, OX40 and CD45RB, and are suppressive in vitro. They originate from $CD4^+CD25^-$ T cells by conversion, as shown by transferring $Thy1.1^+CD4^+CD25^-$ T cells into syngeneic tumor bearing mice and recovering $Thy1.1^+CD4^+CD25^+$ T cells which are double in number in draining lymph node and spleen compared to contro-lateral lymph node. Our data show that although T reg depletion can initially favor the induction of an effective anti-tumor immune response, tumor immunotherapy strategies should use approaches able to inhibit rather than deplete T reg.

B16

TRANSDUCTION OF T LYMPHOCYTES WITH CCR7 CHEMOKINE RECEPTOR TO INCRESASE THEIR HOMING CAPABILITY

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We have recently shown that C57 B/6 mice vaccinated with T lymphocytes transduced with retroviral vector coding for the model antigen Ovalbumin (OVA), are capable of developing a strong and specific immune responcse against OVA, which ultimately controls tumor growth and estabilish es protective long-term memory in mice subcutaneously transplanted with B16-OVA-expressing melanoma.
T lymphocytes homing analysis demonstrated that only 0,6% and 6% of transduced cells had migrated to lymphonodes and spleen respectively.
In order to increase the homing capability of antigen-expressing T cells to SLOs, we transduced them with a retroviral vector coding for human CCR7, which cross-reacts with the murine chemokines (ELC, SLC), and also carries the DNGFr gene (neurvous Growth Factor receptor truncated at the cytoplasmic tail) as a reporter gene.
However, when we transduced murine T cells we did not obtain any cell surface expression of the CCR7 receptor, although they expressed the reporter gene product.
Subsequent analysis on several cell lines of different histological type (U937, Jurkat, K562, T2, CEM A, MSR3-mel, SK23-mel) showed an heterogegenous pattern of CCR7 surface expression, although they all had high CCR7 m-RNA levels.
This observation led us to explore the mechanisms involved in CCR7 regulation.

B17

C1-INHIBITOR PROTECTS AGAINST BRAIN ISCHEMIA/REPERFUSION INJURY VIA INHIBITION OF CELL RECRUITMENT AND INFLAMMATION

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We have previously demonstrated that C1-inhibitor (C1-INH), a major inhibitor of complement and contact-kinin systems, is neuroprotective in cerebral ischemia. In order to clarify the mechanism of this action we evaluated in the present study the expression of neurodegeneration and inflammation related factors in CD1 mice, treated with C1-INH (15U) or saline and subjected to 2h of ischemia (MCAO) and 2 or 46h of reperfusion. mRNA expression was measured by RT-PCR in brain cortex.
C1-INH significantly dampened the mRNA expression of the adhesion molecules *P-selectin* and *ICAM-1* induced by the ischemic insult. These data has been confirmed by immunohistochemistry analysis: C1-INH markedly inhibited the activation and/or recruitment of microglia/macrophage, as well as the infiltration of leukocyte. It significantly decreased the pro-inflammatory cytokine (*TNFα*, *IL-18)* and increased the protective cytokine (*IL-6*, *IL-10*) gene expression. C1-INH treatment prevented the decrease of *NFH* gene, a marker of cellular integrity and counteracted the increase of *pro-caspase 3*, an apoptosis index.
In conclusion, C1-INH exerts an anti-inflammatory and anti-apoptotic action on ischemia/reperfusion injury. Our present and past data support a major effect of C1-INH on cell recruitment from the vasculature to the ischemic site.

B18

WISKOTT-ALDRICH SYNDROME PROTEIN IS REQUIRED FOR THE PRODUCTION OF TH1 CYTOKINES BY $CD4^+$ T CELLS AND FOR THE ACTIVATION OF $CD8^+$ T CELLS

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Wiskott-Aldrich syndrome protein (WASP) belongs to a family of proteins which are involved in actin cytoskeleton reorganization in haematopoietic cells. In T cells, WASP is required for lipid rafts clustering and immunological synapse organization. Mutations in the WAS gene can lead to a severe immunodeficiency, called Wiskott-Aldrich syndrome (WAS). $CD4^+$ T cell lines, established from WAS patients ; have a selective impairment in the production of Th1 cytokines, in response to TCR-mediated signals. This impairment is due to a blockade in Th1 citokines gene transcription. In contrast, the ability to produce Th2 cytokines is only minimally impaired.
$CD8^+$ T cells from WAS patients show severe impairment of both proliferation and cytokines production in response to TCR stimulation. However, they retain normal lytitc activity.
Studies on TCR/CD28 signaling in WAS $CD4^+$ T cells reveal no alteration of the main cytosolic pathways leading to IL-2 and IFN-γ gene transcription (Ca^{2+} flux, MAPKs activation; IkB-α degradation). However, activity of NFAT1 trascription factor is reduced in the nucleus. The aim of the ongoing studies is the defeinition of the molecular mechanisms leading to WASP-deficient T cells functional defects.

LECTURES

Anton Berns
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Renzo Tomatis, M.D., Writer

SESSION C: CANCER BIOLOGY

Angelo Cicalese
Valeria Cambiaghi
Francesca De Franco
Barbara Ortensi
Sabina Sangaletti
Luigi Maddaluno

JANUARY 25TH - ABSTRACTS

ORAL PRESENTATIONS

BIOLOGICAL CHARACTERIZATION OF MOUSE BREAST STEM CELLS FROM NORMAL AND NEOPLASTIC TISSUES

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Recent findings suggest that stem/progenitor cells are frequent targets of transformation and that different tumors contain a small subset of cells endowed with the property of supporting tumor growth (cancer stem cells). We are currently investigating the biological properties of normal and transformed breast stem cells. We set-up protocols for the *in vitro* propagation of breast stem cells from the mouse mammary gland (BSCs), based on their ability to survive in suspension as 'mammospheres', and to differentiate into myo-epithelial and epithelial cells. Murine mammospheres do not result from passive cell aggregation and have clonal origin, as assessed by labelling cell membranes with different epifluorescent dyes. To prove the "staminality" of these cellular populations we performed *in vivo* reconstitution experiments, by inoculating single cell suspensions of mammospheres in the mouse cleared fat pad. Using the same experimental approach, we have generated mammospheres from adenocarcinomas of MMTV-ErbB2(cNeu) transgenic mice. The mammospheres arising from MMTV-ErbB2 tumors, as compared to matched WT samples, are bigger (1000 *vs*. 200-400 cells/sphere) and show a dramatically prolonged lifespan upon serial passages. To prove that cancer mammospheres are enriched in cancer BSC, we are currently performing *in vivo* tissue reconstitution experiments.

PRELEUKEMIC PHASE ANALYSIS TO UNDERSTAND THE BIOLOGICAL CONTRIBUTION OF PML-RARα TO THE LEUKEMOGENIC PROCESS

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Acute Myeloid Leukemia (AML) associated fusion proteins induce a preleukemic state. The t(15;17) is found exclusively in acute promyelocytic leukaemia (APL), a subtype of AML, and codes for the PML-RARα fusion protein which incorporate the DNA-binding domain of the RARα transcription factor and that functions as a constitutive repressor of retinoic acid (RA)-target genes.
In transgenic mice expressing PML-RARα in the bone marrow, preleukemia occurs after accumulation of additional genetic lesions. In this case preleukemic bone marrow appears morphologically normal before the second hit event. The biological mechanisms underlying the contribution of fusion proteins to the preleukemic phase and to the maintenance of the transformed leukemic phenotype are unknown.
Preliminary data demonstrate how PML-RARα *in vivo* drive the expansion of the stem cell compartment: in fact preleukemic bone marrow is characterized by an overproliferation in Lin- c-Kit+ Sca-1+ subpopulation associated with an overexpression of stem cell genes. Moreover in bone marrow competition experiments, preleukemic cells have a proliferative advantage over normal cells. Now we are investigating which is the mechanism used by PML-RARα to induce genome instability: the answer seems to be a slow mechanism of telomere shortening during the prelukemic phase.

