IRE
ISTITUTO NAZIONALE TUMORI
REGINA ELENA

Università di Torino

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

MECHANISMS OF SIGNAL TRANSDUCTION IN CELL ADHESION AND DIFFERENTIATION

Certosa di Pontignano, 28 e 29 Marzo 2008

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

MECHANISMS OF SIGNAL TRANSDUCTION IN CELL ADHESION AND DIFFERENTIATION

Certosa di Pontignano, 28 e 29 Marzo 2008

Organized by:

Rita Falcioni – Regina Elena Cancer Institute

Emilio Hirsch – University of Turin

We are particularly grateful to:

Società Italiana Chimici S.r.l.

We thank for his help

Roberto Bernardi – Regina Elena Cancer Institute

Spring 2008 ABCD Meeting: "Mechanisms of Signal Transduction in Cell Adhesion and Differentiation" Certosa di Pontignano, 28-29 March 2008

Friday 28.3

Registration up to 12:30

12:30-13.45 **Lunch**

13:45–14:00 – Introduction: Rita Falcioni and Emilio Hirsch

14:00-15:40 SIGNALING IN CELL ADHESION

Chairperson: Mauro Torti

14:00-14:20
A novel and selective Src kinase-independent pathway for phospholipase Cgamma2 activation downstream integrin alpha2beta1 involving Rac1 GTPase.
Gianni F. Guidetti, Bruno Bernardi, Cesare Balduini, Mauro Torti
Department of Biochemistry, University of Pavia, via Bassi 21, 27100 Pavia

14:20-14:40
p27Kip1 contributes to the control of adhesion-dependent signal transduction via modulation of vesicular trafficking
Linda Fabris, Barbara Belletti, Ilenia Pellizzari and Gustavo Baldassarre.
Experimental Oncology 2 Centro di Riferimento Oncologico, Aviano.

14:40-15:00
p140Cap controls early EGFR signalling and cell-cell contact adhesion
Laura Damiano, Paola Di Stefano, Matteo Barba, *Fabrizio Mainiero, Emilia Turco, Paola Defilippi
Molecular Biotechnology Centre and Dept. of Genetics, Biology and Biochemistry, University of Torino; *Dept. of Experimental Medicine and Pathology, University "La Sapienza", Roma

15:00-15:20
Cell adhesion on Collagen 1 promotes c-Src phosphorylation of VEGFR-3
Pennacchini S., Salameh A., Galvagni F. and Oliviero S.
Università di Siena

15:20-15:40
Role of extracellular matrix and its receptors in sodium channel clustering at nodes of Ranvier
C. Colombelli, D. Zambroni, S. Occhi, L. Wrabetz, M.L. Feltri
San Raffaele Scientific Institute, DIBIT, Milano, Italy

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

16:20-18:00 SIGNALING IN CELL MIGRATION AND INVASION

Chairperson: Ivan de Curtis

16:20-16:40

β-Arrestin links endothelin A receptor to β-catenin signaling to induce ovarian cancer cell invasion

Laura Rosanò, Roberta Cianfrocca, Stefano Masi, Francesca Spinella, Valeriana Di Castro, Annamaria Biroccio, Erica Salvati, Maria Rita Nicotra, Pier Giorgio Natali and Anna Bagnato

Laboratory of Molecular Pathology and Ultrastructure, Regina Elena Cancer Institute Via delle Messi D'Oro 156, 00158 Rome, Italy;

16.40-17:00

p130Cas adaptor is critical for oncogenic ErbB2 transformation

Cabodi S., Tinnirello A., Bisarò B., Di Stefano P., Turco E. and Defilippi P

MBC, Dipartimento di Genetica, biologia e biochimica, Università di Torino, Via Santena 5 bis, Torino

17:00-17:20

Study of the role of GIT1 complex in cytoskeletal remodelling adhesion and membrane traffic during cell motility

Gavina M., Za L., Molteni R., Pardi R., de Curtis I.

Cell adhesion Unit,San Raffaele Scientific Institute, Milan , Italy; Leucocyte biology Unit, San raffaele University School of Medicine, Milano Italy

17:20-17:40

Regulation of D6 chemokine scavenging activity by ligand and Rab11-dependent surface upregulation

Raffaella Bonecchi, Elena M. Borroni, Achille Anselmo, Andrea Doni, Benedetta Savino, Massimiliano Mirolo, Monica Fabbri, Venkatakrishna R. Jala, Bodduluri Haribabu, Alberto Mantovani and Massimo Locati.

Istituto Clinico Humanitas IRCCS, Rozzano, Italy; Istituto di Patologia Generale, University of Milan, Milan, Italy; Unit of Leukocyte Biology, DIBIT-Scientific Institute San Raffaele, Milan, Italy.; James Graham Brown Cancer Center, University of Louisville Health Sciences, Louisville, USA.

17:40-18:00

The Prostate Specific Membrane Antigen (PSMA) activates the RAC-MAPK pathway in prostate cancer cells and entertains functional relationships with beta1 integrin.

Silvia Grasso, Alessandra Porzia, Giulio Fracasso, Martin Heine, Hassan Naim, Fabrizio Mainiero, Marco Colombatti, Dunia Ramarli

Dept. of Pathology, University of Verona, Italy, Clinical Immunology, G.B. Rossi Hospital, Verona, Italy, Dept. of Experimental Medicine and Pathology, Institute Pasteur-Fondazione Cenci Bolognetti, University of Rome "La Sapienza", Italy, Dept of Physiological Chemistry-University of Veterinary Medicine-Hannover, Germany

18:00-19:00 PLENARY PRESENTATION

Chairperson: Guido Tarone

Pier Paolo Di Fiore
IFOM Fondazione Istituto FIRC di Oncologia Molecolare

"...really, how many things does endocytosis do?"

20:00 **Social dinner**

Saturday 29.3

7:30 **Breakfast**

8:30-10:10 INTEGRATING SIGNALS IN THE NUCLEUS

Chairperson: Giulia Piaggio

8:30-8:50
Unrestricted NF-Y activity leads to p53-dependent apoptosis directly regulating E2F-1 expression.
Paola Fuschi, Aymone Gurtner, Fabio Martelli, Matthias Dobbelstein, Ada Sacchi and Giulia Piaggio
Experimental Oncology Department, Istituto Regina Elena, Via delle Messi D'Oro 156, 00158 Rome, Italy.

8:50-9:10
The AP2-alpha and AP2-gamma transcription factors regulate tumor formation and progression via a specific genetic program
Francesca Orso, Elisa Penna, Davide Corà, Michele Caselle, Piero Sismondi, Michele De Bortoli and Daniela Taverna
MBC, Dept. Onc. Sci., University of Torino, Via Nizza 52, 10126 Torino, Italy. Dept. Theoretical Physics, University of Torino and INFN, Via P. Giuria 1, 10125 Torino, Italy. Center for Complex Systems in Molecular Biology and Medicine, University of Torino, Via Accademia Albertina, 13, 10123 Torino, Italy. 4Dept. Onc. Sci., University of Torino and IRCC, Str Prov 142 - Km 3.95, 10060 Candiolo (Turin), Italy.

9:10-9:30
STAT5 and PPARγ coopreate in mediating circulating angiogenic cell proliferation
Patrizia Dentelli, Gabriele Togliatto, Antonella Trombetta, Annarita Zeoli, Arturo Rosso, Barbara Uberti, Luigi Pegoraro and Maria Felice Brizzi.
Department of Internal Medicine, University of Torino, corso Dogliotti 14, 10126, Torino Italy

9:30-9:50
Transcriptional regulation of alpha6beta4, an integrin that drives tumor progression

Giulia Bon, Selene E. Di Carlo, Valentina Folgiero, Gabriella D'Orazi, Ada Sacchi, Giulia Fontemaggi, Giovanni Blandino, Rita Falcioni.
Regina Elena Cancer Institute, Molecular Oncogenesis Laboratory, Via delle Messi D'Oro, 156 – 00158 Rome, Italy.

9:50-10:10
STAT3 and Her2 cooperate in mammary tumorigenesis
Sara Pensa, I. Barbieri, T. Pannellini, V. Fagiano, P. Provero, P. Musiani, V. Poli
Molecular Biotechnology Center, Univ. Turin (Turin); Ce.S.I., Anatomia Patologica, Univ. G. D'Annunzio (Chieti); Dept of Theoretical Physics, University of Turin and INFN, Turin

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

10:30-12:10 INTEGRATING SIGNALS PROMOTING CELL PROLIFERATION

Chairperson: Annarosa Arcangeli

10:30-10:50
Overcoming chemotherapy resistance in childhood Acute Lymphoblastic Leukemia by targeting ion channels
Pillozzi Serena, Masselli Marika, Crociani Olivia, De Lorenzo Emanuele, Veltroni Marinella, Amedeo Amedei, Gaipa Giuseppe, and Arcangeli Annarosa .
Department of Experimental Pathology and Oncology, University of Firenze; Children's Hospital A. Meyer, Firenze, Italy; Department of Internal Medicine, University of Florence, Florence, Italy; Centro Ricerca M. Tettamanti, Università di Milano-Bicocca, Ospedale San Gerardo, Monza, Italy.

10:50-11:10
Preliminary investigation on the conditional deletion of CSN5/JAB1 in hepatocytes
Giugliano R , Panattoni M and Pardi R.
Leukocyte Biology Unit, San Raffaele University School of Medicine, Milan, Italy

11:10-11:30
Phosphoinositide 3-Kinase p110beta activity: Key Role in Metabolism and Cancer but not Development.
Elisa Ciraolo, Claudia Curcio, Romina Marone, Stefano Marengo, Manuela Iezzi, Carlotta Costa, Ornella Azzolino, Cristiano Gonella, Cristina Rubinetto, Haiyan Wu, Walter Dastrù, Erica L. Martin, Lorenzo Silengo , Fiorella Altruda , Emilia Turco, Letizia Lanzetti, Piero Musiani, Thomas Rückle, Christian Rommel, Jonathan M. Backer, Guido Forni, Matthias P. Wymann & Emilio Hirsch .
Molecular Biotechnology Center. Department of Genetics, Biology and Biochemistry. University of Torino. Via Nizza 52. 10126 Torino. Italy.

11:30-11:50
Clathrin-mediated internalisation is essential for sustained EGFR signalling but dispensable for degradation

Sara Sigismund, Elisabetta Argenzio, Gilda Nappo, Daniela Tosoni, Elena Cavallaro, Simona Polo, Pier Paolo di Fiore
IFOM, the FIRC Institute for Molecular Oncology, Via Adamello 16, 20139, Milan, Italy, European Institute of Oncology, Via Ripamonti 435, 20141 Milan, Italy, Dipartimento di Medicina, Chirurgia ed Odontoiatria, Università degli Studi di Milano, Via di Rudinì 8, 20122 Milan, Italy.

11:50-12:10
Diacylglycerol kinase alpha is required for proliferation and invasion induced by growth factors and chemokines
Paolo E. Porporato, Elena Rainero, Federica Chianale, Gabriella Ranaldo, Chiara Ambrogio, Irene Locatelli, Miriam Gaggianesi, Gianluca Baldanzi, Sara Traini, Andrea Graziani
Department of Medical Sciences, University of Piemonte Orientale "A. Avogadro", 28100 Novara, Italy.

Concluding remarks: Rita Falcioni and Emilio Hirsch

12:30-14:00 Lunch

ABSTRACTS

Transcriptional regulation of alpha6beta4, an integrin that drives tumor progression

Giulia Bon, Selene E. Di Carlo, Valentina Folgiero, Gabriella D'Orazi, Ada Sacchi, Giulia Fontemaggi, Giovanni Blandino, Rita Falcioni.
Regina Elena Cancer Institute, Molecular Oncogenesis Laboratory, Via delle Messi D'Oro, 156 – 00158 Rome.

