386 The role of DUSP1 in angiogenesis and lung cancer metastases

V. Moncho[1], I. Ibañez de Cáceres[2], E. Bandres[3], B. Martinez-Poveda[4], I. Sanchez-Perez[1], A. Rovira[5], J. Albanell[5], B. Jimenez-Cuenca[4], F. Rojo[6], R. Perona[1]. [1]*Alberto Sols Biomedical Research Center, Experimental models for human diseases studies, Madrid, Spain,* [2]*Research Unit Hospital La Paz, Oncology Service, Madrid, Spain,* [3]*CIMA, Medical Oncology, Pamplona, Spain,* [4]*Alberto Sols Biomedical Research Center, Molecular and Cellular Biology of Cancer, Madrid, Spain,* [5]*Hospital del Mar, Oncology, Barcelona, Spain,* [6]*Fundacion Jimenez Diaz, Pathology Anatomy, Madrid, Spain*

MAPK constitute a family of proteins that control cell proliferation, cell migration and apoptosis. Their activation requires phosphorylation of the threonine and tyrosine residues, while dephosphorylation is mediated by a dual specific family of protein phosphatases (DUSPs). The first member identified in this family was DUSP1 and its expression is stimulated by inflammation, oxidative stress or growth factors.

The aim of this study is to gain insight into the cellular pathways involving *DUSP1* actions using a double strategy that combines siRNA and microarray technologies. Specifically, gene expression profiles of H460v (empty vector) and H460cri (DUSP1 siRNA) cells were compared using an array platform. After data analysis, gene expression was validated by qPCR and functional assays were performed. Thus, angiogenesis was tested both *in vitro* and *in vivo* using H460v or H460cri conditioned media, and *cell migration and invasion* assays were carried out using the wounding healing technique and invasiveness, through Boyden chambers. For the *tumourogenesis and metastasis* analysis, nu^-/nu^- mice were inoculated with both cell lines. Finally, we validated our results on human non small cell lung cancer (NCSLC) specimens.

Results showed that some of statistically significant genes were involved in angiogenesis and tumour progression. Angiogenesis studies revealed in H460cri conditioned media a decrease in the migration of HMVEC cells and *in vivo* assays performed with this media in matrigel plugs, showed lower vessel density. This was also observed in solid tumours derived from H460cri cells, which correlated with the decrease observed on VEGFC expression in tumours. On the other hand, we observed less migration and invasive capacity of H460cri compared to H460v cells. When cells were inoculated in nu^-/nu^- mice, H460cri cells induced fewer tumours and metastasis. Metastasis induced by H460cri cells were only found in lungs. However, the animals inoculated with H460v cells developed tumours in several tissues and lower median survival time was observed. Finally, in human NSCLC specimens we found a significant correlation between DUSP1 and VEGFC expression.

All together, our results suggest that DUSP1 targeting in NSCLC, would be a good strategy to inhibit tumour angiogenesis and progression and therefore DUSP1 might represent a good biomaker for combined treatment with chemotherapeutic drugs and angiogenic inhibitors.

Supported by FIS project numbers: PI08/1485 and PS09/00472.

387 An internal domain of dyskerin as a gene therapy approach in X-linked dyskeratosis congenita

J. Carrillo García[1], G. Güenechea-Amurrio[2], P.J. Mason[3], R. Machado-Pinilla[1], J.A. Bueren[2], R. Perona Abellón[1]. [1]*Instituto de Investigaciones Biomédicas, CIBER de Enfermedades Raras, Madrid, Spain,* [2]*CIEMAT, Hematopoietic Gene Therapy, Division and CIBER de Enfermedades Raras, Madrid, Spain,* [3]*Washington University School of Medicine, Division of Hematology, Department of Medicine, St. Louis, USA*

Dyskeratosis congenita is an inheritable syndrome, which is characterised by mucocutaneous abnormalities, cancer predisposition and bone marrow failure. These symptoms are consequence of mutations, which affect different members of the telomerase or shelterin complex, and as result, the length of telomeres is progressively reduced. We have described that GSE24.2, an internal domain of DKC1 gene (dyskerin, a component of telomerase complex) reactivates telomerase activity in X-linked dyskeratosis congenita (X-DC) patient cells.