ROLE OF QUIESCIENT STEM CELLS IN THE MAINTENANCE OF LEUKAEMIA CELL POPULATIONS

Francesca De Franco, Andrea Viale, Annette Orleth, Valeria Cabiaghi, Simona Ronzoni, Saverio Minucci, Myram Alcalay, Pier Giuseppe Pelicci.
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Stem/progenitor cells ensure for tissue and organism homeostasis and might represent a frequent target of transformation.Stem cells are relatively quiescent, while their more differentiated progeny have dramatic proliferative ability.In the absence of the cell cycle inhibitor p21, increased cell cycle leads to hematopoietic stem cell exhaustion and, under stress conditions haematopoietic cell depletion.We are investigating the existence, in leukemia animal models, of quiescent leukemic stem cells (LSC) and their role in the maintenance of the leukemic clone.We found that leukemic fusion proteins (AML1/ETO, PML/RAR, PLZF/RAR) induce up-regulation of p21 expression.Strikingly, AML1/ETO is unable to induce leukaemia in p21-/- mice and PML-RAR leukemias engineered into a p21-/- background fail to transplant into syngeneic animals.Ectopic expression of p21 into WT hematopoietic stem cells induces the expansion of the stem cell compartment, as evaluated by the long-term culture initiating-cell (LTC-IC) assay.Using moreover a membrane stable fluorescent dye (PKH), we isolated a quiescent/slow-cycling leukemic cell population characterized by a stem cell phenotype and capable to transplant leukemia.

RAI (SHC-C) REGULATES NEURONAL STEM CELL FUNCTION

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Rai (Shc-C) is a neuron specific member of the Shc-family of adaptor proteins and functions as an upstream activator of PI3K-Akt pathway, stimulating neuronal cell survival upon environmental stresses or limited availability of GDNF, the ligand for the Ret receptor-kinase. In *vivo*, Rai expression protects against neuronal loss during development and, in adult mouse, following ischemic brain injury.
The PI3K-Akt signalling pathway is a critical determinant of proliferation and survival of mammalian cells and constitutively activated in human glioblastomas (GBMs).
Rai is expressed in neural stem cells (NSCs), in mature neurons but not in glial cells. Unexpectedly, however, tumors of glial origin (GBMs) express high levels of Rai protein, suggesting that ectopic expression of Rai in GBM might be relevant for the transformed phenotype. The mechanisms, however, responsible for the expression of Rai in GBM, as well as the phenotypic consequences of the ectopic expression are unknown.
In order to understand the role of Rai in gliomagenesis I am studying both normal and cancer stem cells, isolating the normal ones from Rai wt and -/- mice and the tumoral ones from human GBMs. Preliminary results suggest that Rai expression exerts a negative effect on the growth properties of NSCs and has a role in their differentiation towards neurons.

STROMAL-DERIVED SPARC REGULATES STROMA CELLS-TUMOR CELLS INTERACTION TOWARD METASTASIS.

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SPARC (secreted protein acidic and rich in cysteine) is a matricellular glycoprotein involved in cancers although with contradictory results. One possible explanation is that SPARC is produced by both tumor and stromal cells perhaps with different functions. We have now evidence that SPARC produced by stromal cells is necessary for their interactions with tumor cells toward *in vivo* lung metastases. 4T1 carcinoma cells injected into BALB/c and SPARC KO mice produce lung metastasis only in the wild type mice while growing subcutaneously without difference. Since 4T1 cells produce SPARC directly, we hypothesized that the absence of SPARC from stromal cells hampers the metastatic process. To prove such hypothesis we injected 4T1 cells in chimeric mice expressing or not SPARC in donor bone-marrow (BM). Chimeric mice expressing SPARC only in BM–derived cells (CB6>SPARC KO) forms the same number of metastases of control chimeras (CB6>BALB/c), whereas reciprocal chimeras (SPARC KO >CB6) show a dramatic decrease in number of lung colonies. Thus host- rather than tumor-produced SPARC is needed in the metastatic process. Chimeric mice receiving BM from transgenic mice carrying a suicide fusion protein under csf-1 promoter will prove the role of macrophages through their specific ablation upon drug treatment.

L1CAM: A NEW REGULATOR OF DENDRITIC CELL TRAFFICKING?

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The lymphatic vasculature is the system of thin-walled, low-pressure vessels that collects and transports excess interstitial fluid and macromolecules unidirectionally from tissues back to the blood circulation. Moreover, lymphatic vessels play a crucial role in the immune system because lymphocytes and dendritic cells enter lymphatic capillaries in the periphery and migrate, through the lymphatic vessel, to the lymph nodes where they elicit the immuno responses to non-self antigens. Clinico-pathological studies have suggested that lymphatic vessels serve also as the primary route for the metastatic spread of tumor cells to regional lymph nodes. However, the mechanisms and the molecules that regulate the passage of immune and cancer cells across the lymphatic endothelium remain poorly understood. Our preliminary data show that one of the molecules that can play a key role in this process is L1, a cell-adhesion molecule (CAM) previously characterized in the nervous system. L1 is expressed at high levels in both human and mouse dendritic cells, lymphatic endothelial cells and it is up-regulated in highly invasive tumors like melanoma, ovarian and colon cancer. By employing the Cre-loxP technology, we have generated L1-deficient mouse bone marrow derived dendritic cells (BMDCs) and our preliminary experiments indicate that the absence of L1– drammatically affects the ability of BMDCs to adhere to and migrate through the lymphatic endothelium. Our results point to a novel role of L1 in dendritic cell function, a hypothesis that we are currently verifying in genetically manipulated mouse models.

SESSION C- CANCER BIOLOGY

JANUARY 25TH - ABSTRACTS

POSTERS

C1

GENOME WIDE ANNOTATION OF EPIGENETIC MARKERS: HYPERSENSITIVE SITES IN HUMAN CD34+ CELLS

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DNA Hypersensitives sites have been used as an hallmark of potentially regulatory regions since late eighties. Recently new high-throughput tecniques have been applied to the identification of such sites in Human cell lines. In summary: extraction of chromatin from cells is followed by DNAseI digestion and the fragments are than cloned, sequenced and in silico mapped on the genome. Direct validation of the clones showed very high rates of false positives (DNA shearing, overdigestion, cleavage of partially protected sites) which are directly inherited by the bionformatic pipeline.
Here we show the initial results of a new tecnique, developed in collaboration with Sangamo Biosciences (see G.Gargiulo et Al., 4th PhD meeting Riva Del Garda 23-25 January 2005) on Human CD34+ cells. Even in this early stages our data clearly suggest a wide spread open-chromatin state, reflecting a typical germ line pattern. Interestingly half of the sites mapped inside or nerby a RefSeq gene and the second half is some Kb far away from any known gene. More over these regions show distinctive features if compared with an in silico digestion with the same nuclease we used in vivo.
The first 3314 mapping clones have been included in the 'Expression and Regulation' track in the UCSC genome browser and are avaiable online at http://genome.ucsc.edu/.

