

**24° Convegno Annuale  
Associazione Italiana di Colture Cellulari  
(ONLUS-AICC)**

**STRUTTURA, MODIFICAZIONI EPIGENETICHE E  
MECCANISMI DI RIPARO DEL DNA COME BERSAGLIO PER  
TERAPIE INNOVATIVE ANTITUMORALI**

**International Satellite Symposium AICC-GISM**

**MESENCHYMAL STEM CELLS:  
CHALLENGES IN TRANSLATING RESEARCH INTO CLINIC**

**21-23 Novembre 2011 - Roma**

**Sede del Convegno:**

**Centro Congressi R. Bastianelli**

**Istituto Nazionale dei Tumori "Regina Elena"**

**Via Fermo Ognibene 23 - 00144 Roma**

## PRESENTAZIONE DEL CONVEGNO

Lo sviluppo di terapie mirate e la conoscenza dei meccanismi responsabili della mancata risposta delle cellule tumorali ai trattamenti rappresentano alcune delle sfide più impegnative per la ricerca oncologica. Nel corso di questo convegno, l'AICC propone di discutere di alcune tematiche legate a terapie emergenti, chimiche e/o fisiche, che hanno come bersaglio il DNA.

L'induzione del danno al DNA come potenziale terapeutico è stato uno dei maggiori obiettivi fin dagli albori dell'oncologia, mediante l'uso delle radiazioni ionizzanti e con la scoperta casuale, nella metà degli anni cinquanta del secolo scorso, della capacità antitumorale delle mostarde azotate. L'importanza del danno al DNA e dei relativi meccanismi di riparo è dimostrata dal fatto che il cisplatino e i suoi derivati, gli agenti alchilanti e le radiazioni ionizzanti sono tra i presidi terapeutici più usati nell'oncologia medica e, a tutt'oggi, rappresentano il trattamento d'elezione per la maggiore parte dei tumori solidi, dei linfomi e di altre neoplasie ematologiche.

Nel corso degli ultimi anni questa area di ricerca ha avuto un grande impulso, favorito dalle maggiori conoscenze della struttura e della topologia del DNA, dei meccanismi di riparo e delle alterazioni a carico di questi meccanismi. Tutto ciò ha portato alla sintesi di nuovi composti alcuni dei quali hanno ormai raggiunto le fasi più avanzate delle sperimentazioni cliniche. In particolare, gli inibitori di PARP utilizzati sia in presenza di alterazioni a carico dei geni BRCA, nell'ambito del cosiddetto concetto di letalità sintetica, che in combinazione con agenti danneggianti il DNA, hanno dato risultati interessanti e favorito il disegno di studi clinici sperimentali. Un'altra possibilità è data dall'impiego di combinazioni di agenti chimico/fisici in grado di danneggiare la replicazione del DNA arrestando la proliferazione di cellule ad alto indice mitotico e dalla modulazione dell'espressione genica attraverso modificazioni post-traslazionali delle proteine istoniche. In questo ambito, il controllo dell'acetilazione degli istoni appare di enorme interesse allo scopo di modulare l'assetto fenotipico delle cellule tumorali e di regolarne il programma differenziativo.

Un altro aspetto importante è rappresentato dalla necessità di individuare biomarcatori correlati al danno al DNA che siano in grado di fornire una misura dell'efficacia delle terapie genotossiche. A questo proposito,  $\gamma$ H2AX, PAR e la metilazione di MGMT sembrano essere i biomarcatori più promettenti sia per la valutazione dell'efficacia antitumorale che per l'identificazione di eventuali danni ai tessuti normali.

Nell'ambito di questo Convegno, il Gruppo Italiano Staminali Mesenchimali (GISM) di AICC propone un simposio internazionale le cui sessioni affrontano aspetti problematici fondamentali generati nella cosiddetta "ricerca traslazionale". La potenzialità terapeutica di queste cellule ha suscitato grande interesse e speranze e l'approfondimento della loro biologia e dei problemi connessi alla loro espansione permetterà di percorrere strade veramente nuove e arrivare ad applicazioni terapeutiche veramente sicure. Accanto alla necessaria conoscenza delle diverse fonti tissutali per ottenere cellule mesenchimali (alcune delle quali ancora poco note), il loro potenziale uso terapeutico sarà approfondito e discusso nell'ambito di alcune importanti esemplificazioni. Infine, il confronto critico basato sulla esperienza di Cell Factories, potrà permettere di valutare i problemi connessi alla produzione di cellule in condizioni GMP anche alla luce delle attuali normative di legge.

L'AICC si augura che questo convegno sia una proficua occasione di incontro e discussione tra ricercatori clinici, traslazionali e di base per il miglioramento delle conoscenze ed utile per pianificare sinergie tra le diverse professionalità fondamentali per la ricerca biomedica.