As we are interested in exploring the therapeutic use of GSE24.2, we have cloned it in a lentiviral vector under the control of a weak promoter (PGK), which also has the EGFP reporter gene. As a control, we have used an EGFP lentiviral vector. We have demonstrated by quantitative-PCR, that transfection of GSE24.2 lentiviral vector in X-DC fibroblasts slightly increases the levels of c-myc versus cells transfect with control vector, as we have previously described. Moreover, in these fibroblasts, expressing GSE24.2, the telomerase activity is increased. We have produced viruses by transfecting this lentiviral vector with packaging vectors in 293T cells, and with use them to infect patient X-DC fibroblasts to stable express GSE24.2. We have observed that the proliferation level of these GSE24.2 expressing fibroblasts is increased versus control fibroblasts. Furthermore, we have concentrated lentivirus by ultracentrifugation to obtain viruses with high titer to infect patient X-DC lymphoblasts with high efficiency and observed that GSE24.2 expression increases telomerase activity in X-DC lymphoblasts. Finally, we have also infected mouse bone marrow stem cells with high efficiency, to be implanted in the bone marrow of a dyskeratosis congenita mouse model. We are currently studying the ability of X-DC stem cells infected with GSE24.2 lentivirus to repopulate the bone marrow and to maintain other tissues affected by this disease.

Supported by FIS project numbers: PI081485 and CIBER de Enfermedades Raras.

388 Positive inter-regulation between beta-catenin and endothelin signaling: critical role of beta-arrestin-1 in the epigenetic regulation of gene transcription in ovarian cancer cells

L. Rosanò[1], R. Cianfrocca[1], F. Spinella[1], V. Di Castro[1], P.G. Natali[1], A. Bagnato[1]. [1]*Regina Elena Cancer Institute, Experimental Research Center, Rome, Italy*

Activation of the endothelin A receptor (ET_AR) by endothelin-1 (ET-1) has a critical role in ovarian cancer progression. We previously demonstrated that β-arrestin is recruited to ET_AR to form molecular signaling complexes activating β-catenin/TCF4 transcriptional activity and epithelial to mesenchymal transition. However, the mechanism of β-arrestin-dependent mediated β-catenin transcriptional activation remains poor understood. Here, we show that in ovarian cancer cells, ET_AR promotes epigenetic modification through β-arrestin-1. Thus, as shown by immunoprecipitation, ET-1 promotes the interaction between endogenous β-arrestin-1 and β-catenin in the cytoplasm and in the nucleus. In these cells, ET-1 is a target gene of β-catenin, as shown by effects of gain/loss function of β-catenin on ET-1 expression and activity. Chromatin immunoprecipitation (ChIP) and reporter gene assays revealed that in the nucleus, β-arrestin-1 is selectively enriched at specific ET-1 promoter, and is directly involved in β-catenin transcriptional activity. Moreover, β-arrestin-1 silencing induces the loss of ET-1 mRNA expression and ET-1 secretion, as well as a significant inhibition of ET-1 promoter activity, thus controlling the positive inter-regulation between β-catenin and ET-1/ET_AR autocrine loop in ovarian cancer cells. Moreover, ET-1 promotes the nuclear association between histone acetyltransferase p300 and β-arrestin and the recruitment of p300 on the ET-1 promoter, resulting in H3 and H4 histone acetylation and enhanced ET-1 transcription. Besides ET-1, β-arrestin-1 represents a platform for achieving signal specificity that converges on β-catenin-mediated transcription of defined genes, such as cyclin D1 and matrix metalloprotease (MMP)-2, but not MMP-9. ET_AR blockade with the specific ET_AR antagonist, ZD4054, abrogates the engagement of β-arrestin in the interplay between ET_AR and the β-catenin pathway in controlling gene transcription. In an i.p. model of ovarian cancer metastasis, HEY cells expressing the mutant β-arrestin-1, or treated with ZD4054, metastasized at a reduced rate associated with loss of β-catenin expression, highlighting the importance of β-arrestin-mediated signaling in metastasis formation. Altogether these results reveal a novel function for b-arrestin-1 as a nuclear messenger in ET_AR signalling driving ovarian cancer progression, underpinned by an epigenetic mechanism controlling β-catenin-mediated ET-1 transcription.