C2

IDENTIFICATION OF GENES ASSOCIATED WITH THE TOXICITY FROM RADIATION THERAPY USING A cDNA MICROARRAY APPROACH

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On the base of the hypothesis that radiation toxicity may be associated with an abnormal transcriptional response to DNA damage, the aim of this work is the identification of genes associated with the toxicity from radiation therapy that could be used to predict response to radiation treatment (Rieger et al. 2004). We have collected 30 blood samples from patients affected from prostate cancer who undergone 3-D conformal radiation at high doses (>74 Gy). Patients belonged to three different categories, measured according to a dose volume histogram: a) low risk but who developed radiation toxicity with grade 2-3 (RTOG/EORTC) chronic toxicity (10 patients), b) high risk, who developed toxicity (10 patients) and c) high risk but who did not show any toxicity (10 patients). To determine which cell type use for this study, we performed a preliminary cDNA microarray experiment on RNA extracted either from LCLs (used by Rieger et al, 2004) or PHA-PBLs from 10 healthy blood donors before and after 5 Gy radiation treatment. RNA extracted from them was used to evaluate the variation in gene expression pattern in these two cell types. This first experiment will allow determining if PHA-PBLs can be used for the comparison, otherwise we will work with LCL. To identify genes associated with radiation toxicity we will analyze these cases using two different approaches: the validation by quantitative real-time PCR of Rieger's findings and whole genome expression profile using cDNA microarrays.

C3

GENE EXPRESSION PROFILING IN PATIENTS WITH ESTROGEN-RECEPTOR POSITIVE BREAST CANCER TREATED WITH TAMOXIFEN

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Although hormonal therapy with the anti-estrogen tamoxifen is effective for the adjuvant therapy of estrogen receptor (ER)-positive breast cancer, some patients fail to respond to the treatment and become resistant. To investigate the role of ER modulated genes and their association in clinical outcome, we analyzed the gene expression profile of a group of 113 breast cancer cases consisting in 45 ER-positive breast tumors from patients who relapsed during hormone treatment and 68 ER-positive breast tumors from patients who resulted disease-free over 70 months of follow-up. Complete clinical data were available for each sample. The two groups were balanced for age, type of surgery and tumor histotype, whereas relapsed cases were more frequently associated with a tumor diameter >2 cm (65%) and >4 metastatic lymph nodes (39%) with respect to disease-free patients. Gene expression profiles were investigated using the cDNA IFOM-chips containing a total of 15424 genes. Preliminary data indicated that about a hundred genes were significantly correlated with ER expression and tamoxifen response. Analysis of the identified genes is at present ongoing and the results will be presented and discussed.

C4

EPIGENETIC EVENTS INDUCED BY PML/RARα ON p21 PROMOTER

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Acute Promyelocytic Leukemias (APL) are characterized by the 15;17 chromosome translocation (Faretta et al., 2001) generating a chimeric gene (PML/RARα) that involves PML (chromosome 15) and RARα, the Retinoic Acid Receptor α (chromosome 17) (Melnick and Licht, 1999). In absence of Retinoic Acid (RA), wild receptors (RARs) bind to specific DNA sequences (called RA Responsive Elements or RARE) and repress transcription by recruiting a HDAC bound co-repressor complex, SMRT/NCoR in a 1:1 molar ratio.
This effect can be reversed by pharmacological doses of RA, which release the HDAC complex from the PML/RARα target promoters, thus promoting transcriptional de-repression and induction, both in vivo and in vitro, of terminal differentiation of the APL blasts. (Minucci et al., 2000)
Among RARE containing promoters, p21 shows some interesting features concerning transcriptional regulation and chromatin dynamic (histones modification and DNA methylation). We have demonstrated by Chromatin Immunoprecipitation and Real Time PCR that there are 2 preferential sites for PML/RARα binding on p21 promoter. After its binding PML/RARα is able to induce interesting epigenetic modifications that lead to transcriptional regulation. The in vivo occupancy of p21 promoter with or without PML/RARα analyzed by genomic footprinting (DNaseI digestion) revealed a very complex pattern confirmed also by nucleosome positioning in the TATA box region.

C5

MYC-T58 PHOSPHORYLATION BY GSK3 IS REGULATED BY THE APC-AXIN TUMOR SUPPRESSOR COMPLEX

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The c-Myc transcription factor is an essential regulator of cell proliferation and apoptosis. Myc is an unstable protein and its levels are tightly regulated within the cell. Recent work in the field has started to unravel the network that controls Myc stability. Myc is degraded via the ubiquitin-proteasome pathway, in a process that is largely dependent upon the phosphorylation of Thr 58 (T58) by glycogen synthase kinase-3 (GSK-3). T58 is a mutational hotspot in Burkitt's lymphomas, and Myc mutants harboring T58 alterations show prolonged half-life and increased oncogenic potential. GSK-3 also phosphorylates beta-catenin, the intracellular effector of the Wnt pathway. Phosphorylation targets beta-catenin to ubiquitination and degradation, and occurs within a multi-protein complex that comprises, among others, the tumor suppressor proteins APC and Axin. Our findings suggest that the phosphorylation of Myc by GSK-3 also relies upon the APC/AXIN complex. First, Myc and APC can be co-precipitated from transfected cells. Second, Myc T58 phosphorylation by GSK-3 is impaired in colon cancer or hepatocarcinoma cell lines that contain mutations in APC or AXIN, respectively. T58 phosphorylation is also altered when the APC protein is experimentally eliminated from a cell. Our progress in characterizing this physical and functional interaction between Myc and APC will be presented.

C6

IDENTIFICATION OF CANCER ASSOCIATED GENE ISOFORMS THROUGH BIOINFORMATICS

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A number of genes that exhibit cancer-associated isoforms has been characterized. We are investigating cancer-associated gene isoforms as novel biomarkers of tumor prognosis and outcome. It consists in a bioinformatics part and in an experimental part. We focus on four gene collections: apoptosis-related, transcription factors, cancer genes and lung cancer genes. These gene lists are derived from the intersection of different sources like literature and experimental data. We are interested in the identification of new isoforms using a dedicated software (ASPIC) and in the construction of a comprehensive catalogue of known transcript variants collected from 3 different transcriptomes: ENSEMBL, REFSEQ and ACEVIEW, with the aim of identifying a cluster of transcripts for each genes. This will help the analysis of the UTR region of each cluster to identify signalling sequence for isoform specificity, and to detect skipped exons that will be used for the experimental validation step. Aligning a collection of EST belonging to three different tissues (lung, skin and brain) to the collection of clustered transcript, we will be able to predict a possible variation of a given gene isoform between normal and cancer. The identified splice variant will then be validated in clinical tumor samples, using two different approaches, Real-time quantitative PCR analysis and Tissue microarray analysis (TMA), to understand wheter there might be changes in splicing between the form found in normal tissues versus the tumors.

C7

TOWARDS A BIOMOLECULAR CLASSIFICATION OF HUMAN THYROID CANCER

Cristina Ferrario, L. De Cecco, P. Bressan, M. Gariboldi, M. Frattini, S. Lagonigro, P. Mondellini, J.F. Reid, I. Bongarzone, A. Greco, M.A. Pierotti
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Papillary (PTC) and follicular (FTC) carcinomas represent the most common forms of differentiated thyroid cancer accounting for more than 80% of all thyroid malignancies. Specific lesions have been associated to the different histotypes, however the molecular events leading to thyroid carcinogenesis are not completely understood and the identification of new accurate markers is important for differential diagnosis and treatment decision.
To better understand the molecular mechanisms of thyroid carcinogenesis, we investigated the gene expression pattern of several thyroid specimens using cDNA microarrays prepared in our laboratory and spotted with 5K annotated clones. Our collection consists of 8 follicular adenomas, 31 PTCs, 10 FTCs and 7 normal thyroid tissues
A preliminary analysis of the expression data shows that it is possible to group the different histotypes on the base of their gene expression profile. Moreover, using supervised analyses, we identified specific genes differentially expressed among the histotypes. Further analyses are in progress to clarify the biological significance of these genes in thyroid carcinogenesis.
We also compared our results to public microarray datasets.