The alpha6beta4 integrin is up-regulated in different tumors of epithelial origin where it participates to signalling pathways that contribute to malignancy. Alpha6beta4 combines with and enhances the signalling function of several receptor tyrosine kinases such as ErbB-2, ErbB-3, EGFR and Met, facilitating key functions of carcinoma cells including their ability to migrate, invade, and evade apoptosis. The mechanism involves a profound alpha6beta4 effect on the activation of PI3-K/Akt pathway. This effect is counteracted by p53 that inhibiting alpha6beta4 integrin survival signaling promotes caspase-dependent cleavage of AKT/PKB.
It has been recently found that the transcription factor p63 is a key regulator of cellular adhesion and survival and has a direct role in the regulation of ITGB4 gene (the beta4 integrin subunit) transcription in normal cells, thereby suggesting that beta4 expression is regulated, at least in part, at the transcription level. In order to elucidate the mechanism responsible for beta4 gene expression and the transcriptional basis for cancer development and invasion, we found that HIPK2 (Homeodomain-Interacting Protein kinase 2) participates to the regulation of beta4 integrin transcription. Using a panel of human cancer cell lines carrying a wild type or a mutated p53 molecule we demonstrate that HIPK2 down-regulates beta4 transcription. Indeed, the depletion of HIPK2 causes a strong activation of beta4 transcription that results in a strong increases of MAPK activation, increases of anchorage independent grow and invasion. The stabilization of HIPK2 by Adryamacin or UV treat
ment down-regulates beta4 only in a wild type p53 context, but not in cell lines expressing a mutant p53 or in p53 null cells. HDAC1, that forms a co-repressor complex with HIPK2, binds and inhibits beta4 promoter activity in the presence of HIPK2, but not in the same cells upon HIPK2 interference. Indeed the histone deacetylase inhibitor TSA rescues beta4 transcriptional activity after adriamycin treatment.
In attempt to investigate the role of the other p53 family members in the regulation of beta4 transcription, we found that in the absence of HIPK2, TAp73 is the strongest activator of beta4 transcription in epithelial tumour cells. To a lesser extent also TAp63 and DNp63 are able to activate beta4 transcription while, DNp73 is a potent down regulator of beta4 transcription.
In conclusion, our data have demonstrated that HIPK2/p53 participate to the regulation of beta4 transcription in tumors and have explained for the first time that proteins involved in the apoptosis counteracting beta4 transcription inhibit the expression of a gene which function is strongly related to cell survival.

Regulation of D6 chemokine scavenging activity by ligand and Rab11-dependent surface upregulation

Raffaella Bonecchi[1,2,*], Elena M. Borroni[1,2,*], Achille Anselmo[1], Andrea Doni[1], Benedetta Savino[1,2], Massimiliano Mirolo[1], Monica Fabbri[3], Venkatakrishna R. Jala[4], Bodduluri Haribabu[4], Alberto Mantovani[1,2] and Massimo Locati[1,2].
[1]Istituto Clinico Humanitas IRCCS, Rozzano, Italy; [2]Istituto di Patologia Generale, University of Milan, Milan, Italy; [3]Unit of Leukocyte Biology, DIBIT-Scientific Institute San Raffaele, Milan, Italy.; [4]James Graham Brown Cancer Center, University of Louisville Health Sciences, Louisville, USA.

The decoy receptor D6 plays a non-redundant role in the control of inflammatory processes through scavenging of inflammatory chemokines. However it remains unclear how it is regulated. Here we show that D6 scavenging activity relies on unique trafficking properties. Under resting conditions, D6 constitutively recycled through both a rapid wortmannin (WM)-sensitive and a slower brefeldin A (BFA)-sensitive pathway maintaining low levels of surface expression that required both Rab4 and Rab11 activities. In contrast to "conventional" chemokine receptors that are downregulated by cognate ligands, chemokine engagement induced a dose-dependent brefeldin BFA-sensitive Rab11-dependent D6 redistribution to the cell membrane and a corresponding increase in chemokine degradation rate. Thus, the energy-expensive constitutive D6 cycling through Rab11 vesicles allows a rapid, ligand concentration-dependent, increase of chemokine scavenging activity by means of receptor redistribution to the plasma membrane. D6 is not regulated at a transcriptional level in a variety of cellular contexts, thus ligand-dependent optimization of its scavenger performance represents a rapid and unique mechanism allowing D6 to control inflammation.

p130Cas adaptor is critical for oncogenic ErbB2 transformation

Cabodi S., Tinnirello A., Bisarò B., Di Stefano P., Turco E. and Defilippi P.
MBC, Dipartimento di Genetica, biologia e biochimica, Università di Torino, Via Santena 5 bis, Torino

We recently provide evidences that the p130Cas adaptor, involved in integrin and growth factor signalling, co-operates with Her2-Neu in cell transformation both in humans and mice models. Here we show that in a foci formation assay in NIH3T3 fibroblasts, down-regulation of p130Cas expression by retro or lentiviral shRNA constructs was able to halve the number of foci induced by transformation with ErbB2, adding new evidences for the relevance of p130Cas in ErbB2 transformation. To further assess the involvement of the p130Cas adaptor in ErbB2 (V/E) transformation, mouse embryo fibroblasts derived from p130Cas knock-out or wild-type animals were stably transduced with ErbB2 (V/E) retroviral particles. ErbB2 (V/E) Cas -/- and +/+ over-expressing cells were first tested both in anchorage-dependent and independent growth. Within 5 days of cell culture the number of viable growing ErbB2 (V/E) Cas -/- cells in adherent conditions was strongly decreased compared to ErbB2 (V/E) Cas +/+ cells. More importantly, in soft agar assays, ErbB2 (V/E) Cas -/- cells were severely impaired in colony formation, showing a 90% reduction in the number of colonies. Re-expression of p130Cas in ErbB2 (V/E) Cas -/- cells was able to rescue the growth of colonies in soft agar assays, meaning that the capacity of ErbB2 (V/E) to induce anchorage-independent growth is strictly dependent by the presence of p130Cas. By biochemical studies, p130Cas and ErbB2 were found to co-immunoprecipitate in a molecular signalling complex both in transformed fibroblasts and in epithelial breast cancer cells. Taken together these data provide the first evidences on the critical role for p130Cas in oncogenic ErbB2 transformation.

Peptide Mimicking the Met Receptor Multifunctional Docking Site Impairs in vitro HGF-Dependent Responses and in vivo Growth

Cantelmo A.R. [1], Morini M.[2], Pietronave S.[3] , Cammarota R. [1] , Prat M.[3], Albini A. [1]
[1]Oncological Research Area, IRCCS Multimedica, Milano [2] Tumor Progression Unit, National Cancer Research Institute, Genova [3]Dept of Medical Sciences, University of Piemonte Orientale, Novara

Tyrosine kinase receptors are responsible for a wide variety of cellular responses, essential for physiological development including mitogenesis, morphogenesis and differentiation, and also

implicated in human diseases including cancer, metabolic disorders, and developmental defects. Interaction of the hepatocyte growth factor (HGF) with its receptor, the Met tyrosine kinase, results in growth, invasion, and tumor metastasis.
All Met-mediated biological responses depend on trans/autophosphorylation of the receptor on two tyrosines localized in the kinase activation loop (Y1234-Y1235) and in the C-terminal tail docking site (Y1349-Y1356) respectively.
The latter tyrosines are strictly required for Met-mediated invasive growth. In fact, their substitution with phenylalanine, completely abolishes proliferation, motility, invasion, and tubulogenesis (Ponzetto et al., 1994; Zhu et al., 1994; Fixman et al., 1995).
Molecules interfering with the biological behavior of the tumor cells could represent valuable therapeutic agents.
In this study we investigate the possibility that synthetic peptides, mimicking intracellular Met tail involved in activation and signal transduction, can antagonize Met-mediated responses, both in vitro and in vivo.
There is evidence that HGF and its receptor Met may be involved in the pathogenesis of Kaposi's sarcoma and therefore we used KS-Imm cells as a model system in our experiments.
Synthetic peptides derived from the Met docking site, when delivered into intact cells by fusion with the internalization domain of Antennapedia homeoprotein, have been reported to inhibit some HGF-induced effects in vitro (Bardelli et al., 1999).
We found that the peptide mimicking the docking site at micromolar concentrations was able to inhibit HGF-dependent downstream signaling, impairing MAPK activation and reducing the Akt phosphorylation.
We have also demonstrated inhibitory effects of anti docking site peptide on cell proliferation, cell migration and invasiveness in vitro, using the crystal violet assay, the "wound healing" assay and the "transwell" system respectively.
Moreover, the peptide mimicking the docking site was able to inhibit cytoskeleton organization of migrating KS-Imm cells, as demonstrated by the alteration of actin polymerization in cells stained with phalloidin.
In vivo, in a KS xenograft model, the same peptide significantly retarded KS-Imm tumor growth, when injected every second day both from the beginning of the experiment and after 10 days of tumor growth.
Although preliminary, these data show that carboxyl-terminal sequences of the Met receptor impair the biological response triggered by HGF/Met interaction, suggesting the development of anti docking site compounds to be employed in malignant diseases and in tumor progression.

Is HDAC4 involved in the control of cell motility?

Nadia Cernotta, Andrea Clocchiatti and Claudio Brancolini
Dip.Scienze e Tecnologie Biomediche, Università degli Studi di Udine

Histone deacetylases are well-known transcriptional regulators. They exert their enzymatic activity on ?-amino groups of lysine residues of histones, inducing epigenetic gene silencing. In particular, HDAC4 belongs to the class IIa subfamily: the members of this class are able to shuttle between the nucleus and the cytoplasm because of a Nuclear Localization Signal (NLS) located at the N-terminus and a Nuclear Export Sequence (NES) present at the C-terminus. HDAC4 nuclear-cytoplasmic shuttling is regulated by phosphorylation, that is promoted by a number of different kinases including PKD, CaMKs, ERKs, PKCs and (MARK)-Par-1 kinases. Moreover, during apoptosis HDAC4 is processed by caspases and its cleavage generates an amino-terminal fragment that accumulates into the nucleus. HDAC4 represent an important link between intra- and extra-cellular stimuli and transcriptional regulation.

We have recently observed that small GTPases of the Rho subfamily can regulate HDAC4 nuclear/cytoplasmic shuttling. RhoA is involved in the formation of stress fibers and in the regulation of the transcription factor SRF (Serum Response Factor). Interestingly SRF, in turn, is bound and repressed by HDAC4. In particular we have observed that RhoA (in its iperactive form) and Rac1 (in its dominant negative form) promote HDAC4 cytoplasmic localization whereas Rac1 promotes its nuclear accumulation. In parallel we studied the effect of HDAC4 over-expression on cell motility. We performed a wound-healing assay on U2OS cell expressing HDAC4 in an inducible manner and we observed that cells treated with Ponasterone were two-times faster than untreated cells in the recovery of the scratched surface. siRNA experiments are ongoing to confirm the role of HDAC4 on cell motility

Phosphoinositide 3-Kinase p110beta activity: Key Role in Metabolism and Cancer but not Development.

Elisa Ciraolo[1], Claudia Curcio[2], Romina Marone[3], Stefano Marengo[1], Manuela Iezzi[4], Carlotta Costa[1], Ornella Azzolino[1], Cristiano Gonella[1], Cristina Rubinetto[1], Haiyan Wu[5], Walter Dastrù[6], Erica L. Martin[7], Lorenzo Silengo[1], Fiorella Altruda[1], Emilia Turco[1], Letizia Lanzetti[8], Piero Musiani[4], Thomas Rückle[9], Christian Rommel[9], Jonathan M. Backer[5], Guido Forni2, Matthias P. Wymann3 & Emilio Hirsch[1].
[1]Molecular Biotechnology Center. Department of Genetics, Biology and Biochemistry. University of Torino. Via Nizza 52. 10126 Torino. Italy.

Class IA phosphoinositide 3-kinases (PI3K) are protein and lipid kinases involved in receptor-mediated signal transduction. Upon receptor activation they produce the phosphorylated lipid product PtdIns(3,4,5)P3, which initiates multiple biological responses including cell growth and proliferation. In mammals, the catalytic subunits of these PI3Ks (p110alpha, beta, and gamma) are encoded by individual genes (Pik3ca, Pik3cb and Pik3cd). Genetic and pharmacological dissection of class IA PI3K function have defined that these genes play distinct, non-redundant roles. Deletion in the germ line of Pik3cb leads to an embryonic lethal phenotype, thus previously precluding a full characterization of p110beta function in adults. Here we report that mice homozygous for the mutant allele of Pik3cb (Pik3cbK805R/K805R) expressing a catalytically inactive p110beta (p110betaK805R) can survive to adulthood. We found that the mutation is hypomorphic with variable expressivity and that survival of mutant embryos correlates with the amount of p110betaK805R expression. Homozygotes showed growth retardation and developed mild insulin resistance with age. Pharmacologic and genetic analysis of p110beta function in liver revealed that p110beta catalytic activity is needed to sustain signalling after insulin stimulation. To understand if p110beta was similarly involved with downstream oncogenic growth factor receptors, Pik3cbK805R/K805R mice were studied in a model of breast cancer triggered by the expression of activated Erbb2. In these mice the Pik3cbK805R mutation strongly protected from tumor development. Consistently, p110beta-selective inhibitors blocked proliferation of Erbb2-transformed Pik3cbWT/WT (wild-type) mammary gland cancer cells. Therefore, our data indicate that p110beta kinase activity is not required for embryonic development but is involved in prolonged insulin signalling and is essential for proliferation of Erbb2-driven mammary gland cancer; these findings thus open new perspectives for the modulation of p110beta activity in the treatment of diabetes or cancer.