Supported by AIRC, AstraZeneca

389 Transition of murine leukemic cells to epithelial-like cells under different growth conditions

Z. Ben-Ishay[1], R. Panarsky[1], F. Reichert[1], D. Cheishvili[2], M. Tarshis[3]. [1]*Hebrew University Medical School, Anatomy and Cell Biology, Jerusalem, Israel,* [2]*Hebrew University Medical School, Developmental Biology and Cancer Research, Jerusalem, Israel,* [3]*Hebrew University Medical School, The Core Research Facility, Jerusalem, Israel*

Epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET) are basic biological processes during embryogenesis. Recently, it was recognized that EMT/MET are associated with the metastasis of solid malignant tumours to distant sites.

We report that murine leukemic cells of mesenchymal origin acquire an epithelial-like appearance and epithelial cell surface markers upon transfer from in vitro culture to subcutaneous sites in mice. Specifically, C-1498 Acute Myeloid Leukemia (AML) cells of C-57Bl mice origin (NCI Friedrick Institute, MD, USA) inserted subcutaneously in C-57Bl mice form local tumours consisting mainly of cuboidal cells, as observed by light microscopy. (1) The Western blot analysis of tumour samples indicates expression of the epithelial marker, Epidermal Growth Factor Receptor (EGFR), the mesenchymal markers, Vimentin (VIM) and Matrix metalloproteinase 2 (MMP2), as well as Hypoxia Inducible Factor 1a (HIF1a), indicating tumour hypoxia. Thus, the tumour cells express both epithelial and mesenchymal markers. (2) In contrast, cultured AML cells do not express epithelial markers and HIF1a, whereas they do express VIM but not MMP2. Expression of MMP2 in tumours but not in AML cells suggests that stromal cells within tumours contribute to the marker expression. (3) A control murine epithelial tumour cell line expresses EGFR and E-cadherin (E-cad), and lacks VIM, MMP2 and HIF1a. Of note, the subcutaneous tumours do not express E-cad.

We conclude that the tumour cells derived from C-1498 AML cells are not genuine epithelial cells despite EGFR expression. Conversion of AML cells

from culture to epithelial-like cells expressing EGFR in tumours indicates deregulation of cell surface molecules possibly induced by epigenetic stress such as hypoxia and local factors in a different environment.
This study was conducted abiding by the regulations of the Ethic Committee of the Hebrew University – Hadassah Medical School, Jerusalem, Israel.

390 Enhanced suppression of Raf-1 activity by K vitamins mediates their synergy with Sorafenib to inhibit HCC cell growth in vitro and in vivo