C8

THE ROLE OF NEURAL-CELL ADHESION MOLECULE (NCAM) IN FGFR SIGNALING DURING TUMOR PROGRESSION.

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Cell adhesion molecules (CAMs), including integrins, cadherins, immunogobulin-like CAMs and selectins, are crucial for many cellular functions, such as adhesion, migration or proliferation. Changes in the expression or function of CAMs are frequently observed during tumour development, often leading to poor prognosis. We are characterizing the molecular mechanisms underlying the role of CAMs in tumour progression. In particular, since our group have recently described a functional cross-talk between neural-cell adhesion molecule (NCAM), FGFRs and N-cadherin, we wanted to investigate the biological relevance of this complex during tumour progression. Our results implicate NCAM as a key modulator of FGFR signalling: the direct interaction between NCAM and FGFR is required for NCAM-mediated MAPK phosphorylation and cell-matrix adhesion in tumour cells. Moreover, a soluble form of NCAM, but not NCAM lacking the putative FGFR-binding domain, is able to induce FGFR-mediated signalling in cells that do not express NCAM. We are also investigating whether NCAM affects the response of FGFR to its canonical ligand, the FGFs. We hypothesize that the presence of NCAM influences the duration of FGF-dependent signalling or the binding of FGF to its receptor. Finally, we are testing whether NCAM and FGF-mediated pathways have common effector molecules or whether they activate different FGFR-mediated cascades. The preliminary results of these studies will be discussed.

C9

ACETYLATION AND CANCER: DECIPHERING THE ACETYLOME

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The transformation from normal to cancer cells involves multiple etiologic pathways and is a genetic, epigenetic and cytogenetic process.
One of the epigenetic route to carcinogenesis involves the aberrant pattern of histone modifications (acetylation, methylation, phosphorylation and ubiquination). The best-studied histone modification is the acetylation of lysine residues (Ac-K) of histones H3 and H4. This is a dynamic, reversible and highly regulated chemical modification mainly controlled by the opposing activities of 2 groups of enzymes: HATs and HDACs. Disruption of HAT or HDAC activity has been associated with the development of cancer.
However, several studies have demonstrated that also non-histone proteins (i.e. transcription factors, nuclear import proteins, signal transduction molecules and DNA repair enzymes etc.) can be substrate for HATs and HDACs, involving reversible acetylation in a broad range of biological functions.
Aim of our work is the developing of a method for a systematic analysis of Ac-K proteins. An antibody against Ac-K has been used to affinity purify all the acetylated proteins in the cell. Mass spectrometry analysis of the purified proteins revealed newly putative acetylated proteins that are under validation.
Furthermore, we would study the global dynamics of acetylation-based signaling events using mass spectrometry methods that convert temporal changes to differences in peptide isotopic abundance (SILAC, stable isotope labeling with aminoacids in cell culture).

C10

FUNCTIONAL CHARACTERIZATION OF ALLELIC VARIANTS OF PAS1 GENES FOR THEIR CANCER MODIFIER ACTIVITY

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Lung cancer is the leading cause of cancer mortality in the Western world. Its leading cause is cigarette smoking but family studies indicate a complex inheritance of predisposition. The present project is aimed to cloning candidate genes for the Pulmonary adenoma susceptibility 1 (*Pas1*) locus, that represents the major quantitative trait locus (QTL) affecting inherited predisposition to lung tumorigenesis in mouse. I have carried out a linkage disequilibrium (LD) study in 31 mouse strains of known susceptibility and resistance to lung tumorigenesis in order to narrow the mapping region of *Pas1*. This approach allowed us to restrict the chromosomal segment containing the Pas1 locus from 1 Mb to 517 Kb. I have characterized the four candidates genes (*Lrmp*, *Las1*, *Ghiso* and *Lmna-rs1*), that map in this region, looking for the presence of nucleotide polymorphisms affecting amino acid synthesis in the susceptible (A/J) and resistant and (B6) strains. I continued the analysis of the four candidates genes by subcloning them in mammalian expression vectors and transfecting into human lung cancer cell lines to study the possible cancer modifier activity in vitro by clonogenic assay experiment, cells count experiment and by studying the gene level expression. In vitro colony formation assay revealed allele-specific modulations of colony numbers by *Lmna-rs1* and *Las1*, but not by *Lrmp* and *Ghiso*. This effect provides evidence that alleles of candidate cancer modifier genes can modulate growth of human cancer cells.

C11

WHOLE-GENOME SCANNING BY DNA SUBTRACTION FOR THE RAPID MAPPING OF MOUSE CANCER MODIFIER LOCI

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We aimed to identify loci linked with skin and lung tumor susceptibility/resistance in a SWR/J x (SWR/J x Car-R) backcross population containing an outbred line (Car-R). To provide whole genome dense coverage by genetic markers, we designed a DNA subtraction approach on DNA pools prepared from highly tumor susceptible and resistance mice, respectively. Then, we performed restriction digestion and genome fractionation, followed by amplification of a specific fractionated subset of the genome to allow reduction in genome complexity. Subsequent subtractive hybridization was carried out on the fractionated genome and the resulting DNA fragments were subcloned, sequenced and mapped in silico. Computer simulation analysis indicated that our approach may be equivalent to the analysis of about 2,000 SNPs. We observed a cluster of subtracted clones mapping on Chromosome 1, between 75 and 91 Mb. Genotyping of individual mice of the backcross population confirmed the success of the genomic DNA subtraction and indicated the mapping of a new skin and lung tumor modifier locus on Chromosome 1. In conclusion, the proposed method may represent a convenient alternative to the dense SNP mapping of experimental populations.

C12

TRANSCRIPTIONAL DE-REGULATION IN LEUKEMIA: AML1/ETO IS PLAYING TWICE

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Acute myelogenous leukemias (AMLs) are characterized by chromosomal rearrangements that generate fusion proteins with aberrant transcriptional regulatory activity. The most frequent translocation in AML is the t(8;21), which gives origin to the AML1/ETO fusion protein. AML1 is a master gene of hemopoiesis able to bind DNA while ETO has the capacity to recruit HDAC and N-Cor complexes. Therefore the aberrant fusion protein is thought to function as a transcriptional repressor.
We previously analyzed the gene expression profile of U937 cells expressing AML1/ETO, and identified a set of putative target genes. Two surprising observations emerged: i) several genes that are part of the same functional pathways are co-regulated; ii) in contrast to what is expected from a transcriptional repressor, the number of genes that are induced by AML1/ETO is equivalent to the number of genes that are repressed. We then performed a genome-wide chromatin immunoprecipitation analysis to look for the direct target genes. By crossing expression data with the binding profile of AML1/ETO to human promoters, we sorted out a list of 225 genes whose expression is strongly deregulated by direct binding of fusion protein to regulatory regions. These include myeloid differentiation genes, apoptosis and cell cycle regulators. Surprisingly, 30% of these genes are upregulated, suggesting that AML1/ETO can also act as a transcriptional activator, possibly by interacting with a different protein complex. We are currently performing proteomics analysis and studying epigenetic modifications.

C13

GENOME-WIDE CHORMATIN-BASED ISOLATION OF ACTIVE *cis*-REGULATORY DNA ELEMENTS FROM HUMAN CELLS

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Development, cell proliferation, and cellular responses to environmental signals are all orchestrated by coordinated patterns of gene expression. DNA-encoded genetic information is packaged into a chromatin polymer, which must be opened to allow increased accessibility for gene regulatory factors, or compacted to restrict access of the transcriptional machinery to target genes. In general, these "opened" and "closed" chromatin states correspond to gene activation versus gene repression, respectively, and are often modulated by the direct binding of a large spectrum of transcription factors to DNA regulatory elements.
We describe a rapid, simple, generally applicable method to identify regulatory DNA elements in human cells genome-wide: mild treatment of cells with a restriction enzyme followed by the isolation of the DNA ends generated by the nuclease in chromatin. Ninety percent of the fragments isolated this way from human tissue culture cells and from CD34+ hematopoietic stem cells correspond to *bona fide* regulatory DNA. Only 40% of such regulatory DNA elements correspond to gene promoters.