Role of extracellular matrix and its receptors in sodium channel clustering at nodes of Ranvier

C. Colombelli, D. Zambroni, S. Occhi, L. Wrabetz, M.L. Feltri
San Raffaele Scientific Institute, DIBIT, Milano, Italy

Nodes of Ranvier are specialized axonal domains where voltage gated sodium channels (Nav) cluster, ensuring saltatory propagation of action potentials in myelinated nerves. Clustering of molecules in discrete domains of axons depends on axon-glia interactions. In peripheral nerves, we have shown that both laminin and dystroglycan, a laminin receptor, are necessary for proper Nav clustering. Mice lacking Schwann cell dystroglycan or laminins have poorly clustered Nav and reduced conduction velocities. Similar alterations are found in a patient with Merosin Deficient Congenital Muscular Dystrophy (Occhi et al., J. Neuroscience 2005). We also showed that different laminin isoforms and different dystroglycan complexes have specific localization around nodes of Ranvier. Indeed, laminin ?5 is concentrated in the Schwann cell basal lamina above the nodal and the paranodal region, and dystroglycan-Dp116 complex is enriched at nodes. To understand how these molecules a
id Nav clustering, we first asked if their localization at nodes precedes or follows Nav clusters. We find that, whereas Dp116 is localized at nodes as early as ERM proteins during early postnatal development, laminin ?5 becomes polarized after Nav clusters are formed, suggesting different roles for these two molecules in early axonal differentiation. Instead, it has been recently shown that gliomedin, a microvillar Schwann cell molecule that induces Nav clustering in vitro, is cleaved and secreted at the axon-glial surface, where it is incorporated in the ECM in a heparin-dependent manner (Eshed et al. Journal Cell Biology 2007). Thus, gliomedin may form a complex with heparan-sulfate proteoglycans (HSPG) at the nodal gap. We report that agrin and perlecan, two HSPGs that can bind dystroglycan, are found at nodes of Ranvier, suggesting that dystroglycan might interact with and present a putative gliomedin-HSPG complex to the axonal membrane.

Analysis of the function of ArhGAP15 in leukocyte migration and more

Carlotta Costa[1], Giulia Germena[1], Chiara Ambrogio[2], Roberto Chiarle[2] and Emilio Hirsch[1]
[1]Dipartimento di Genetica, Biologia e Biochimica. Molecular Biotechnology Center. University of Torino. Via Nizza 52. Torino 10126. Italy [2]Department of Biomedical Sciences and Human Oncology and CeRMS, University of Torino, Via Santena 7, Torino 10126, Italy.

The Rho GTPases such as Cdc42, Rac1 and RhoA regulate diverse biological processes including actin cytoskeletal dynamics, cell adhesion, cell polarity and migration. Through their intrinsic GTPase activity, small GTPases switch off by converting this GTP to GDP. Under cellular conditions the intrinsic rate of exchange of bound nucleotide is low and small GTPases are inhibited by factors enhancing their GTPase activity (GAPs).
ArhGAP15 is a recently cloned RacGAP with a pleckstrin homology (PH) domain at C-terminal and a RhoGAP domain at N-terminal. The GAP domain of ArhGAP15 showed specificity towards Rac1 in vitro. The PH domain is required for ArhGAP15 to localize on plasma membrane and cells that overexpress ArhGAP15 resulted more rounded than control. Coexpression analysis with bioinformatic tools revealed that ArhGAP15 coexists in leukocytes with PI3Kgamma, the unique class IB of PI3K that is specifically activated by G protein-coupled receptor and that has a central role in leukocyte migration. The expression of a myc-tagged ArhGAP15 by lentiviral transduction in bone marrow-derived macrophages revealed that this protein appears localized in the cytoplasm in resting conditions and is translocated to the plasma membrane upon C5a stimulation. Treatment with the PI3K inhibitor LY294002 significantly reduced ArhGAP15 membrane localization in stimulated cells, indicating that is membrane localiza
tion and activation is PI3K-dependent. These results indicate ArhGAP15 as a possible PI3K effector. We are currently investigating the physiological function of ArhGAP15 in leukocyte migration.

p140Cap controls early EGFR signalling and cell-cell contact adhesion

Laura Damiano, Paola Di Stefano, Matteo Barba*, Fabrizio Mainiero*, Emilia Turco, Paola Defilippi

Molecular Biotechnology Centre and Dept. of Genetics, Biology and Biochemistry, University of Torino; * Dept. of Experimental Medicine and Pathology, University “La Sapienza”, Roma

We recently described p140Cap as a novel protein regulating Src kinase activity through Csk recruitment. p140Cap controls Src kinase activity through Csk induction and its over-expression impairs “in vivo” tumour growth (Di Stefano et al., 2007). To analyse the molecular mechanisms underlying the defect in cell proliferation due to p140Cap over-expression, here we study the role of p140Cap in EGF-mediated signalling and in cell-cell contact stability in MCF7 cells.
In normal culture conditions, p140Cap over-expressing cells grow in small dense islets, show decreased expression of Cyclin D1 and impaired proliferation. Consistently, in response to EGF, p140Cap over-expressing cells show decreased activation of EGFR and of its downstream effectors such as Ras and ERK. Interestingly, EGF is capable of inducing ERK activity in p140Cap over-expressing cells plated as single cells, suggesting that the down-regulation of EGF signalling in these cells is due to a cell-cell adhesion mediated mechanism.
Moreover we show that in MCF7 cells p140Cap co-localizes and immunoprecipitates with adherens junction proteins, E-cadherin, p120catenin and -catenin, indicating the existence of a macromolecular complex at the level of adherens junctions. Furthermore in p140Cap over-expressing cells adherens junction disassembly and E-cadherin internalization induced by EGTA or cytochalasinD treatment are impaired, providing evidences for a role of p140Cap in stabilization of cell-cell contacts.
These data indicate that p140Cap complexes with cadherins and catenins leading to increased cell-cell contact stability. Moreover p140Cap affects EGF receptor signalling and cell proliferation, likely through a density-dependent growth inhibition.

STAT5 AND PPARγ COOPERATE IN MEDIATING CIRCULATING ANGIOGENIC CELL PROLIFERATION

Patrizia Dentelli, Gabriele Togliatto, Antonella Trombetta, Annarita Zeoli, Arturo Rosso, Barbara Uberti, Luigi Pegoraro and Maria Felice Brizzi.
Department of Internal Medicine, University of Torino, corso Dogliotti 14, 10126, Torino Italy

Objective- Expansion and differentiation of angiogenic cells (AC) are multistage processes that require sequential activation of particular signalling molecules still poorly defined. We have recently identify STAT5 as a crucial signalling molecule involved in AC expansion. Herein we try to characterize the molecular targets of STAT5 in regulating this event. In particular the involvement of PPARγ1 was investigated.
Methods and Results- IL-3- or EGM2-cultured AC showed a different kinetic of expansion, however, in both culture conditions STAT5 activation correlates with cell expansion as shown by siRNA technology. In both culture conditions PPARγ1 expression, temporally related to cell expansion, is controlled by STAT5 transcripional activity as demonstrated by EMSA analysis and by knocking down STAT5. FACS analysis on AC silenced for STAT5 demonstrates that either STAT5 activation and PPARγ1 expression are required for cell expansion. Unlike growth factor-mediated AC expansion, agonist-dependent PPARγ1 expression fails to promote proliferation, indicating that this event requires signals upstream to PPARγ1. By co-immunoprecipitation experiments a STAT5/PPARγ1 molecular complex was detected. PPARγ1 knocked down demonstrates that this molecular complex is required to promote both cell expansion and cyclin D1 expression. Finally by ChIP assay we demonstrate that the STAT5/PPARγ1 molecular complex is involved in the control of cyclin D1 expression and AC expansion.
Conclusions-These data identify the STAT5/PPARγ1 molecular complex as the main regulator of cyclin D1 expression and AC expansion . The characterization of this pathway provides evidences for upstream signaling to PPAR γ1 in controlling the fate of AC.

p27Kip1 contributes to the control of adhesion-dependent signal transduction via modulation of vesicular trafficking

Linda Fabris, Barbara Belletti, Ilenia Pellizzari and Gustavo Baldassarre.
Experimental Oncology 2 Centro di Riferimento Oncologico, Aviano.

p27Kip1 (hereafter p27) is a member of the Cip/Kip family of inhibitors of cyclin-dependent kinase (CDK). To execute this function as cell cycle regulator p27 has to be localized in the nucleus. Emerging studies suggest that p27 plays additional functions, and we and others have reported that it is involved in the control of cell motility. Previous studies of our laboratory demonstrated an inhibitory role of p27 in cell migration, due to its interaction with the microtubule (MT) destabilizing protein Stathmin. To better characterize this p27 function, we analyzed the activation of the signal transduction pathways during the first phases of cell-ECM contact, in fibroblasts WT or KO for p27 gene.
Our data show that in p27 KO fibroblasts the adhesion-mediated signalling is altered respect to the WT counterpart. This effect is specifically dependent on p27 expression, since it can be reverted by re-expression of p27 in p27null fibroblasts. Focusing on the MAPK cascade, we observed that it is activated more rapidly in p27null than in WT cells. In particular, ELK, a specific target of the MAPK cascade, is hyperphosphorylated and hence more active as transcriptional factor, in cells lacking p27. Interestingly, the concomitant absence of Stathmin in these cells (fibroblasts double knock out for both p27 and Stathmin) reverts the observed phenotype. Given that p27 is able to inhibit Stathmin activity and that this in turn increases the MT stability, we tested whether MT-stability could influence the activation of MAPK-ELK pathway. To this aim, we treated cells with low doses of the MT stabilizing agent Taxol and demonstrated that treatment of p27 KO fibroblasts with Taxol resulted in a dose-dependent inhibition of ELK phosphorylation. It is well accepted that the MT network is essential for the vesicular trafficking, and that interfering with its dynamics can affect vesicular trafficking and thereby alter membrane recycling and receptor signalling. To assess whether this could be the case in p27 KO cells, we examined the rates of adhesion-dependent endocytosis/esocytosis of lipid raft components in our model, using a well-characterized lipid raft marker, the Cholera Toxin subunit B (CTB). Labelling of p27 WT and KO fibroblasts with CTB demonstrated that indeed p27 KO cells displayed an enhanced rate of lipid raft trafficking. Consistently with the above results on the signal transduction, this phenotype is reverted by co-depletion of Stathmin or by taxol treatment. Further experiments are needed to definitely demonstrate whether the accelerated rate of recycling observed in absence of p27 is, as suggested by our preliminary observations, directly linked to the activation of the MAPK signalling pathway.

PDGF-RECEPTOR ALPHA INHIBITS MELANOMA PROLIFERATION

D. Faraone, M.S Aguzzi, G. Toietta, A.M. Facchiano, F. Facchiano, A. Magenta, F. Martelli, S. Truffa, D. Ribatti, M.C. Capogrossi, A. Facchiano
Istituto Dermopatico della Immacolata, IDI-IRCCS, Rome; Institute of Food Science, CNR, Avellino;3 Istituto Superiore di Sanità, Rome;Policlinico, Bari.

Melanoma is the most aggressive skin cancer. It is highly metastatic and has poor prognosis at late clinical stages, due to the poor response to the current treatments. Several factors control melanoma growth, including growth factors acting with autocrine and paracrine effects. Platelet-derived growth factors (PDGFs) and their receptors (PDGF-Receptors) play key roles controlling different features at different levels, in solid tumors. We have shown previously that PDGF-BB and FGF-2 dimerize and induce hetero-dimerization of the respective receptors, with strong inhibitory effects on their biological functions. In the present study, we investigated the functional consequences of PDGF-R alpha over-expression in a human melanoma model in vitro and in a mouse melanoma model in vivo. A novel role of PDGF-AA and his receptor PDGF-R alpha was observed. Over-expression of PDGF-R alpha was found to markedly reduce proliferation and metastatic potential and to induce apoptosis of human melanoma cells in vitro. Moreover, PDGF-R alpha was found to

mediate the toxic effects of staurosporine, a known pro-apoptotic molecule, currently under evalation as anti-cancer agent. Furthermore, in a mouse model, in vivo primary melanoma growth was strongly inhibited by over-expressing PDGF-R alpha in the melanoma cells. These findings demonstrate that the expression of PDGF-R alpha inversely correlates with melanoma growth in vitro and in vivo and may suggest a novel mechanism underlying melanoma development.