B. Carr[1], Z. Wang[1], M. Wang[1]. [1]*Thomas Jefferson University, Medical Oncology, Philadelphia PA, USA*

Sorafenib is a multi-kinase inhibitor that has been approved for treatment of human hepatocellular carcinoma (HCC). However, Sorafenib-induced tumour shrinkage is minor. Naturally occurring K vitamins are blood coagulation cofactors, which have also been reported to possess weak inhibitory effects on hepatoma cell growth. To enhance Sorafenib inhibition of HCC growth, we developed a new strategy to combine K vitamins with Sorafenib, and found that K vitamins synergized with Sorafenib to inhibit HCC cell growth in culture and in a rat transplantable HCC model. To explore the mechanisms involved, we examined the role of Raf-1 kinase, because it has been reported that Sorafenib inhibits tumour cell growth as a direct inhibitor of Raf, whereas vitamins K can induce PKA activity, which is a Raf-1 upstream kinase. We found that while lower doses of vitamin K1 or K2 (10 mM) or Sorafenib (2.5 mM) alone slightly induced Raf-1 phosphorylation at both Ser-43 and Ser-259 in Hep3B cells, combination of vitamin K1 or K2 plus Sorafenib treatment resulted in strong Raf-1 phosphorylation at these two Raf-inhibitory serine residues. Raf-1 protein kinase activity assay showed that lower concentrations of vitamin K1 or K2 or Sorafenib alone had little, if any effects on Raf-1 activity, whereas combination of vitamin K1 or K2 plus Sorafenib at the same low concentrations significantly inhibited Raf-1 activity, judged by strong inhibition of MEK, and of ERK phosphorylation, the Raf downstream targets. Since Raf-1 phosphorylation at Ser-43 and Ser-259 can be regulated by either PKA or Akt kinase, we examined the effects of both vitamin K1 or K2 or Sorafenib on their phosphorylation. Although both vitamin K1 or K2 or Sorafenib alone induced PKA phosphorylation, no synergistic phosphorylation effects on PKA were found using the combination. However, vitamin K1 or K2 enhanced Sorafenib-induced Akt phosphorylation, which was associated with enhanced c-Met tyrosine phosphorylation at Tyr-1349, a known phospho-Akt regulator. Our data suggest that enhanced inhibitory Raf-1 phosphorylation at Ser-43 and Ser-259 may play an important role in K vitamin plus Sorafenib synergy in inhibiting HCC cell growth.

391 Heterogeneous tumourogenic ability of human brain tumour derived neurospheres

F. Colleoni[1], M. Belicchi[1], P. Razini[1], R. Galli[2], S. Brunelli[2], M. Di Segni[3], L. Bello[4], D. Coviello[3], N. Bresolin[1], Y. Torrente[1]. [1]*Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico, Università di Milano Centro Dino Ferrari, Neurological Sciences, Milano, Italy*, [2]*Hospital S. Raffaele, Stem Cell Research Institute (SCRI) DIBIT, Milano, Italy,* [3]*Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico, Laboratory of Medical Genetics, Milano, Italy,* [4]*Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico, Università di Milano, Neurological Sciences, Milano, Italy*

Background: Gliomas constitute an highly heterogeneous group of tumours. To date, glioblastoma (GBM) remains one of the most difficult cancer to treat. Several evidences emerged in the last years suggest that tumours growth initiate and is sustained by a subpopulation of cells called cancer stem cells (CSCs). Recent findings adfirm that tumours consist of heterogeneous populations of cells differing in markers expression and growth capacities.
Materials and Methods: Tumours sample were dissociated and the cells obtained cultured in proliferative or differentiating medium. CD133 cells were selected by immunomagnetic separation and clones obtained by limiting dilution assay. Cytogenetic was performed on metaphases chromosome obtained by adding colchicine to the medium. Cells were incubated in hypotonic solution, fixed and chromosomes were Q-banded. Chicken chorio-allantoic membrane (CAM) experiments: GBM growth was obtained grafting the cells on the membrane surface. 7 days after implantation, CAMs were harvested and frozen for immunofluorescence analysis. Intracranial experiments: nude mice received stereotactical injection of different groups of cells into the left forebrain; at different time point animals were sacrificed and the brain frozen for subsequent analysis.
Results: Tumour-derived spheres express high levels of nestin and differentiate in the 3 neural lineages. Cytogenetic analyses revealed a near tetraploid karyotype with an average of 75−90 chromosomes. After intracranial transplantation in nude mice in different sets of experiments, in toto neurospheres derived from one GBM and CD133+/CD133− cells showed different degrees of brain modifications. To understand this biological heterogeneity we derived single cell clones from the tumour derived neurospheres. Among these clones we noticed different growth rates even if they retain the same ability to differentiate. We then implanted the clones in CAM model and after in nude mice. The results confirmed the different growth rate among the clones in both the animal models.
Conclusion: These findings demonstrated the existence of different cell types within tumour spheres confirmed by the presence of different proliferation rates that characterize every single cell. These data reveal that these heterogeneity matches exactly the CSCs hypothesis, that describe the tumours as hierarchical organization of malignant cells, with the clonal evolution, which determines uncontrolled proliferation and differentiation of tumour cells.