C14

CHROMATIN MEDIATED REGULATION OF V(D)J RECOMBINATION

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During lymphocyte development a vast array of antigen specific receptor expressed by B and T cell receptor molecules is generated by a mechanism known as V(D)J recombination. This DNA site specific reaction assembles antigen receptor genes into a fuctional coding unit by the imprecise joining of multiple gene segments. Recombination activity is targeted by conserved recombination signal sequences (RSS) which are recognized by two lymphoid-specific proteins, named RAG1 and RAG2. A tight regulation of the processes which control RAGs expression and their accessibility to the genome is required: inefficient recombination results in immunodeficiency, while inaccurate joining can results in oncogenic chromosome rearrangements. Using reconstituted chromatin and purified Rags proteins I developed an in vitro approach to investigate the role of chromatin modifying activities in modulating RAGs accessibility to RSS. In my preliminary results I saw that RAG proteins themselves play a role as co-factors in regulating accessibility to RSS in chromatin. Mutation or deletion of the amino-terminal region of RAG1 or the carboxy-terminal region of RAG2 reduce the accessibility to RSS on chromatinized templates suggesting that these regions are involved in interacting with some remodeling activities or with chromatin structures.

C15

ROLE OF THE PIRIN PROTEIN IN PROGRAMMED CELL DEATH AND TUMORIGENESIS

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Pirin is a novel highly conserved protein first isolated by a yeast two-hybrid screen as an interactor of NFI/CTF1 transcription factor. Orthologs of the human gene have been reported to be induced under stress conditions: the cyanobacterial gene is up-regulated in high salinity, the tomato and the A. Thaliana homologues during camptothecin-induced programmed cell death. In accordance with a possible role of the protein in pro-apoptotic processes, we have found the pirin gene to be target of repression by oncogenic transcription factors involved in acute myeloid leukemogenesis and confirmed this data in a murine APL model. Moreover, we established by different approaches (both at mRNA and protein level) that pirin expression is specific to melanocytes and that it is repressed in melanoma cell lines but not in the metastatic counterparts. We confirmed by immuno-fluorescence the nuclear localization of pirin.
An interaction has been described with Bcl3, a member of the NF-kB pathway, in which pirin stabilizes the complex formed by Bcl3 with p50 and DNA.
This project is aimed at the characterization of the role of human Pirin in tumorigenesis in mammalian models. In the next future we are willing to: i) overexpress and delete Pirin to characterize its role in the response to pro-apoptotic stimuli and chemotherapeutic agents; ii) identify Pirin interactors by proteomic approaches using the polyclonal antibody that we have generated and that has been proven functional in the main immuno-based assays.

C16

TRANSCRIPTIONAL REGULATION OF C-MYC TARGET GENES

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The *c-myc* proto-oncogene encodes a transcription factor that can both activate and repress specific target genes. One of the essential process in regulating transcriptional activation are the modifications of nucleosomal histones in which the best characterized are lysine acetylation and methylation. These modifications are under control of several histone acetyltransferase (HATs) and histone methyltransferase (HMTs) which are part of an intricate game to organize the chromatin structure and to regulate DNA-associated events. By using quantitative chromatin immunoprecipitation (q-ChIP) we analysed the correlation among histone marks and the Myc binding on several human promoters. The goal is to characterize the determinants of Myc binding to chromatin by analyzing histone marks before the Myc binding as opposed to those induced by Myc. Indeed, several HATs are likely to be recruited by Myc itself in order to mediate different transcriptional and/or biological events. However, their functional role and possible overlapping remains unclear, for this reason we are performing q-ChIP combined with RNA interference which will allow us to analyse the Myc-dependent recruitment of specific factors and the corresponding modifications on chromatin.

C17

DELOCALIZATION AND DESTABILIZATION OF THE ARF TUMOR SUPPRESSOR BY THE LEUKEMIA-ASSOCIATED NPM MUTANT

Paola Martinelli, Emanuela Colombo, Raffaella Zamponi, Danielle Shing, Paola Bonetti, Lucilla Luzi, Sara Volorio, Loris Bernard, Giancarlo Pruneri, Myriam Alcalay, and Pier Giuseppe Pelicci.
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One third of acute myeloid leukemias (AML) are characterized by the aberrant cytoplasmic localization of Nucleophosmin (NPM), due to mutations within its putative nucleolar localization signal. NPM mutations are mutually exclusive with major AML-associated chromosome rearrangements, and are frequently associated with normal karyotype, suggesting that they are critical during leukemogenesis. The underlying molecular mechanisms are, however, unknown. NPM is a nucleus-cytoplasmic shuttling protein that has been implicated in several cellular processes, including ribosome biogenesis, centrosome duplication, cell cycle progression and stress response. It has been recently shown that NPM is required for the stabilization and proper localization of the tumor suppressor p19Arf. We report here that the AML-associated NPM mutant localizes mainly in the cytoplasm, due to an alteration of its nucleus-cytoplasmic shuttling equilibrium, forms a direct complex with p19Arf, but is unable to protect it from degradation. As a consequence, cells or leukemic blasts expressing the NPM mutant have low levels of cytoplasmic Arf. Inactivation of p19Arf, a key regulator of the p53-dependent cellular response to oncogene expression, might therefore contribute to leukemogenesis in AMLs with mutated NPM.

C18

MOLECULAR CHARACTERIZATION OF EARLY NON SMALL CELL LUNG CANCERS (NSCLCs) AND ISOLATION OF LUNG STEM CELLS: A FIRST STEP IN THE UNDERSTANDING OF LUNG CANCEROGENESIS

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My project has the aim to identify early lung tumors from a selected population, for the genetic characterization of Non Small Cell Lung Cancer (NSCLC) followed by the isolation of lung stem cells, in order to get a complete knowledge of lung tumorigenesis.
The COSMOS screening program set up at IEO has been conceived to study 5000 heavy smokers volunteers (age older than 50) by low dose spiral CT scan. It is expected to find about 1% affected by early NSCLC, among the enrolled population. My aim is to analyze the tumoral lung tissue from these patients, removed by surgical resection.
As a first step, I check the genomic content of each case, by determining the karyotype.
For those cases in which the karyotype is normal, the following step is to verify this result by means Comparative Genomic Hybridization (CGH).
Those samples confirmed normal, are going to be studied further with an approach based on the analysis of Single Nucleotide Polymorphisms (SNPs).
To date, 27 samples have already been collected and their analysis is in progress.

C19

HUMAN ANTIBODIES FOR DISCOVERY OF NOVEL PROSTATE CARCINOMA MARKERS

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Prostate carcinoma (CaP) represents the second cause of male death due to neoplasia in industrialized countries. The discovery and validation of new target molecules and human antibodies directed against them could provide to the clinic early diagnostic-prognostic tools and potentially more suitable therapeutic reagents.
During this first year, we set up all methodologies and reagents needed. The first aim of the project was the creation of an antibody library from patients with CaP. We have selected three groups of patients at different stage of the disease to reach a wide variability of immune response against different antigen profiles, correlated with the stage of the disease. Blood samples from 10 patients were already obtained and processed. To date, the VK light chains have been inserted in the phagemid vector pMIMO prepared in our laboratory. To validate the antibody library the selection will be performed on hPSMA (human prostate specific membrane antigen), which is an already known tumor associated antigen. In order to have a suitable positive control we converted a murine monoclonal antibody in a scFv format. This antibody fragment will be also used to generate an immunotoxin for therapeutic preclinical approaches. Furthermore we analyzed patients' sera to evaluate the presence of circulating antibodies reacting against PSMA and we characterized a panel of CaP cell lines.