Induction of ErbB-3 expression by alpha6beta4 integrin contributes to Tamoxifen resistance in ERbeta1-negative breast carcinomas

Valentina Folgiero, Paolo Avetrani, Giulia Bon, Selene E. Di Carlo, Alessandra Fabi, Cecilia Nisticò, Patrizia Vici, Ada Sacchi, Marcella Mottolese, and Rita Falcioni
Regina Elena Cancer Institute, Molecular Oncogenesis Laboratory, Via delle Messi D'Oro, 156 – 00158 Rome

Tamoxifen is still the most widely used drug in hormone therapy for the treatment of breast cancer. Its benefits in adjuvant treatment are well documented in controlled and randomized clinical studies, which have demonstrated an increase in disease-free intervals of patients with positive hormonal receptors. However, the mechanisms involved in endocrine resistance are not clear. Laboratory and clinical data now indicate that bi-directional molecular cross-talk between nuclear or membrane ER and growth factor receptor pathways may be involved in endocrine resistance. We recently found a functional interaction between alpha6beta4 integrin and ErbB-3 receptor to maintain the PI3K/Akt survival pathway of mammary tumour cells. We sought to improve understanding of this process in order to provide the involvement of both receptors insight into mechanism of Tamoxifen resistance.
Using a panel of human breast cancer cell lines displaying different levels of alpha6beta4 and ErbB-3 receptors and a series of 232 breast cancer biopsies from patients submitted to adjuvant Tamoxifen monotherapy for five years, we evaluated the functional interaction between both receptors in relationship to Tamoxifen responsiveness. In mammary carcinoma cells, we evidenced that the alpha6beta4 integrin strongly influence Akt phosphorylation through ErbB-3 protein regulation. Moreover, the ErbB-3 inactivation inhibits Akt phosphorylation, induces apoptosis and inhibits in vitro invasion favouring Tamoxifen responsiveness. The analysis of human tumors revealed a significant relationship between alpha6beta4 and ErbB-3 in P-Akt-positive and ERbeta1-negative breast cancers derived from patients with lower disease free survival.
In conclusion, we provided evidence that a strong relationship occurs between alpha6beta4 and ErbB-3 positivity in ERbeta1-negative breast cancers. We also found that the association between ErbB-3 and P-Akt positivity mainly occurs in ERbeta1-negative breast cancer derived from patients with lower DFS indicating that both receptors are clinically relevant in predicting the response to Tamoxifen.

Study of the role of GIT1 complex in cytoskeletal remodelling adhesion and membrane traffic during cell motility

Gavina M. [1], Za L.1, Molteni R. [2], Pardi R. [2], de Curtis I.[1]
[1]Cell adhesion Unit,San Raffaele Scientific Institute, Milan , Italy; [2]Leucocyte biology Unit, San raffaele University School of Medicine, Milano Italy

RBL-2H3 (rat basophilic leukemia) cells are a good model to study directional cell motility. These cells stably express the G-protein-coupled formyl peptide receptor (FPR) and fMLP-stimulation induces adhesion, spreading and a marked dose-dependent chemotactic response. Peptide binding is followed by receptor conversion to a form that retains high affinity for ligand, and then by FPR association with the cytoskeleton. RBL-2H3 cells express both GIT1 and GIT2, in contrast to neutrophils that have been shown to express only GIT2 mRNA by RT-PCR. Both proteins stably

interact with bPIX. GIT proteins have long been implicated in the internalization of 7-transmembrane receptors upon ligand binding. However their precise role in this process is still not clear. RBL-2H3 cells were used to study by RNAi the function of GIT1, GIT2 and bPIX in the trafficking of chemokine receptors and in the resulting effects on cell motility. In these cells the adhesion to fibronectin was increased in cells treated with the GIT1-, GIT2-, bPIX-specific siRNA in comparison with control cells. The improvement was observed in both stimulated and not-stimulated cells, but stronger effects were detected in presence of fMLP.
Incubation of RBL-2H3 cells with fMLP stimulated FPR internalization by 30 minutes. A significant effect on the internalization was observed in the RBL-2H3 after treatment with siRNA. GIT1-, GIT2- and bPIX-specific siRNA stimulated FPR internalization at short times of cytokine exposition.
Finally, the results obtained in RBL-2H3 cells transfected with siRNAs specific for GIT1, GIT2 or bPIX indicate that down-regulation of these proteins does not affect fMLP-induced cell migration on fibronectin. We are presently addressing the effects of the down-regulation of GIT and PIX proteins on fMLP-induced spreading with the aim of clarify the mechanisms regulating cytokine induced cell migration.

Preliminary investigation on the conditional deletion of CSN5/JAB1 in hepatocytes

Giugliano R [1], Panattoni M [1] and Pardi R[1].
[1]Leukocyte Biology Unit, San Raffaele University School of Medicine, Milan, Italy

Integrins mediate cell adhesion and transduce activation signals that affect gene expression controlling cell proliferation, differentiation and survival. We found that integrin-dependent adhesion controls the function and subcellular localization of CSN5/JAB1. CSN5/JAB1 is the catalytic subunit of the "COP9 signalosome" (CSN), a conserved multi-molecular complex that regulates protein ubiquitination and proteasome-dependent degradation by controlling the activity of cullin-based ubiquitin ligase complexes. The aim of our project is to dissect the role of CSN5/JAB1 and the associated CSN this purpose; we conditionally deleted CSN5/JAB1 in hepatocytes.
The gross morphologic appearance of CSN5/JAB1-deficient livers shows areas of tissue damage and disordered regeneration. Histological analysis confirmed the presence of areas of cell death followed by strong proliferation of progenitor-like cells as well as of morphologically normal hepatocytes, expressing CSN5/JAB1 presumably as a result of leakiness of the Alb-Cre excision process. The increased susceptibility to death of deficient CSN5/JAB1 hepatocytes is under investigation. Preliminary results using a model of synchronized regeneration following CCl4 injury suggest the existence of a deregulation of critical checkpoints either in cell cycle progression or as part of an adaptive response to stress, both leading to induction of p53 and enhanced apoptosis.

The Prostate Specific Membrane Antigen (PSMA) activates the RAC-MAPK pathway in prostate cancer cells and entertains functional relationships with beta1 integrin.

Silvia Grasso*, Alessandra Porzia§, Giulio Fracasso*, Martin Heine^, Hassan Naim^, Fabrizio Mainiero§, Marco Colombatti°*, Dunia Ramarli°,
* Dept. of Pathology, University of Verona, Italy, °Clinical Immunology, G.B. Rossi Hospital, Verona, Italy, §Dept. of Experimental Medicine and Pathology, Institute Pasteur-Fondazione Cenci Bolognetti, University of Rome "La Sapienza", Italy, ^Dept of Physiological Chemistry-University of Veterinary Medicine-Hannover, Germany

The Prostate Specific Membrane Antigen (PSMA) is a transmembrane protein (100 kDa) endowed with enzymatic activity which has recently emerged as one of the most promising biomarkers in diagnosis and treatment of prostate cancer and is presently a target of immunotherapy trials. Its low expression in normal prostate epithelial cells greatly increases in high-grade prostate cancers,

metastasis, as well as in the endotheliun of the neo-vasculature of tumors of different histotype. PSMA enzymatic activity appears to counteract invasiveness. Moreover, a more complex function has been unveiled in normal endothelial cells (HUVEC) where, thanks to the adaptor function of filamin, PSMA participates in integrin signaling and in the regulation of cytoskeletal dynamics via activation of PAK-1.
These observations prompted us to investigate whether PSMA may display signalling potentials and may entertain functional relationships with β1 integrin also in prostate cancer cell (LnCap cell line). As no natural ligand is so far available, PSMA was recruited at LnCap cell surface by cross-linking of mAbs directed against its extracellular domain. As a read-out of our experimental system we selected the production of IL-6 and CCL5, both known to regulate proliferation of LnCap and prostate cancer cells and for this reason specially interesting in a neoplastic cell setting. By biochemical and functional analysis we demonstrated that PSMA cross-linking rapidly and consistently induces an almost overlapping kinetic of phosphorylation of RAC1 and p38 and ERK1/2 MAPK, starting at 5 min and declining at 20 min after stimulation. Consistently, the poor level of IL-6 produced basally by LnCap cells was found increased three-fold after 24h. The same applied to CCL5. Functional blockade with specific pharmacological inhibitors put into evidence the pivotal role exerted in this phenomenon by the PSMA-activated p38 and, to a lesser extent, ERK1/2. Having assessed for the first time that PSMA has signalling abilities in cancer cells, we next investigated whether a PSMA-β1 integrin interaction could occurr at the plasma membrane. Preliminary studies with isolated Lubrol and Tween 20 rafts revealed the presence of both β1 and PSMA, suggesting that the two molecules associate at very early stages during bio-synthesis. Confocal Microscopy analysis of adherent, non stimulated LnCap cells confirmed the co-localization of the two molecules, that, in addition, could be co-immune precipitated together with filamin A. Noteworthy, the PSMA-cross-linking determined the conformational change of β1 molecules leading to the exposure of the activation epitopes recognized by the HUT5-21 mAb (kindly provided by Francisco Sanchez-Madrid) and the cross-linking of the two molecules at the same time raised maximizes the production of IL-6 and CCL5 by LnCap cells.
Although the role of filamin in PSMA-β1 interactions and the molecular assembly at the plasma membrane still need to be investigated, we think that this newly discovered functions of PSMA and its possible collaboration with β1 integrin may help 17n elucidating the biological effects of PSMA, implicating this molecule as an important regulator of prostate tumor cell survival and proliferation.
Silvia Grasso and Alessandra Porzia contributed equally to this work

A novel and selective Src kinase-independent pathway for phospholipase Cgamma2 activation downstream integrin alpha2beta1 involving Rac1 GTPase.

Gianni F. Guidetti, Bruno Bernardi, Cesare Balduini, Mauro Torti
Department of Biochemistry, University of Pavia, via Bassi 21, 27100 Pavia

Integrin alpha2beta1-mediated interaction with collagen plays a pivotal role in platelet activation and thrombus formation. We have previously demonstrated that platelet adhesion via integrin alpha2beta1 leads to the activation of integrin alphaIIbbeta3 and that the subsequent binding of fibrinogen supports platelet aggregation (Bernardi B. et al, 2006, Blood 107:2728-2735). Such a cross-talk between platelet integrins is not mediated by released ADP and thromboxane A2, but involves the activation of the small GTPase Rap1b. We have also found that integrin alpha2beta1-mediated activation of Rap1b, and the consequent stimulation of integrin alphaIIbbeta3, is mediated by the guanine nucleotide exchange factor CalDAG-GEFI, and occurs downstream of phospholipase C (PLC). Nevertheless, the identity of the specific PLC isoform involved and the mechanism of regulation are still unknown. In this study, we focused our attention on PLCgamma2 isozyme, which is known to play a major role in platelet activation. Since PLCgamma2 is supposed to be activated through tyrosine phosphorylation by Src kinases, we initially evaluated the effects of Src-kinases inhibitor PP2 in the cross-talk between integrin alpha2beta1 and integrin alphaIIbbeta3.

Inhibition of Src-kinases was actually able to suppress integrin alpha2beta1-mediated tyrosine phosphorylation of PLCgamma2, but the phospholipase activity, measured as phosphorylation of protein kinase C (PKC) substrates, was unaffected. In addition, treatment with PP2 was not able to inhibit the activation Rap1b and the binding of fibrinogen to integrin alphaIIbbeta3 in collagen-adherent platelets. In order to verify the real importance of PLCgamma2 isoform in integrin alpha2beta1-mediated outside-in signaling we analyzed platelets PLCgamma2-knockout mice. In contrast to wild-type cells, PLCgamma2-deficient platelets showed a defective activation of both Rap1b and integrin alphaIIbbeta3 upon adhesion to collagen, associated with an undetectable PLC activation. These results demonstrated that PLCgamma2 is actually the only PLC isoform activated downstream integrin alpha2beta1, and that such activation may occur independently of its tyrosine phosphorylation. Importantly, this behaviour was found to be peculiar of integrin alpha2beta1-mediated signalling, as we found that activation of PLCgamma2 induced by adhesion to fibrinogen via integrin alphaIIbbeta3 or by stimulation of ITAM-bearing receptors was strictly dependent on Src-kinases-mediated tyrosine phosphorylation. In search of a possible alternative mechanism for PLCgamma2 regulation, we analyzed the role of the small GTPase Rac1, which was shown to activate PLCgamma2 in vitro (Piechuleck T. et al. 2005, J Biol Chem 280:38923-38931), using the compound NSC23766 that blocks guanine nucleotide exchange factors for Rac. NSC23766 alone had little effect on integrin alpha2beta1-mediated activation of PLCgamma2, Rap1 and integrin alphaIIbbeta3. However, when added to PP2-treated platelets the Rac1 inhibitor prevented all these events. We conclude that PLCgamma2 is absolutely required for the cross-talk between platelet integrin alpha2beta1 and integrin alphaIIbbeta3, and that it can be activated by a novel pathway, independent of tyrosine phosphorylation, but mediated by the Rac1 GTPase.