392 Isolation and characterisation of lymphoma stem cells

N. Moti[1], S.D. Turner[1]. [1]*University of Cambridge, Department of Pathology, Cambridge, United Kingdom*

Cancer stem cells (CSCs) or tumour-initiating cells (TICs) have been described for a number of malignancies and are deemed responsible for continued growth and propagation as well as for disease relapse and in some cases metastatic growth of many cancers. It has yet to be determined whether CSCs/TICs exist for Non-Hodgkin lymphoma (NHL), a heterogeneous disease affecting the cells of the immune system. There are greater than 30 sub-types of NHL and our initial focus is on the Peripheral T-cell Lymphoma-Not Specified Otherwise (PTCL-NOS) and Anaplastic Large Cell Lymphoma (ALCL) categories, both representing mature T-cell malignancies. In this study, we provide evidence for the presence of a distinct sub-population of cells with stem-like properties within the bulk tumour population. We have employed the Side Population (SP) technique to identify cells with enhanced drug efflux capacity, an inherent property of stem cells and hence believed to be enriched in the SP population. We show that a distinct population of SP cells exist in ALCL cell lines and that this population contains cells positive for the Haemopoietic Stem Cell (HSC) surface markers CD117 (2−5%), CD90 (8−10%) and CD34 (1−3%) which are absent in the main population (MP). Furthermore, *in-vitro* differentiation assays show that the isolated SP population proliferates at a significantly greater rate than the MP cells. We have also identified a verapamil-sensitive SP population in primary human ALCL and PTCL-NOS patient samples and furthermore we demonstrate that 2−3% of these cells express CD117 on their surface. These data suggest that there may be a population of CSCs/TICs within NHL populations. It remains to be determined whether these cells whilst resembling tissue-specific stem cells function in a similar manner, i.e. differentiate to give rise to the bulk tumour population. However, limiting dilutions of primary patient biopsy specimens on engraftment into immunodeficient mice produce tumours with a latency reflective of the lymphoma cell dilution. The tumours that develop also contain a small population of CD117-expressing cells. Hence, we propose that at least some NHL may constitute a small population of CSCs/TICs.

393 NPAS3 is a novel late-stage acting progression factor in gliomas with tumour suppressive functions

D. Kamnasaran[1], N. Ajewung[1], M. Rana[1], P. Gould[2]. [1]*Université Laval, Paediatrics, Québec, Canada,* [2]*Université Laval, Pathology, Québec, Canada*

Background: In our effort to better comprehend the genetics of gliomas, we explored new therapeutic targets. We previously cloned NPAS3, a transcription factor which maps to human chromosome 14. Our principal aim is to comprehend the disease associations of NPAS3, since we recently identified expression in human astrocytes. We investigated NPAS3 as a candidate for astrocytomas based on findings archived from the Cancer Genome Project demonstrating a loss of NPAS3 expression and with loss-of-function deletions of human chromosome 14 with NPAS3 in 30−50% of astrocytomas.
Methods and Results: After undertaking extensive functional analyses, *we now have novel evidence supporting NPAS3 as an astrocytoma tumour suppressor involved in late-stage tumour progression*, based on: (1) Absent NPAS3 expression is predominant in high grade astrocytomas (79−83%), in comparison to low grade astrocytomas (29−35%). (2) Loss of function mutations of NPAS3, which are associated with a loss of heterozygosity of the NPAS3 locus are identified in GBMs. (3) Absent NPAS3 expression is predominant in >60% of malignant human glioma cell lines. (4) An over-expressed NPAS3 in malignant glioma cell lines suppresses the transformation potential, while the converse reduced expression promotes an increase in transformation potential. (5) A reduced NPAS3 expression (efficiency >90%) in concert with other gliomagenesis genes can transform a well characterized TERT immortalized human astrocyte cell line and promote the growth of anaplastic astrocytomas.
Conclusions: Our data provide compelling findings of NPAS3 as a novel gene involved in the cause of astrocytomas, with tumour suppressive and late-stage acting progression factor roles.