C20

MELANOMA STEM CELLS

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Based on recent evidences on solid tumors and hemopoietic malignancies, we hypothesize that melanoma originates from, and is maintained by, transformed stem cells. Stem/precursor cells of the melanocyte lineage have been identified *in vivo*, within the bulge area of mouse hair follicles, and can be propagated *in vitro*. Little is still known in the human system.
RaLP is a newly isolated member of the Shc family of adaptor protein. We have recently demonstrated that high levels of specific RaLP transcripts are detected in 50% of all the invasive and metastatic melanomas, suggesting that RaLP expression correlates with the acquisition of cell migration properties during tumor progression. Moreover we have proved RaLP being a physiological substrate of receptor tyrosine kinases in melanomas. Preliminary data suggest that RaLP is expressed in neural stem cells and melanoblasts and that its expression decreases along with differentiation. It is then of interest to study the involvement of RaLP in melanomagenesis, setting up cultures of normal and neoplastic melanocytic stem cells.
To this end, we have started isolating precursor cells in normal skin and melanoma biopsis by disaggregation of the tissue and subsequent growth of the cells by means of their capacity to survive in suspension as spheres. “Melanospheres” can also be obtained from freshly isolated melanoma cultures, as part of a slow-diving population. The spheres will be characterized for self-renewal capacity, differentiation potential and tumor formation in vivo.

C21

INVESTIGATION OF THE OLIGOMERIZATION PROCESS IN THE CONTEXT OF ACUTE PROMYELOCYTIC LEUKEMIA

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The hallmark of acute promyelocytic leukemia (APL) is the expression of a fusion protein between the retinoic acid receptor □(RAR□ and different partners, among which the most frequent are: promyelocytic leukemia protein (PML), promyelocytic leukemia zinc finger (PLZF) and nucleophosmin (NPM). They contain an oligomerization domain that is retained in the chimeric version and that is responsible for the formation of oligomers of the fusion protein. These assemblies lead to the aberrant gain of function of the RAR□□□□□□□□, due to the constitutive recruitment of the N-CoR/SMART/HDAC nuclear corepressor complex on its target genes, with the subsequent differentiation block of myeloid precursor. We want to evaluate if oligomerization per se is sufficient to trigger leukemia development. We are testing constructs characterized by the presence of an oligomerization domain fused to RAR protein: CC-RAR (CC: Coiled-Coil region of PML), p53-RAR (p53: tetramerization domain of p53), GCN4-RAR (GCN4: homodimerization portion of the yeast protein GCN4) to evaluate their biological effect upon bone marrow progenitors in terms of proliferative potential, differentiation and survival upon stress. Moreover we are testing the ability of transduced cells to induce APL in mice.

C22

IDENTIFICATION AND CHARACTERIZATION OF NOVEL TARGET GENES OF pRB-E2F PATHWAY

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The mutation of pRB pathway is a hallmark of human cancer. The core members of this pathway include the pRB tumor suppressor protein which through binding to a number of cellular proteins, most notably members of the E2F transcription factor family, regulates progression through the cell division cycle. With the aim of identifying new target genes of the pRB pathway, we have used a gene expression screening of cell lines that conditionally express a constitutively active phosphorylation site mutant of pRB (pRBΔCDK) or p16INK4A (p16).
We noticed an enrichment of a set of novel motifs in the promoter of these genes, which were used as an in-silico filter for identification of novel p16-pRB-E2F pathway target genes. I'm focusing my attention on an identified gene: Lap2α The immunohistochemical analysis of this gene revealed its overexpression in many cancer tissues compared with normal ones.

C23

ROLE OF MYC IN TRANSCRIPTIONAL RESPONSES INDUCED BY A VARIETY OF KEY SIGNALING PATHWAYS

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The Myc oncoprotein is a transcription factor that can either activate or repress gene expression. Global analysis of gene expression, including microarray-based screenings and other approaches, has failed to reveal any universal signature. Furthermore, it has been suggested that Myc is required but not sufficient for gene activation and that other signals must be involved. Myc is emerging as a permissive factor, a "transcriptional switch" which modifies the state of responsiveness of many genes to other signals. The aim of this project is to challenge this hypothesis with a particular focus on a variety of key signaling pathways, such as TGF-β, TNF-α and serum response. We will test if Myc cooperates with these pathways in regulating transcription. Using Affymetrix microarrays, we are currently collecting profiling data in $3T9^{f/f}$ fibroblasts, derived from conditional "flox-myc" mice, in which c-myc has been deleted or not with a OHT-inducible form of Cre. From preliminary data coming from the TGF-β profiling, it is clear that the pathway remains functional upon Myc deletion. With a system of tree-clustering analysis we found clusters of genes that behave differently and they are now being validated by RT-PCR. We will use bioinformatic tools to look for common regulatory modules in the promoter sequences of these clusters and then we will test these findings in living cells using ChIP assays. Finally, the genes identified as Myc-dependent will be studied in physiological models such as tumorigenesis and EMT.

C24

IDENTIFICATION OF AMPK EXPRESSION LEVELS IN MELANOMA CELLS.

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AMP-dependent kinase (AMPK) is involved in the regulation of lipid and protein cell metabolism and is regulated by changes in cellular energy state. AMPK has also been associated to replicative senescence in human fibroblasts, leading to cell cycle arrest in either a p53-dependent or independent manner. Recent studies suggest that AMPK is related to either cancer cell growth or survival involving mediators such as FAS, mTOR, LKB1, TSC2, p53, p21. So far, no information is available about the expression of AMPK and melanoma. Interestingly, the expression of activated AMPK was found upregulated in senescent melanoma cells induced to co-express oncogenic NRAS and BRAF *in vitro*. In contrast, AMPK was expressed at low levels in melanoma cells singularly expressing either NRAS or BRAF, together with wild type p53. AMPK expression was not detectable in melanoma cells co-expressing oncogenic BRAF and a mutated p53. Despite the selective activation of AMPK, neither mTOR nor phospho-p53 (Ser15) expression levels were modified, suggesting that AMPK may signal through other pathways. A wider panel of melanoma cell lines, derived from patients at different stages of the disease, is being screened for the expression of both AMPK and a panel of AMPK-interacting molecules. Activation of AMPK will be further correlated to the presence of a senescent phenotype, representing a tumor suppressive mechanism and possibly a protective process regulating tumor progression. These results will help in determining whether AMPK activity is modulated in melanoma and how it is associated to tumor behavior.

C25

IN VITRO PROPAGATION AND FUNCTIONAL CHARACTERIZATION OF BREAST TUMOR-INITIATING CELLS.

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Breast cancer-initiating cells (BC-ICs) have been identified as $CD44^{+}/CD24^{-/low}$ cells which exclusively retain the ability to repopulate breast tumors when injected into the mammary fat pad of immunocompromised mice. In addition to the repopulating ability, $CD44^{+}/CD24^{-/low}$ breast cancer cells share with stem cells the key features of self-renewal and multi-lineage differentiation. By applying a previously described method with minor modifications, we have recently developed a culture system for BC-ICs to be selectively isolated and propagated *in vitro* as non-adherent spherical clusters of relatively undifferentiated cells. We provided evidence that BC-ICs propagated *in vitro* display stem/progenitor cells properties and are highly tumorigenic. According to literature data, we also demonstrated that BC-ICs can be purified from the well-known breast carcinoma cell line MCF7, suggesting that established cell lines may represent an easy-access source of BC-ICs. To consolidate this idea, we have established and functionally characterized new long-term cell lines of BC-ICs from two different established breast carcinoma cell lines, respectively 734B and T47D. The availability of long-term cultures of BC-ICs derived from both post-surgery tumor specimens and from established cell lines may provide a complete panel of tools to gain new insights into the biology of the tumorigenic sub-population of breast cancer.