NF-Y Dependent Epigenetic Events Discriminate Between Mitotic and Postmitotic Status

Aymone Gurtner, Paola Fuschi, Fiorenza Magi, Claudia Colussi, Carlo Gaetano, Matthias Dobbelstein, Ada Sacchi and Giulia Piaggio
Molecular Oncogenesis Laboratory, Experimental Oncology Department, Regina Elena Cancer Institute, Via delle Messi D'Oro 156, 00158, Rome, Italy.

Transcriptional regulation of gene expression requires posttranslational modification of histone proteins, which in concert with chromatin remodeling factors, modulate chromatin structure. How transcription machinery actually modulate chromatin structure; and how this modulation is regulated during proliferation and differentiation is an attractive field of study. Compelling evidence suggests that the transcription factor NF-Y acts as a master regulator of cell cycle progression activating transcription of many cell cycle regulatory genes. However, the molecular mechanism through which it exerts its activity is not yet completely understood.
We here demonstrated that the transcription factor NF-Y exerts its transcriptional activity regulating the histone "code". NF-Y colocalizes with nascent RNA, RPII phosphorylated on serine 2 of CTD repeat YSPTSPS and histones carrying modifications that represent activation signals of gene expression (H3K9ac and PAN-H4ac). Comparing postmitotic muscle tissue from normal mice and proliferating muscles from mdx mice, we demonstrate, by ChIP experiments, that NF-Y binding activity correlates with the accumulation of acetylated histones H3 and H4 on promoters of key cell cycle regulatory genes when they are actively transcribed. Accordingly, p300 is recruited onto chromatin of NF-Y target genes in a NF-Y-dependent manner as demonstrated by Re-ChIP. Conversely, the loss of NF-Y binding correlate with a decrease of acetylated histones, the recruitment of HDAC1 and leads to a repressed heterochromatic state, with enrichment of histones carrying modifications known to mediate silencing of gene expression (H3K9me3, H3K27me2 and H4K20me3), and consequent downregulation of transcription of NF-Y target genes.

Unrestricted NF-Y activity leads to p53-dependent apoptosis directly regulating E2F-1 expression.

Paola Fuschi, Aymone Gurtner, Fabio Martelli, Matthias Dobbelstein, Ada Sacchi and Giulia Piaggio
Experimental Oncology Department, Istituto Regina Elena, Via delle Messi D'Oro 156, 00158 Rome, Italy.

The CCAAT binding transcription factor NF-Y plays a central role in regulating cellular proliferation by controlling the expression of genes required for cell cycle progression such as cyclin A, cyclin B1, cyclin B2, cdc25A, cdc25C and cdk1. Here we show that unrestricted NF-Y activity leads to apoptosis in an E2F1-dependent manner. Consistent with this we demonstrate that NF-Y directly regulated the expression of E2F1 at transcriptional level. NF-Y binds in vivo E2F1 promoter and this correlats with the recruitment of open chromatin marks. Unrestricted NF-Y activity induces an increase in E2F1 mRNA and protein levels that is accompanied by an increase in serine 15 p53 phosphorylation. In agreement with this, NF-Y induces apoptosis in a p53-dependent manner. Moreover, in cells lacking E2F1, the ability of NF-Y to induce the phosphorylation of p53 is impaired, indicating that NF-Y acts upstream to E2F1 in p53-mediated apoptosis and linking NF-Y in the oncogenic stress signaling pathway between E2F1 and p53.

FUNCTIONAL CHARACTERIZATION OF AN EVOLUTIONARY CONSERVED PRO-RICH DOCKING SITE IN BETA-ARRESTINS

Carolina L. Crespo, Monica Fabbri, Raffaella Molteni and Ruggero Pardi
Leukocyte Biology Unit, S. Raffaele University School of Medicine, Milan, Italy

The GPCR/GRK/beta-arrestin signalosome is well-known to be required for receptor desensitization and turnover. In addition, beta-arrestins have recently emerged as multifunctional adaptors/scaffold proteins thereby contributing as signal transducers to GPCR signaling.
We observed by intravital microscopy that leukocytes from β-arrestin2 KO mice fail to arrest to- and migrate across the capillary endothelium upon keratinocyte-derived chemokine (KC) stimulation. Accordingly, β-arrestin2 depletion on RBL-2H3 cell line stably expressing CXCR2 decrease KC-induced Rap1 activation as well as short term activation of both Akt and Erk1/2.
Based on these findings, we are currently investigating the possible non-redundant roles of β-arrestins as modulators of adhesion-related enzymatic cascades. We used site-directed mutagenesis to generate specific mutations within a putative SH3 binding region localized in a solvent exposed loop of beta-arrestin2. Functional assays have been performed after over expressing CXCR2 together with β-arrestin2 WT or mutant. Interestingly, we observed a dominant-negative effect of β-arrestin2 mutants in both KC-induced PI3K and MAPK activation.
To discover newly beta-arrestins' molecular interactors, purified beta-arrestins WT and proline-rich mutants were incubated over SH3 domain Arrays. The possible migration-associated interactions are currently being characterized within a cell context.
Our results suggest a positive regulatory role for β-arrestin2 proline-rich domain in KC-induced signal transduction.

ROLE OF PROTEOLYTIC ENZYMES AND ECM REMODELLING IN THE MOUSE MODEL FOR DUCHENNE MUSCULAR DYSTROPHY (DMD), MDX.

[1]Leandro Lo Cascio, [1]Monica Salamone, [2]Antonella Bongiovanni and [1]Giulio Ghersi
[1]Dipartimento di Biologia Cellulare e dello Sviluppo, Università di Palermo, Viale delle Scienze, Edificio 16; 90128 Palermo, Italy. [2]Istituto di Biomedicina e di Immunologia Molecolare, Consiglio Nazionale delle Ricerche, Via Ugo La Malfa, 153 - 90146 Palermo, Italy.

In muscle pathological condition such as DMD, the aberrant and recurrent degeneration-regeneration processes lead to loss of muscle mass, locomotive deficiency and lethality. In dystrophinopathies, the muscle repair process is accompanied by anomalous remodeling of

extracellular matrix (ECM), which leads to fibrosis. Several families of proteins are involved in ECM remodeling; these molecules belong mainly to three classes: matrix metalloproteases (MMPs), serine-proteases and adhesion molecules, i.e. integrin. Other proteins involved in this event are the constituents of ECM. Several studies point to a relationship between the physiological process of angiogenesis and pathological alteration and destruction of ECM (Sternlicht, M. D. et al. (2001). Annu. Rev. Cell Dev. Biol. 17, 463–516).
The aim of our work is to determine whether proteolytic enzymes and adhesion molecules are deregulated and play a part in the pathogenesis of degenerative muscle disorders. Furthermore, we will evaluate how different resident and/or infiltrating muscle cell populations (mesenchimal and satellite cells) isolated from wild-type and dystrophic (mdx) mice play a part to the degeneration/regeneration processes, via proteolytic cascade/s.
In our experiments, we have used a mouse model for DMD, mdx; as the human counterpart it carries a mutation in the dystrophin gene, mapped on X-chromosome (Bogdanovich S. et al. (2004). J Mol Med 82:102–115). Dystrophin is a member of a multi-proteic complex that allows a physical link between actin cytoskeleton and members of ECM (Yamada, H. et al. (2001). Hum.Mol.Genet. 10: 1563-1569). An aberrant complex produces a less strong resistance to mechanical stress but also alter permeability to ions in extracellular environment. Dystrophic patients undergo to a progressive degeneration of muscle tissue, which is substituted by fibrotic tissue and several adipocytes. ECM usually is abnormally degraded, and there are present many inflammation foci.
In our work we use different experimental approaches to understand which molecular events underlying the muscle degeneration, and how a single genetic mutation is correlated to the degradations pathways observed. Zymographic analyses of cellular protein extracts from mdx muscles showed an increased expression of the pro-form of two gelatinases, MMP-2 and MMP-9, compared to normal muscle. These enzymes are well known to act in angiogenesis and cancer invasion. Immunoblotting assays demonstrated also augmented levels of the MT1-MMP and the β1-integrin; both molecules usually are also over-expressed in specific membrane domain of cells involved into angiogenesis. Moreover, substrate digestion assays performed with freshly isolated mesenchymal primary culture cells from mdx and normal muscles, showed an higher degradative capability of some mdx cells compared to normal.

Lifetime imaging of FRET signals: the best way to look at interacting membrane receptors.
Masi A[1], Cicchi R[3], Morello V[2], Broggio C[3], Pavone FS[3], Arcangeli A[1] and Defilippi P[2]
[1]Dipartimento di Patologia ed Oncologia Sperimentali, Università di Firenze.

Forster Resonance Energy Transfer (FRET) is defined as a non-radiational transfer of energy from an excited fluorophore (named donor) to another fluorophore (named acceptor) which undergoes a transition to an excited state rapidly followed by the emission of a photon. For the process to be efficient, the donor and acceptor molecules, called a FRET pair, must: (1) have overlapping spectra and (2) be in close vicinity to one another (5-10 nm). When these conditions are satisfied, the excitation of the donor will result in the partial quenching of its own emission by the acceptor. As a direct consequence, the acceptor's emission will be intensified proportionally. A major experimental application to life science is the determination of intra- and inter-molecular distances. FRET can be detected and measured in two ways: (1) by calculating the amount of energy transferred (either by measuring the quenching of the donor or the increase of the acceptor's emission), or (2) by measuring the donor's fluorescence lifetime. We have used both approaches to investigate the physical interaction of EGFR and integrin alphavbeta1 upon integrin activation. EGFR and integrin beta1 were labelled in live cells by using Alexa 488/546-conjugated monoclonal antibodies. Early experiments were performed by using an intensified CCD camera to image ECV 304 cells in presence of Alexa 488 anti-EGFR alone, then after addition of Alexa 546 TS2/16, an activating anti-beta1 antibody which is known to trigger the formation of EGFR-integrin membrane complex. Signal intensity was taken in the donor and acceptor's detection channels and used to obtain a time course of FRET. This method, apparently straightforward, has some major drawbacks:

(1) bleaching of the donor always occurs extensively and may result in an overestimation of FRET, (2) absolute intensities are largely affected by antibody binding efficiency, (3) significant overlap between the donor and acceptor's absorption spectra leads to the need for complicated corrections. All the issues listed above concur to reduce the sensitivity of this method, making it difficult to make an accurate analysis, hence to clearly state whether FRET is or is not occurring. On the other hand, donor's lifetime imaging (FLIM-FRET) relies solely on the measure of the donor's fluorescence lifetime. Donor's fluorescence lifetime is shortened by quenching in a measurable manner. Lifetime measures are intrinsically independent from absolute fluorescence intensities, hence they are not affected by the issues reported above. Using FLIM we have detected FRET in a reproducible a controlled way. Measures obtained in cells labelled as described before show that (1) FRET occurs between labelled EGFR and activated integrin receptors, (2) this process is Src kinase-dependent and (3) the process is enhanced in presence of EGF. Results are greatly encouraging and prompt us to explore other experimental mode
ls and other methods of labelling proteins. In particular, CFP and YFP fusion protein-based approach represents a complementary approach that is being attempted in on going experiments in our laboratory.

Non-Clathrin Endocytosis of the Epidermal Growth Factor Receptor

Gilda Nappo[1], Sara Sigismund[1], Simona Polo[1], Pier Paolo di Fiore[1,2]
[1]Fondazione IFOM, [2] European Institute of Oncology

Receptor Tyrosine Kinases (RTKs), such as EGFR, can be endocytosed through different entry routes, including clathrin-dependent and -independent pathways. Our group found a differential recruitment of the EGFR into the two pathways as a function of EGF concentration, and this correlated with the ubiquitination state of the receptor. Indeed, at low doses of EGF, the receptor is not ubiquitinated and is internalized exclusively through the clathrin pathway (here referred as CME, clathrin-mediated endocytosis). At higher concentrations of ligand, however, a substantial fraction of the receptor is endocytosed through a clathrin-independent, cholesterol-dependent, route (here referred as NCE, non-clathrin endocytosis), as the receptor becomes ubiquitinated.
The molecular mechanisms involved in NCE of the EGFR are mainly unknown. Essentially, NCE is defined by its insensitivity to functional ablation (KD) of clathrin and for its sensitivity to cholesterol-interfering drugs, hence its definition as a "raft-dependent pathway".
In order to identify the molecular components of NCE, we are employing a large-scale proteomic approach. To this end, i) we will follow the internalization of the EGFR through NCE, under condition of clathrin KD; ii) a vesicular fraction will then be prepared by using differential centrifugation technique, and iii) subjected to a double-step immuno-purification with anti-EGFR and anti-ubiquitin antibody; iv) the final step will be the mass-spec analysis of the immuno-purified fraction, in order to identify the components of vesicles carrying the EGFR internalized through NCE.
Preliminary results on the setting-up of the different steps will be presented. In particular, we are developing an inducible KD for clathrin in HeLa cells, using lentiviral vectors of new generation, which have the advantage of carrying all the genes required for inducibility in one single vector (Shin et al., 2006). Moreover, the comparison between different protocols to isolate the vesicular fraction will be shown

THE AP-2ALPHA AND AP-2GAMMA TRANSCRIPTION FACTORS REGULATE TUMOR FORMATION AND PROGRESSION VIA A SPECIFIC GENETIC PROGRAM

Francesca Orso[1,3], Elisa Penna[1], Davide Corà[2,3], Michele Caselle[2,3], Piero Sismondi[4], Michele De Bortoli[3,4] and Daniela Taverna[1,3]
[1]MBC, Dept. Onc. Sci., University of Torino, Via Nizza 52, 10126 Torino, Italy. [2]Dept. Theoretical Physics, University of Torino and INFN, Via P. Giuria 1, 10125 Torino, Italy. [3]Center for

Complex Systems in Molecular Biology and Medicine, University of Torino, Via Accademia Albertina, 13, 10123 Torino, Italy. [4]Dept. Onc. Sci., University of Torino and IRCC, Str Prov 142 - Km 3.95, 10060 Candiolo (Turin), Italy.

AP-2 transcription factors are a family of developmentally regulated DNA binding proteins. They are encoded by five different genes (alpha, beta, gamma, delta and epsilon) but they share a very common structure. They can act as homo- or heterodimers and bind to GC-rich DNA sequences apparently without any specificity for the different isoforms. AP-2 play relevant roles in growth, differentiation, adhesion and migration by regulating specific genes. Many evidences suggest that AP-2 act as tumor suppressors in particular in melanomas and mammary carcinomas. Here we are investigating the roles of AP-2alpha and AP-2gamma proteins in cancer formation and progression. We down-modulated AP-2 expression in tumor cells and obtained enhanced tumor growth and reduced chemotherapy-induced apoptosis as well as migration and invasion. These biological modulations were rescued by AP-2 overexpression experiments. In order to identify a genetic program that could explain how A P-2alpha could regulate tumor growth, apoptosis and progression we performed microarray analysis of control and AP-2alpha low expressing cells (Whole Human Genome 44K, Agilent) and found 719 differentially up- or down-regulated genes (FC> 1.5 pv < 0.01). We validated 14 of these genes by qRT-PCR and performed Gene Ontology analysis. We identified highly modulated genes involved in cell cycle (i.e. CCNDBP1, SESN1, SESN3, CDKN1A and HRASL3), apoptosis (i.e. HIPK3, FASTK and BIRC3) and migration or adhesion (i.e. ESDN, EREG, CXCL1, CXCL2, IL11 and ITGBL1). For some of these genes we identified AP-2 binding sites in their regulatory regions and demonstrated AP-2alpha binding by ChIP experiments. We are currently looking for putative binding sites (signatures) for other transcription factors that could cooperate with AP-2 in the regulation of these genes. Moreover we studied the defect in migration in more details and observed that it is, at least in part, mediated by secreted factors. We then proved that ESDN and EREG play a major role in migration regulation.

Which alpha integrin pairs with beta1 to form the Schwann cell receptor required for axonal sorting?

Marta Pellegatta[1], Alessandra D'Urso[1], Desirée Zambroni[1], Alessandro Nodari[2], Adèle De Arcangelis[3], Elisabeth Georges-Labouesse[3], Lawrence Wrabetz[1] and Maria Laura Feltri[1].
[1]San Raffaele Scientific Institute, Milano. [2]National Institutes of Health (NIH), Bethesda. [3]Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France.

During peripheral nervous system development Schwann cells segregate large caliber axons destined to be myelinated during the process of "axonal sorting". Axonal sorting is necessary for subsequent myelination, and requires signals deriving from both axons and the extracellular matrix. Genetic alterations in mice have shown that laminins and beta1 integrin are required for axonal sorting. Thus a receptor containing beta1 integrin links laminin to the Schwann cell cytoskeleton to enable them to ensheathe axons. To form laminin receptors, beta1 integrin can pair with different alpha integrin chains, many of which are expressed by Schwann cells. The goal of this study is to identify the partner of beta1 integrin in the receptor involved in axonal sorting.
Alpha6 integrin is highly expressed in Schwann cells during axonal sorting and alpha6beta1 integrin is a laminin receptor. Thus, we ablated alpha6 integrin specifically in Schwann cells using the Cre/LoxP system in mice. Alpha6 conditional null mice are viable and fertile. Despite complete recombination of the alpha6 gene in sciatic nerves, and absence of the alpha6 protein in Schwann cells, sciatic nerves and spinal roots surprisingly do not present sorting abnormalities. To address possible redundancy/compensation we are analyzing the expression of other alpha integrins (alpha1, 2, 3, 5, and 7) and we will produce appropriate single or double-null mice. This study will elucidate the general mechanism of Schwann cell sorting of axons, a fundamental step for peripheral nervous system development and myelination.

Role of p27Kip1/Stathmin interaction in the control of cell cycle progression

Ilenia Pellizzari, Barbara Belletti, Linda Fabris, and Gustavo Baldassarre
Experimental Oncology 2-Centro di Riferimento Oncologico, Aviano

The mitotic cell cycle is a tightly regulated process that ensures the correct division of one cell into two daughter cells. p27kip1 (hereafter p27) is a universal CDK inhibitor and an important tumor suppressor. More recently, we and others have demonstrated that p27 may exert additional functions when localized in the cytoplasm. Particularly, we showed an inhibitory role of p27 in the regulation of cellular migration, through its interaction with Stathmin. Stathmin is a phosphorylation-regulated microtubule (MT) destabilizing protein and is also involved in the cell cycle regulation and cell transformation.

To investigate whether p27 and Stathmin interaction plays a role also in the control of proliferation, we used primary mouse embryo fibroblasts from wild type (MEF WT), p27 knockout (MEF p27KO), Stathmin knockout (MEF STM-KO) and p27 and Stathmin double knockout (MEF DKO) animals. Growth curve experiments confirmed that MEF p27KO display a proliferation rate higher than that of MEF WT, as already reported by others, but, surprisingly this growth advantage was abolished in MEF DKO. Conversely, MEF STM-KO proliferated at similar rate of MEF WT.

Similarly, the proliferation of MEFs after starvation and release with serum revealed that fibroblasts p27KO reentry the cell cycle sooner (peak of S phase after 15 hours of release) than MEF WT, MEF STM-KO and MEF DKO (20 hours), suggesting that p27/Stathmin interaction could play a major effect in the control of the G1/S transition.

To better characterize these results, we analyzed the cell cycle of the different MEF populations under molecular and biochemical profiles. To this aim we performed Western Blot analysis coupled with Kinase Assay on many different markers of the S phase. Results demonstrated that MEF p27KO exhibited a higher Cyclin/CDKs activity, compared to all other analyzed MEFs. These data demonstrate that the simultaneous absence of p27 and Stathmin in MEFs is able to reduce the proliferative advantage of fibroblasts p27KO.

On the other hand, we observed that Stathmin absence in a p27 null background strongly correlates with an increased number of polynucleated cells. Time lapse microscopy revealed that p27/Stathmin DKO cells failed to properly complete DNA segregation and cytokinesis.

Here, we report that p27 and Stathmin interaction is important also in the control of proliferation and, in particular, that Stathmin ablation in a p27 KO background reverts the hyperproliferative phenotype p27 null cells but induces alterations in the mitotic division. The way whereby p27/Stathmin interaction controls cell cycle progression is currently under investigation.

CELL ADHESION ON COLLAGEN I PROMOTES C-SRC PHOSPHORYLATION OF VEGFR-3

Pennacchini S., Salameh A., Galvagni F. and Oliviero S.
Dipartimento di Biologia Molecolare, Università degli Studi di Siena, Via Torre Fiorentina 1, Siena, Italy.

Signalling cooperation between integrins and growth factor receptors appears to regulate complex processes including blood vessels development during embryogenesis as well as tumor growth and angiogenesis in the adult. Integrins can directly associate with growth factor receptors regulating dowstream signaling pathways.

We investigated upon the activation of VEGFR-3 in a ligand-independent way and iduced by cell stimulation with extracellular matrix proteins. Cell adhesion on collagen I leads to phosphorylation of both VEGFR-3 wild type (VEGFR-3 WT) and VEGFR-3 kinase dead mutant (VEGFR-3 KD). Phosphorylation of VEGFR-3 induced by cell adhesion is independent from its intrinsic kinase

activity since treatment with receptor pharmacological inhibitor MAZ51 do not affect receptor activation by collagen I.
We show that during cell adhesion on collagen I c-Src is directly involved in VEGFR-3 activation since use of c-Src inhibitor PP2 as well as in adhesion assay of fibroblasts cell line knock out for Src-Yes-Fyn (SYF) tyrosine phosphorylation of VEGFR-3 is totally abrogated. Kinase assay with recombinant active c-Src and pull down assay confirm the direct phosphorylation of VEGFR-3 on active sites. Moreover we observed that phosphorylation of VEGFR-3 by c-Src leads to receptor recruitment of adaptor proteis such as CRK, GRB2 and SHC involved in survival and mitogenic signalling pathways.

STAT3 and Her2 cooperate in mammary tumorigenesis

S. Pensa[1], I. Barbieri[1], T. Pannellini2, V. Fagiano[1], P. Provero[1,3], P. Musiani2, V. Poli[1]
[1]Molecular Biotechnology Center, Univ. Turin (Turin); [2]Ce.S.I., Anatomia Patologica, Univ. G. D'annunzio (Chieti); [3]Dept of Theoretical Physics, University of Turin and INFN, Turin

The transcriptional activator STAT3 is constitutively activated in nearly 70% of solid and haematological tumours, and is therefore considered an oncogene. STAT3 contributes to several mechanisms of the tumorigenesis by inducing its target genes, which are involved in apoptosis and proliferation, angiogenesis, tissue invasion and immune evasion. However, direct in vivo oncogenic properties have yet to be clearly demonstrated and the molecular mechanisms involved are not fully understood. The ErBb2 receptor is amplified in a high percentage of mammary tumours and is known to activate STAT3. To investigate the role of STAT3 in mammary tumorigenesis, we intercrossed MMTV-Her2Neu transgenic mice (NeuT), which develop multifocal mammary adenocarcinomas at high multiplicity, with mice expressing a constitutively active form of STAT3 (STAT3C). We observed that NeuT mice carrying a STAT3C allele develop faster growing, more invasive, less differentiated tumours with dramatically decreased levels of apoptotic cells. In order to analyze the molecular mechanisms and target genes involved we derived cell lines from the mammary tumours of NeuT, STAT3C or WT mice and analyzed several clones and subclones. Interestingly, cells derived from STAT3C-expressing tumors (NeuT/STAT3C cells) show strongly increased migratory and invasive capacities. NeuT/STAT3C cells form more dynamic cell-cell contacts and actin fibers, underlining a less differentiated phenotype. Moreover, NeuT/STAT3C cells give rise to a higher number of lung metastasis when injected iv into nude mice. Accordingly, mammary tumour-derived cell lines where STAT3 expression was downregulated by siRNA, form less lung metastasis into singenic mice as compared to non-silenced cells, confirming the idea that in Her2Neu mammary tumorigenesis STAT3 plays a major role in determining invasion and metastasis. To identify specific target genes involved in the STAT3C-mediated tumorigenesis, we performed gene expression profiling of the NeuT, STAT3C or WT cells by microarray analysis. Among the upregulated genes in STAT3C-expressing cells, but also in STAT3C-expressing tumors, there is a number of genes known to be involved in the crosstalk between cells and extracellular environment, suggesting a role for STAT3 in the direct or indirect regulation of these cellular processes. All together, these results indicate that in this context the main role of STAT3 is to promote cell survival and invasivity/metastasis.