C26

EXTRA-CELLULAR HMGB1 PROMOTES BREAST CANCER PROLIFERATION AND INVASION

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HMGB1 is a nuclear protein that positively affects chromatin accessibility in multiple ways. All cells can release passively HMGB1 in the extracellular milieu when they die in an unprogrammed way: extracellular HMGB1 works, through its receptor RAGE, as an inflammatory cytokine and a signal of tissue damage. We have shown that HMGB1 can attract stem cells and promote their proliferation. Several cell types can secrete HMGB1 actively and without dying. Many tumors over-express HMGB1 and/or RAGE, and blockade of their interaction decreases tumor growth and invasiveness. We noticed that, in human breast cancer biopsies, HMGB1 is predominantly located in the cytoplasm of the tumoral cells only, while it is located in the nuclei of normal cells. A cytoplasmic localization is often associated to active secretion of HMGB1. In fact, we found HMGB1 present in the medium of several breast cancer cell lines. Our working hypothesis is that HMGB1 can act as an autocrine stimulus for the tumor itself. Both invasive MDA-MB-231 and non-invasive MCF7 cells proliferated when stimulated with extracellular HMGB1, but only MDA-MB-231 cells responded chemotactically to HMGB1. Responsiveness to HMGB1 might represent a threshold in the transition between invasive and non-invasive tumor phenotypes.

C27

COMBINATION TREATMENT OF HYPOMETHYLATING AGENTS AND BROSTALLICIN IN HUMAN PROSTATIC CANCER CELL LINES

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Brostallicin is a new DNA minor groove binder anticancer drug, whose activity depends on intracellular levels of glutathione/glutathione-S-transferase pi. Prostate cancer is characterized by the absence of the enzyme glutathione-S-transferase pi, due to the strong methylation of the promoter of its gene. More than 90% of human prostate tumors present this molecular characteristic, which is retained by the tumor cell line LNCaP. In vitro brostallicin shows a low antitumor activity on LNCaP cells. These cells were transfected with human GST-pi cDNA and two clones overexpressing GST-pi were selected. Brostallicin antitumor activity on these clones was about 5 folds higher than on LNCaP wild type cells. Combination treatments of demethylating agents and brostallicin were also performed. The best hypomethylating drug, which has shown to sensibilize LNCaP cells to brostallicin, has been the cytidine analog, zebularine. Zebularine has a lower toxicity on LNCaP cells compared to 5-aza-deoxycytidine, which was the first and the strongest hypomethylating agent sinthetyzed. Brostallicin showed a higher antitumor activity on LNCaP cells pretreated with zebularine compared to its activity on controls (MTT assay). GST total activity measurement was performed with cytosolic proteins from LNCaP cells treated with zebularine and using 1-chloro-2,4-dinitrobenzene as substrate. GST total activity was detectable in cells treated with zebularine, but not in the controls. These results indicate that re-expression of the hypermethylated GSTP1 gene increases the activity of brostallicin on LNCaP prostate cancer cells and this could have important clinical relevance for prostate cancer .

C28

INHIBITION OF RET KINASE BY SU5416

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RET is a receptor tyrosine kinase mutated: its unregulated activity causes papillary and medullary thyroid carcinomas. Therefore, RET is a good target for specific therapy of thyroid neoplasms. SU5416 is a member of the 2-indolinone family of small molecule compounds and demonstrates inhibitory properties on VEGFR, c-KIT and FLT3 tyrosine kinases. We found that SU5416 inhibits RET kinase activity *in vitro* with an IC_{50} of 170 nM and prevents RET autophosphorylation in different RET-dependent cells, such as Ba/F3 and NIH-3T3 cells transformed by RET/PTC2 oncogene and TPC-1 cells. In all these cell lines we observed a block of cell proliferation at micromolar concentrations. In TPC-1 cells, the inactivation of RET caused a decrease in ERK and JNK phosphorylation. SU5416 arrested cell cycle in G0/G1 without inducing apoptosis. Recently, it has been shown that a mutant form of RET with the substitution of methionine for valine 804 (RET-V804M) was resistant to known inhibitors of RET. We tested SU5416 on RET-V804M and we couldn't obtain any inhibition, confirming that V804 is a key residue for inhibitor binding. Molecular modelling data predicted that SU5416 forms two H-bonds with the ATP binding site of wild-type RET, whereas no H-bonds were observed in the model of RET-V804M.. In conclusion, SU5416 is a potent inhibitor of RET and may be a good lead structure for the development of specific inhibitors of RET

C29

IDENTIFICATION OF NOVEL GENES INVOLVED IN ACUTE MYELOID LEUKEMOGENESIS USING GENE EXPRESSION PROFILING.

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There is growing interest in the role of hematopoietic stem cells (HSC) in the development of hematopoietic malignancies, particularly in acute myeloid leukemias (AML). AMLs are characterized by the clonal expansion of myeloid precursors that present an increased proliferative potential associated with the incapacity to differentiate into mature blood cells. We have analyzed global gene expression profiles in selected cellular subpopulations purified from the bone marrow derived from wild-type, pre-leukemic and leukemic mice using oligonucleotide microarrays. Comparison of gene expression profiles among the cellular subpopulations within each one of the different mouse models showed that in leukemic blasts, the acquisition of differentiation markers is associated with the expression of genes involved in HSC self-renewal, which are instead gradually repressed during differentiation in WT cells. Comparison of the HSC compartment of different mouse models showed that leukemic stem cells (LSC) express specific "markers" – i.e. genes that are never expressed in the HSC compartment.
We are currently assessing the expression level of selected target genes by Q-PCR in murine subpopulations of different AML models.
Based on these results, we will select genes for functional studies in murine models.

C30

EPSIN3: AN INTERPLAY BETWEEN SIGNALING AND MEMBRANE-TRANSPORT NETWORKS IN CANCER

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Epsin3 belongs to the evolutionary conserved epsin family proteins which are able to bind components of the endocytic machinary and their binding partners. In particular, Epsin1 and Epsin2, are members of the family of adaptors that link various cell-surface receptors to the endocytic machinary (e.g. AP-2, Eps15 and Clathrin), moreover they are both ubiquitously expressed in human tissues. Epsin3 is closely related to human Epn1 and Epn2 in terms of sequence homology and they all share the same domain architecture. However, Epsin3 displays also some peculiar features. Epn3 has never been detected in any normal tissue tested, while it has been found expressed specifically in stress conditions such as in migrating keratinocytes during skin wound healing. Recently, Epsin3 transcript was found to be up regulated in aggressive human breast tumors by Affymetrix screening (our unpublished results) and *in situ* hybridization analysis detected Epsin3 signal in tumor epthelial cells. Moreover, western blot and qPCR experiments showed that several breast tumor cell lines overexpressed Epn3 in comparison to normal cells. Therefore, we have identified a model system to further investigate the role of epsin3 during breast tumor progression.
These preliminary observations, suggest that Epn3 may serve an important function in activated epithelial cells during tissue morphogenesis and in tumor dissemination.

C31

IN VIVO EPIGENETIC PHARMACOTHERAPY AGAINST LEUKEMIAS.