ROLE OF DIACYLGLYCEROL KINASE-ALPHA IN HGF-DEPENDENT BIOLOGICAL EFFECTS OF KAPOSI'S SARCOMA

S. Pietronave, G. Baldanzi, F. Chianale, D. Locarno, P. Porporato, A. Zamperone, A. Graziani, M. Prat
Dipt. Scienze Mediche, Università Piemonte Orientale

Hepatocyte Growth Factor (HGF) is one of the factors involved in the pathogenesis of Kaposi's sarcoma (KS), the most frequent neoplasia in patients with AIDS, characterized by proliferating

spindle cells, infiltrating inflammatory cells, angiogenesis, edema, and invasiveness. In vitro this factor sustains the biological behaviour of KS derived cells, after activation of the its receptor and the downstream MAPK and AKT signals. In the effort to identify new intracellular transducers operative in KS cells, we have investigated the role of diacylglycerol kinase-alpha (DGK-alpha), whose enzymatic activity was reported to be required for the transduction of HGF-induced chemotactic, invasive and proliferative responses in endothelial, epithelial and lymphoma cells. Inhere we report that i) HGF-induced KS motility, proliferation and anchorage-independent growth are inhibited by R59949, a pharmacological inhibitor of class 1 Dgk; ii) motility and anchorage-independent growth are enhanced by overexpression of the wild type enzyme; iii) cell adhesion and spreading on collagen and fibronectin are partially impaired by R59949; iv) DGK-alpha inhibition doesn't affect MAPK and Akt phosphorylation, nor does it induce nuclear translocation of NFkB.
These data show that i) increasing DGK-alpha activity, by overexpression of the enzyme, strongly enhances cell transformation promoting cell migration and anchor-independent growth; ii) in this cell system DGK-alpha inhibition does not affect RAS/MAPK, PI3K/AKT, and NFkB pathways, suggesting that the biological effects observed are due to other so far unidentified signal transducers; iii) pharmacological inhibition of DGK-alpha may be a promising approach for treatment of KS.

OVERCOMING CHEMOTHERAPY RESISTANCE IN CHILDHOOD ACUTE LYMPHOBLATIC LEUKEMIA BY TARGETING ION CHANNELS.

Pillozzi Serena*, Masselli Marika*, Crociani Olivia*, De Lorenzo Emanuele*, Veltroni Marinella°, Amedeo Amedei§, Gaipa Giuseppe^, and Arcangeli Annarosa *.
*Department of Experimental Pathology and Oncology, University of Firenze; °Children's Hospital A. Meyer, Firenze, Italy; §Department of Internal Medicine, University of Florence, Florence, Italy; ^Centro Ricerca M. Tettamanti, Università di Milano-Bicocca, Ospedale San Gerardo, Monza, Italy.

Therapy resistance is still a major obstacle to successful treatment in a significant number of pediatric acute lymphoblastic leukaemia (ALL) patients. It has been previously demonstrated that children with ALL whose leukemia cells exhibit in vitro resistance to single or a combination of drugs have a significantly worse prognosis compared to patients with sensitive leukemic cells. Ion channels are becoming one of the potential targets for cancer therapy and putative biochemical modulators of conventional chemotherapy. In particular, K+ channels belonging to the hERG1 family are attracting most attention, since they are over-expressed in a broad range of primary acute myeloid leukaemias (AML) as well as in both myeloid and lymphoid leukemic cell lines.
In cancer cells, hERG1 channels form macromolecular signalling complexes with adhesion (β1 integrins), growth factor (VEGF receptor 1 (FLT-1)) and chemokine receptors. hERG1 channels in turn modulate signalling pathways triggered by such receptors, and by this way regulate tumour cell proliferation, escape from apoptosis, migration and invasion, and hence malignancy. In AML, hERG1 channels regulate cell proliferation and migration in vitro and in vivo aggressiveness. In adult AML patients, hERG1 channels represent molecular markers of poor prognosis. In ALL, hERG1 channels mediate the protective effect provided by bone marrow microenvironment to escape chemotherapy-induced apoptosis. Such effect relies on pro-survival signals triggered by β1 integrins (mainly VLA4) and chemokine receptors (mainly CXCR4), and further modulated by hERG1 channels. In these cells, the pharmacological block of hERG1 channels shortcomes drug resistance.
It was also recently shown that the expression of hERG1 is related to the chemosensitivity of cancer cells to vincristine, paclitaxel, and hydroxy-camptothecin.
We studied the expression and role of hERG1 channels in various B lymphoid leukaemia cell lines and primary childhood B lymphoid leukaemia samples. It emerged that: i) hERG1 K+ channels are expressed in both all the leukaemia cell lines and primary childhood B leukaemia samples; ii) the N-terminus deleted, herg1b isoform was preferentially expressed in both cell lines and primary

samples. B lymphoid leukaemia cell lines were co-cultured on human bone marrow stromal cells, a system known to enhance leukaemia cell survival and escape from drug-induced apoptosis. In these cultures, the addition of a specific hERG1 inhibitor, E4031, induced a significant apoptosis in leukaemia cells, bypassing the protective effect of the bone marrow microenvironment.
We hypothesise that hERG1 channels can represent a novel molecular device regulating drug sensitivity in childhood acute leukaemia cells, and that targeting of hERG1 channels can restore a proper pro-apoptotic response to chemotherapy in resistant B lymphoid leukemic cells.

DIACYLGLYCEROL KINASE ALPHA IS REQUIRED FOR PROLIFERATION AND INVASION INDUCED BY GROWTH FACTORS AND CHEMOKINES

Paolo E. Porporato, Elena Rainero, Federica Chianale, Gabriella Ranaldo, Chiara Ambrogio, Irene Locatelli, Miriam Gaggianesi, Gianluca Baldanzi, Sara Traini, Andrea Graziani
Department of Medical Sciences, University of Piemonte Orientale "A. Avogadro", 28100 Novara, Italy.

Diacylglycerol kinase enzymes (DGKs) convert diacylglycerol (DG) into phosphatidic acid (PA), thus acting as molecular switches between DG- and PA-mediated signalling. We previously showed that Src-mediated activation of DGKα is required for VEGF- and npm-ALK oncogene sustained cell proliferation respectively in endothelial and leukaemia cells (Baldanzi et al. Oncogene 2004; Bachiocchi et al. Blood 2005). Furthermore DGKα activation is also required for VEGF-induced chemotaxis and angiogenesis in endothelial cells (Cutrupi et al, EMBO J 2000; Baldanzi et al. Oncogene, 2004), and HGF- and v-Src-induced scatter and invasiveness of epithelial cells (Chianale et al. Mol Biol Cell 2007; Baldanzi et al. Oncogene, 2008). We also showed that DGKα regulates HGF-induced membrane ruffling at the leading edge, by mediating Rac activation and membrane targeting and Rac-dependent remodelling of actin cytoskeleton and focal contacts in migrating epithelial cells (Chianale et al. Mol Cell Biol, 2007).
These data suggest that DGKα may play a role in tumorigenesis. In order to set up an experimental system to investigate in vivo the role of DGKα in tumor progression, we developed a lentiviral vector for shRNA-mediated constitutive knocked down of DGKα. LV-shRNA/DGKα infection of highly tumorigenic and metastatic MDA-MB-231 cells breast cancer cells, results in almost complete downregulation of DGKα. Constitutive down-regulation of DGKα results in the 60-70 % reduction of serum-sustained cell proliferation as well as inhibition of EGF-induced DNA synthesis. Expression of murine DGKα, resistant to human shRNA, rescues cell proliferation of MDA-MB-231 demonstrating that the proliferative defect is specifically dependent on DGKα expression.
In addition inhibi26ion or knock-down of DGKα strongly impairs HGF- and SDF-1- induced cell invasion in 3D matrix and secretion of matrix gelatinases. The specificity of this effect is confirmed by the full rescue of invasive response to HGF and SDF-1 upon expression of shRNA resistant murine DGKα.
Recruitment to the invadopodia of both β1-intregrin and PKCz, a PA-regulated atypical PKC, are essential for activation of the invasive machinery and metallo-proteinase (MMPs) regulation. Inhibition of DGK impairs SDF1 and HGF-induced membrane targeting of both β1-integrin and PKCz.
These data strongly suggest that DGKα may play a role in tumor progression of breast carcinomas in vivo, and that MDA-MB-231/ShRNA-DGKα provides a valuable tool to develop an in vivo model to investigate the role of DGKα in tumor progression.

Conditional inactivation of CSN5/JAB1 in myelinanting Schwann cells causes a dysmyelinanting neuropathy.

Porrello Emanuela[1], Triolo Daniela[1], Cerri Federica[1], Dina Giorgia[1], Panattoni Martina[3], Del Carro Ubaldo[2], Paolo Morana[2], ComiGiancarlo[1,2], Wrabetz Lawrence[4], Feltri Maria Laura[4], Pardi Ruggero[3], Quattrini Angelo[1], Previtali Stefano[1]
[1]Neuropathology Unit, Neurology Dept. and INSPE, [2]Neurophysiology Unit, Neurology Dept. and INSPE, [3]Unit of Leukocyte Biology, [4]Biology of Myelin Unit,San Raffaele Scientific Institute and Vita-Salute University, Milan, Italy.

Adhesion is a key event in regulating development, function and repair of the peripheral nerve. Accumulating evidence indicates that when adhesion is perturbed, Schwann cell-axon interaction is impaired causing peripheral nerve disorders and defective regeneration. Although much is known about the role of surface adhesion receptors and extracellular matrix (ECM) components, the downstream events and molecules involved in the signaling pathways generated by their interaction is still poorly understood.
CSN5/JAB1 (Jun activation domain-binding protein 1) is the catalytic component of the COP9 signalosome (CSN), a multiprotein complex involved in the regulation of intracellular signaling pathways, degradation of signaling molecules by the ubiquitin-protease system, cell cycle control and the adaptive response to various types of stress. CSN5/JAB1 may also act as a signaling molecule independently by the CSN, i.e. Integrins (transmembrane adhesive receptors) may directly signal through CSN5/JAB1 following interaction with their ECM receptor.
We describe mice in which Schwann cell-specific disruption of Csn5/Jab1, by Cre-loxP technology, causes abnormal development and function of the peripheral nerve, characterized by impaired Schwann cell-axonal interactions. CSN5/JAB1-null Schwann cells showed abnormal proliferation and defective axonal sorting resulting in dysmyelinating neuropathy. Similar findings have been observed following genetic inactivation of laminin2, beta1 integrin, Rac1, Cdc42 and Fak, which may constitute signaling pathways important to mediate Schwann cell-axon interaction.
Our preliminary data suggest that CSN5/JAB1 is involved in peripheral nerve development and function, and maybe a target of the ECM-integrin signaling pathway in Schwann cells.

Dgk alpha provides the signals for regulating cell migration through PKC zeta, RhoGDI and Rac.

Rainero Elena, Chianale Federica, Cianflone Cristina, Bettio Valentina, Porporato Paolo, Traini Sara, Baldanzi Gianluca, Tur Arlandis Gema and Graziani Andrea.
Clinical and Experimental Medicine Dept., University of Piemonte Orientale, Novara.

Diacylglycerol kinase enzymes (Dgks) convert diacylglycerol (DG) into phosphatidic acid (PA), thus acting as molecular switches between DG- and PA-mediated signalling. We previously showed that Dgka activation is required for VEGF-, HGF- and v-Src-induced cell migration of endothelial and epithelial cells, through a mechanism requiring Dgka phosphorylation on Tyr335 by Src itself (Cutrupi et al, 2000; Baldanzi et al., 2004; Baldanzi et al., 2007). More recently we showed that Dgka regulates HGF-induced membrane ruffling at the leading edge, by mediating Rac activation and membrane targeting and Rac-dependent remodelling of actin cytoskeleton and focal contacts in migrating epithelial cells (Chianale et al., 2007). We also observed that Dgka mediates HGF-induced membrane recruitment of Cdc42. However the molecular mechanism by which Dgka regulates Rac and Cdc42 function still remain to be elucidated.
Upon cell adhesion, Rac targeting to the plasma membrane is controlled by b1 integrin, independently from GTP loading, through its dissociation from RhoGDI (Del Pozo et al., 2002). Rac and Cdc42 dissociation from RhoGDI can be induced through multiple mechanisms, including threonine phosphorylation of RhoGDI mediated by PKCz, an atypical Protein Kinase C, reported to be regulated by PA.
Inhere we show that Dgka is required for HGF-induced targeting of constitutively active Rac-V12, suggesting that Dgka provides a signal promoting Rac targeting to the nascent ruffle independently from its GTP loading. Moreover, we show that inhibition of Dgka impairs HGF-induced Rac

dissociation from RhoGDI, which have been described to be regulated in vitro by acidic lipids such ad PA and PIP2, as well as recruitment of both RhoGDI and PKCz to the plasma membrane at the leading edge. As these data suggest that Dgka may regulate Rac by regulating the localization of both RhoGDI and PKCz function, we investigated whether the expression of a membrane-bound constitutively active Dgka may directly regulate RhoGDI and PKCz. Indeed expression of myr-Dgka stimulates ruffle-like structures containing polymerized actin in MDCK cells at colony edge. Interestingly, expression of myr-Dgka provides a signal sufficient to recruit both RhoGDI and PKCz at cell protrusions.
In conclusion, we envisage a working model in which PA, generated by Dgka in a spatially restricted manner, may directly regulate Rac function at least by two non-alternative pathways. Dgka-generated PA may be both directly responsible for membrane recruitment and activation of PKCz, which in turn phosphorylates RhoGDI, or may contribute directly to the displacement of Rac from RhoDGI, thus promoting Rac release and activation.

β-Arrestin links endothelin A receptor to β-catenin signaling to induce ovarian cancer cell invasion

Laura Rosanò[1], Roberta Cianfrocca[1], Stefano Masi[1], Francesca Spinella[1], Valeriana Di Castro[1], Annamaria Biroccio[3], Erica Salvati[3], Maria Rita Nicotra[2], Pier Giorgio Natali[1, 3] and Anna Bagnato[1]
[1]Laboratory of Molecular Pathology and Ultrastructure, Regina Elena Cancer Institute Via delle Messi D'Oro 156, 00158 Rome, Italy;

Metastatic relapses remain a major challenge in the management of ovarian cancer. In this tumor, the activation of endothelin A receptor (ETAR) by endothelin-1 (ET-1) promotes epithelial to mesenchymal transition (EMT), a metastatic early event. In search of downstream mediators in ET-1-induced EMT, we focused on β-arrestin, as adaptor protein G-protein coupled receptor. Here, we identify a new mechanism, whereby β-arrestin is a novel interaction partner of ETAR to promote β-catenin stabilization and transcriptional activity forming two trimeric signaling complexes, one through the interaction with c-Src and consequent the epidermal growth factor receptor (EGFR) transactivation that tyrosine phosphorylates β-catenin, and another through the direct association with axin. As shown by biochemical and confocal microscopes analyses, ET-1 induced the membrane translocation of β-arrestin and its dephosphorylation on serine-412, leading to the formation of an ETAR/β-arrestin/Src signaling complex ("signalplex"), Using WT- or mutant -arrestin-1 constructs, we showed that this signalplex was crucial for EGFR transactivation, that, in turn, controlled the degree of β-catenin protein stabilization by affecting its tyrosine (Y) phosphorylation. This Y-phospho β-catenin translocated to the nucleus and bound the TCF4 transcription factor, thus representing a transcriptional active pool. Concurrently, ETAR activation leads to the association of the β-arrestin with axin, contributing to release of GSK-3 from axin-containing degradation complex and to β-catenin stabilization. At the functional level, -arrestin siRNA inhibited β-catenin/TCF transcriptional activity and cell invasion, delineating previously unknown biological functions of β-arrestin in EMT-related signaling. Both ZD4054, a small molecule ETAR antagonist and ETAR siRNA prevented the functional role of -arrestin in the interplay between ETAR and β-catenin pathway in invasive signaling. In an i.p. xenograft HEY ovarian cancer model, ETAR blockade by ZD4054 significantly inhibited EMT and metastatic dissemination, that is maximally impaired by combination of ZD4054 and gefitinib, an EGFR inhibitor. Our results indicate that beta-arrestin links ET-1 axis to β-catenin signaling, and hence identify new therapeutic opportunities for ovarian cancer. Supported by AIRC, Ministero della Salute, AstraZeneca.

Clathrin-mediated internalisation is essential for sustained EGFR signalling but dispensable for degradation

Sara Sigismund[1], Elisabetta Argenzio[1], Gilda Nappo[1], Daniela Tosoni[1], Elena Cavallaro[1], Simona Polo[1], Pier Paolo di Fiore[1, 2, 3]
[1]IFOM, the FIRC Institute for Molecular Oncology, Via Adamello 16, 20139, Milan, Italy, [2] European Institute of Oncology, Via Ripamonti 435, 20141 Milan, Italy, [3]Dipartimento di Medicina, Chirurgia ed Odontoiatria, Universita' degli Studi di Milano, Via di Rudini' 8, 20122 Milan, Italy.

Endocytosis has long been regarded as a mechanism of receptor clearance from the cell surface and signal attenuation. In recent years, however, it has become evident that, in addition to the canonical functions, endocytosis provides spatial and temporal dimensions to the execution of signalling pathways. One major level of complexity is added by the fact that signalling receptors might be internalised through different endocytic routes. For instance, we have shown that the EGFR is internalised through both clathrin-mediated endocytosis (CME) and non-clathrin endocytosis (NCE), as a function of ligand dose and ubiquitination status. This raises a major question. Are all endocytic routes equivalent from the functional point of view, or do they rather couple with different receptor functions?
We tackle this issue, for the EGFR system, by elucidating the physiological relevance of the two pathways of internalisation. We found that the clathrin pathway is required for sustained signalling, but dispensable for degradation. EGFRs internalised through CME are by-and-large recycled to the cell surface, thereby allowing continuous cycles of signalling from the plasma membrane and from endosomes. Thus, CME of the EGFR is not an attenuator, but rather a positive modulator of signalling. On the other hand, NCE, which is activated only at high EGF dose, preferentially commits the receptor to degradation.

PERIPHERAL NERVE ABNORMALITIES IN VIMENTIN-DEFICIENT MICE

Daniela Triolo[1], Emanuela Porrello[1], Giorgia Dina[1], Isabella Lorenzetti[1], Federica Cerri[1,3], Ubaldo Del Carro[2,3], Paolo Morana[2,3], Laura Feltri[1], Albee Messing[4], Charles Babinet[5], Giancarlo Comi[1,3], Angelo Quattrini[1,3] and Stefano C. Previtali[1,3]
[1]Neuropathology Unit, [2]Neurophysiology Unit and [3]Department of Neurology, San Raffaele University Hospital, Milan, Italy; [4]Weisman Center and Department of Pathological Sciences, University of Wisconsin-Madison, Madison, USA; and 5INSERM UMR S 546, Paris, France

Several peripheral neuropathies may result from abnormal relationship among Schwann cells, axons and the surrounding extracellular matrix. These interactions are mediated by surface receptors and transduced into cell functions by downstream cytoplasmic proteins such as cytoskeleton constituents.
We are investigating the role of the intermediate filament VIMENTIN, a Schwann cell specific cytoskeleton protein expressed during nerve development and myelination, and up-regulated in nerve regeneration.
Mice lacking vimentin look apparently healthy, since behave and breed normally. However, they present morphological abnormalities of the peripheral nerve and defective attempt to regenerate. Myelin-forming Schwann cells are characterized by the abnormal apposition of the plasma-membrane to the myelin sheath, resembling defects of the periaxin/dystroglycan-associated complex, which mediates adhesion to the ECM and is responsible of human hereditary neuropathies. Vimentin-null mice also show hypermyelinated fibers and delayed regeneration after nerve damage.
Our preliminary results suggest a role for vimentin in mediating Schwann cell-axonal interaction, myelin stabilization and thickness, and in nerve repair. Vimentin might be a candidate gene involved in recessive hereditary neuropathies in humans.

Expression and functional activity of CXCL-8 receptors, CXCR1 and CXCR2, on human malignant melanoma cells

Trisciuoglio D., Gabellini C., Zupi G., Del Bufalo D.
Experimental Chemotherapy Laboratory, Regina Elena Cancer Institute, Rome, Italy

Recent evidences indicate that cancer cells express chemokine receptors whom signaling pathways are crucial for tumor proliferation, migration, and angiogenesis. In the present study, we examined the autocrine/paracrine role of interleukin 8 (CXCL-8) in melanoma growth, migration and invasion by analyzing the expression and functional significance of CXCL-8 receptors.
The expressions of CXCL-8 and its receptors, CXCR1 and CXCR2, were examined in a panel of human malignant melanoma cell lines. Using Western blot and enzyme-linked immunosorbent assay (ELISA), we found that five out of six melanoma cell lines produced detectable levels of CXCL-8 (up to 50 ng/million cells). Expression of CXCR1 and CXCR2 was detected by flow cytometry in five out of six cell lines tested, independently from CXCL8 expression.
Highly metastatic M20 and A375SM cells, expressing similar levels of CXCR1 and CXCR2 but different levels of CXCL-8 protein, were chosen for all the experiments. Treatment of M20 cells, expressing very low levels of CXCL-8, with exogenously added human recombinant CXCL-8 significantly enhanced their proliferation. On the contrary A375SM cells, producing high amount of CXCL-8 protein, were not responsive to exogenously CXCL-8 exposure. We found that cell proliferation of unstimulated A375SM cells and CXCL-8-stimulated M20 cells was significantly reduced in presence of neutralizing antibody against CXCL-8 when compared to cells cultured with medium alone or control antibody, confirming the autocrine/paracrine role of CXCL-8 in melanoma cell proliferation. Furthermore, we investigated which receptor(s) mediated the functions of CXCL-8 in melanoma cells and we observed reduced migration and invasion of M20 and A375SM melanoma cells treated with anti-CXCR2, but not anti-CXCR1 neutralizing antibodies, as compared to controls.
In summary, these data suggest that constitutive expression of CXCR2 plays an important role in regulating the CXCL-8-mediated invasive behaviour of human malignant melanoma cells.

PROTEOLITIC ENZYMES ACTIVATION/EXPRESSION DURING ANGIOGENIC PROCESS

Viviana Vesco, Jana Němečková and Giulio Ghersi.
Dipartimento di Biologia Cellulare e dello Sviluppo, viale delle Scienze, Edificio 16, 90128 Palermo

Angiogenesis (growth of new blood vessels) plays a key role in tissue repair, such as in cancer progression (Folkman 1995. New Engl. J. Med. 333, 1757-63). At the beginning of the process is observed the matrix degradation and endothelial cells (ECs) migration inside to the connective tissue in proximity of vessel walls. During angiogenesis several modifications occurs at plasma membrane level; a redistribution of cell-cell and cell-matrix adhesion molecules (Bazzoni et al 1999), such as an increased expression of several proteolytic enzymes, including MMPs and serine proteases, as plasminogen activators and SIMPs, were observed (Ghersi et al. 2006. Cancer Res. 66, 4652-61; Ghersi 2008 Front Biosci. 13, 2335-55).

Angiogenesis “in vivo” is established by ephitelial-mesenchymal transition of endothelial cells; a similar phenotypic exchange can be induced “in vitro” by growing ECs to low density or by perturbing cell-cell contacts. Cell-cell contacts are mediated by several molecules, enclose: CD31/PECAM-1, CD144/VE-cadherins, N-cadherins and β1-integrin adhesion molecules. These molecules take contacts with actin cytoskeletal components to stabilize epithelial phenotype through specific cytoplasmatic mediators.

In particular a cytoplasmic mediator of cadherin/cytoskeletal interaction is the β-catenin, that apart this function can acts as transcription factor in association with TCF/LEF members family when

cell-cell contacts are not stable. During angiogenesis cadherin-cadherin contacts are modified and endothelial cells acquired mesenchymal phenotype; in this condition β-catenin dissociate from cadherin cytoplasmic tail and can acquire the capability to translocate, into the nucleus working as genes' activator factor.

Our experiments were focalised on the different expression/activation of angiogenic proteolytic enzymes when cell-cell contacts were perturbed in endothelial cells mechanically, in wound hedge system, or site specific, using antibodies against specific cadherin domains. We have investigated in this direction using different approaches:

1- Computational, on the 50.000 bp up-stream non translated sequences of several MMPs and SIMPs genes about the presence of just identify β-catenin/TCF-4 binding sequences hTBE-1 and hTBE-2, that was demonstrated to be MMP-7's transcription regulation sequences (Li et al. 2005. World J.Gastroenterol. 11, 2117-23);

2- Biochemical, on different proteolytic enzymes mRNA (by rt-PCR), proteins and enzymatic activities (by immunoblotting and gelatin zymography) expression when cell-cell contacts were perturbed mechanically or by addition of IgGs and/or Fabs recognizing cadherins extracellular (Cac125) and cytoplasm (Pan-cadherin) domains;

Preliminary results by computational approach, demonstrated that: the transcription binding sequence analysed hTBE-1 and hTBE-2 were present in all analysed non translated 50.000 bp sequences of thirteen proteolytic enzymes genes. Moreover, other sequences containing the "core" (CAAAG) specific for several transcription factors, with 1 or 2 mismatch in the "core" flanking sequences were also identified. The statistic z-score values determined for the hTBE-1 and hTBE-2 were 1,25 and 2,95 respectively. Suggesting the involvement of the hTBE-2 sequence as regulator of investigated enzyme genes. These results were also confirmed by direct biochemical/bimolecular experiments such as by experiments of specific competition.

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3. Li, Y. J., Wei, Z. M., Meng, Y. X., and Ji, X. R. (2005). Beta-catenin up-regulates the expression of cyclinD1, c-myc and MMP-7 in human pancreatic cancer: relationships with carcinogenesis and metastasis. *World J.Gastroenterol.* **11**, 2117-2123.