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The histone deacetilase inhibitor valproic acid (VPA) demonstrated to contrast leukemia progression in PML/RAR leukemic mice by inducing apoptosis via FAS and TRAIL pathways selectively on leukemic blasts. We have demonstrated that another epigenetic drug, 5 aza 2'deoxycytidine (DAC), which targets DNA methyl-transferases, significantly increases leukemic mice survival in a comparable way to VPA. It induces apoptosis of leukemic blast and expression of the same Death Receptors pathway of VPA, and provokes blast depletion in bone marrow and peripheral blood. The combination of the two drugs synergizes both *in vitro* and *in vivo* without further inducing the expression of the considered pathways.
DAC and VPA both improve the all trans retinoic acid (ATRA, a differentiating drug) antileukemic effect. Combination of DAC, VPA and ATRA is really effective, but showed lethal myelosuppression in 66% of treated leukemic mice and in 50% of healthy mice.

C32

IDENTIFICATION OF NOVEL TUMOR SUPPRESSORS GENES BY SHRNA LIBRARY SCREENINGS, IN HUMAN PRIMARY CELLS.

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Cancer is a genetic disease, in which activation of oncogenes and inactivation of tumor suppressor genes are the most recurring alterations.
I'm performing two kind of RNAi-based genetic screens to identify novel tumor suppressor genes in human cell-based models: one to identify tumor suppressors whose inactivation will substitute for p53 inactivation in cellular transformation (p53 screening) and another one to discover shRNAs able to bypass RASV12 induced senescence. Human primary cells can be transformed by five genetic alterations: the inactivation of p53 and pRB or $p16^{INK4A}$; the expression of: hTERT, the SV40 small t antigen, and $H\text{-}RAS^{V12}$.
Human primary fibroblasts had been generated in the Lab, to contain all these alterations, except for p53 inactivation. These cells were infected with a shRNA library to perform a p53 screen, scoring for clones able to grow in anchorage independent fashion. Five transforming shRNAs were identified, which I am currently testing in validation experiments. I'm also generating a new cell-system to perform another p53 screening in which the identification of the shRNAs, and the validation process should be improved.
I'm performing the second kind of screen in cells expressing hTERT, and $H\text{-}RAS^{V12}$. I isolated about 40 clones able to bypass $H\text{-}RAS^{V12}$ – induced senescence, from which I'm recovering the shRNAs for their identification and validation.
The validated genes from both these screens, will then be prioritized for further investigation, according to the existing knowledge, and to their potential involvement in tumorigenesis.

C33

MOLECULAR CLONING AND CHARACTERIZATION OF NEWLY IDENTIFIED BREAKPOINTS IN AML REARRANGEMENTS

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The aim of my project is the cloning of rare rearrangements in Acute Myeloid Leukemias (AMLs) in order to identify new genes involved in leukemogenesis and cancerogenesis. We have collected 14 AML cases: in 8 case we are still in the process of fine mapping the chromosome breakpoints by FISH experiments; in 4 cases we found the gene involved in 1 of the breaks and we are performing PCR experiments in order to find the possible partner gene(s); in the last 2 cases we have already characterized the involved fusion genes and now we will proceed to their functional characterization by means of both *in vitro* and *in vivo* studies. About theese two cases, one carries a t(3;12) translocation generating a fusion between the *ETV6* gene on chromosome 12 and the *EVI1* gene on 3. This fusion gene has already been reported in literature, however our fusion transcript seems to give raise to a fusion protein with peculiar characteristics, which have never been described. The second case harbors a t(X;6) and we have determined that it involves the *c-Myb* gene on chromosome 6 along with the *GATA1* gene on X: they are important for proliferation and for differentiation of hematopietic cells respectively.

C34

THE FUNCTIONAL ROLE OF ACTIVATION LOOP TYROSINES RESIDUES ON THE AUTOREGULATION AND ONCOGENIC SIGNALLING OF NPM-ALK.

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Anaplastic Large Cell Lymphoma (ALCL) is characterized by the expression of the oncogenic fusion protein NPM-ALK (N/A). Since the oncogenicity of N/A is dependent on its kinase activity, N/A is a valid target for anticancer therapy. We are interested in determining the molecular mechanism of autoregulation of the ALK kinase. ALK belongs to the IRK family of kinases, possessing a 'YxxxYY' motif in the activation loop (A-loop). The sequential autophosphorylation of these tyrosines (Y) leads to a conformational change in the A-loop and activation of the kinase. Using purified ALK kinase domain and peptides mimicking the A-loop sequence of ALK, we demonstrated that the 1^{st}Y is phosphorylated preferentially, with respect to the 2^{nd} and 3^{rd}Ys. To determine if a similar situation is observed in full length N/A, we generated a series of N/A mutants in which the A-loop Y were replaced with phenylalanine (F). We observed that the YxxxFF mutant possesses kinase activity and transforms cells both in vitro and in vivo similar to wild type N/A. In contrast, the FxxxYF, FxxxFY and FxxxFF mutants displayed minimal kinase activity and impaired transforming ability. Mutation of only a single Y in the A-loop completely inactivated kinase activity in manner comparable to kinase dead N/A ($K^{210}R$). These results indicate that phosphorylation of the 1^{st}Y alone allows the A-loop to adopt a fully active conformation and that the 2^{nd}/3^{rd}Ys may play an inhibitory role in the phosphorylation of the 1^{st}Y.

C35

IDENTIFICATION OF GENES ASSOCIATED WITH MELANOMA PROGRESSION

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Cutaneous melanoma is an aggressive disease generally resistant to the available therapeutic treatments. The analysis of the genes aberrantly expressed during melanoma progression will lead to the identification of novel molecular markers representing potential therapeutic targets. We analyzed gene expression profiles in a model system of melanoma progression. Among the genes up-regulated in metastatic samples, CCN3 was identified as a possible metastasis associated gene, as its altered expression has been observed in different tumor types. The evaluation of CCN3 expression levels by RT-PCR as well as by Western blot analysis in a panel of 39 melanoma cell lines and of 12 metastatic melanoma specimens indicated that CCN3 is down regulated during progression from primary melanoma to cutaneous metastasis. However, an association between CCN3 expression and disease was observed, with tumors from patients that did not show clinical progression not expressing CCN3. Nuclear localization of the CCN3 truncated form and its secretion by melanoma cells was also determined. Forced expression of CCN3 in melanoma significantly reduced cell proliferation in vitro and growth in anchorage-independent conditions. CCN3-transfected cells showed an increased expression of Fibulin 1C which may be the basis for possible increased migratory and adhesive abilities.

C36

L1: AN ADHESION MOLECULE WITH NOVEL FUNCTIONS IN TUMOR PROGRESSION

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Some of our preliminary observations and a report in the literature prompted us to investigate the expression of the immunoglobulin-like adhesion molecule L1 during ovarian cancer progression. Intriguingly, while L1 is found in normal ovarian epithelium as well as in benign lesions (not shown), in most of the carcinoma samples the expression of L1 is lost. In one third of late-stage tumors, however, the expression of L1 is maintained and is up-regulated at the invasive front. Thus, L1 might play a role in ovarian cancer progression, a hypothesis that we will verify in an appropriate animal model, the MISIIR-Tag transgenic mouse. We are also assessing the biochemical and functional properties of L1 in cell lines established from normal or neoplastic ovarian epithelium. In particular, we have stably downregulated the expression of L1 in ovarian cancer cells by means of the RNA interference technology, while the stable expression of L1 in L1-negative cells was induced by adenoviral gene delivery. The emerging picture is intriguing, with L1 promoting cell motility, invasiveness, and cell-matrix adhesion. Moreover, L1 modulates the response of ovarian cancer cells to FGF, implying a cross-talk with FGFR signalling machinery. These data support the hypothesis that certain Ig-CAMs are implicated in ovarian cancer progression, and will guide our future studies aimed at dissecting these pathogenetic mechanisms at the molecular level, both *in vitro* and in suitable animal models.

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