**Carlo Leonetti, Augusto Pessina e Silvia Soddu**

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**Daniela Bona** ([bona@ifo.it](mailto:bona@ifo.it))

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**Adele Petricca** ([petricca@ifo.it](mailto:petricca@ifo.it))

**Tel.: 0652662537**

**Fax: 0652662592**

### **Grafica**

**Mauro Di Giovanni**, Istituto Regina Elena, Roma

**Ulteriori informazioni sul sito:** [www.onlus-aicc.org](http://www.onlus-aicc.org)

## **PROGRAMMA SCIENTIFICO**

**LUNEDÌ, 21 NOVEMBRE 2011**

**13.00 - 18.00** Registrazione e collocazione poster

**14.00 - 14.30** SALUTO DELLE AUTORITÀ

*Lucio Capurso, Direttore Generale, IFO, Roma*

*Ruggero De Maria, Direttore Scientifico, Istituto Regina Elena, Roma*

**APERTURA DEI LAVORI**

*Carlo Leonetti, Presidente AICC, Istituto Regina Elena, Roma*

*Augusto Pessina, Past President AICC, Università degli Studi di Milano*

*Silvia Soddu, Istituto Regina Elena, Roma*

**14.30 - 15.00** LETTURA MAGISTRALE

**DNA e sue funzioni quali bersagli nella terapia antitumorale.**

*Franco Zunino, Fondazione IRCCS Istituto Nazionale Tumori, Milano*

**15.00 - 18.00** SESSIONE 1: STUDI CLINICI E PROSPETTIVE TRASLAZIONALI

*Moderatori:*

*Daniele Santini, Università Campus-Bio-Medico, Roma*

*Lidia Strigari, Istituto Regina Elena, Roma*

**15.00 - 15.20** Combinazione di gemcitabina e radioterapia per il trattamento del glioblastoma multiforme.

*Alessandra Fabi, Istituto Regina Elena, Roma*

**15.25 - 15.45** Fotemustina e glioblastoma ricorrente: una nuova opportunità per un vecchio farmaco.

*Raffaele Addeo, Ospedale "S. Giovanni di Dio", Frattamaggiore (NA)*

**15.50 - 16.10** Ruolo prognostico/predittivo dell'espressione di geni del riparo nella risposta al trattamento chemioterapico.

*Giovanna Damia, Istituto Mario Negri, Milano*

**16.15 - 16.30** Coffee break

**16.30 - 16.50** Varianti germinali e somatiche dei geni di riparo del DNA: implicazioni prognostiche per i linfomi aggressivi.

*Davide Rossi, Università degli Studi del Piemonte Orientale, Novara*

- 16.55 - 17.15** Neurotossicità dei trattamenti antitumorali e strategie di neuro protezione.  
*Andrea Pace, Istituto Regina Elena, Roma*
- 17.20 - 18.00** Comunicazioni selezionate
- 17.20 - 17.30** O<sup>6</sup> – methylguanine DNA methyltransferase (MGMT): expression in lung neuroendocrine cancer.  
*Mariantonia Carosi, Istituto Regina Elena, Roma*
- 17.30 - 17.40** Regolazione trascrizionale ed epigenetica del gene oncosoppressore kctd11<sup>ren</sup>  
*Daniela Verzella, Università degli Studi de L'Aquila*
- 17.40 - 17.50** Alterations of phosphatidylcholine metabolism in HER2-overexpressing ovarian cancer cells: comparative evaluation of the effects of cisplatin and phospholipase C inhibition.  
*Maria Elena Pisanu, Istituto Superiore di Sanità, Roma*
- 17.50 - 18.00** Epigenetic fingerprint in endometrial carcinogenesis: the hypothesis of an uterine field cancerization.  
*Antonia Feola, Seconda Università di Napoli*
- 18.00** Assemblea dei soci AICC

**MARTEDÌ, 22 NOVEMBRE 2011**

- 9.00 - 11.30**    **SESSIONE 2: SVILUPPO PRECLINICO E CLINICO DI NUOVI FARMACI**  
*Moderatori:*  
**Katia Scotlandi**, Istituto Ortopedico Rizzoli, Bologna  
**Michele Milella**, Istituto Regina Elena, Roma
- 9.00 - 9.20**    **Basi molecolari e nuove opzioni per trattamenti epigenetici alternativi contro il cancro.**  
**Lucia Altucci**, Seconda Università di Napoli
- 9.25 - 9.45**    **I telomeri come bersaglio per lo sviluppo di nuovi farmaci antitumorali.**  
**Annamaria Biroccio**, Istituto Regina Elena, Roma
- 9.50 - 10.10**    **Inibitori di PARP come modulatori della resistenza alla chemioterapia.**  
**Lucio Tentori**, Università "Tor Vergata", Roma
- 10.15 - 10.35**    **Nuovi inibitori di PARP in sviluppo: rationale per la loro associazione con chemioterapici.**  
**Claudio Pisano**, Sigma-Tau, Pomezia
- 10.40 - 11.00**    Coffee break
- 11.00 - 11.30**    **Comunicazioni selezionate**
- 11.00 - 11.10**    **The First Selective DNA G-quadruplex Groove Binders: Evidence For Telomeric DNA Damage and Tumor Cell Death.**  
**Sandro Cosconati**, Seconda Università di Napoli
- 11.10 - 11.20**    **The thiazole-derivative CPTH6, a novel Gcn5 and pCAF HAT inhibitor, induces apoptosis in human leukemia cells.**  
**Ylenia Ragazzoni**, Istituto Regina Elena, Roma
- 11.20 - 11.30**    **A transgenic mouse model to image NF-Y transcriptional activity in physiological processes of living organisms.**  
**Simona Artuso**, Istituto Regina Elena, Roma
- 11.30 - 12.30**    **Cerimonia di consegna dei Premi AICC 2011 - Relazioni dei vincitori**
- 12.30 - 13.00**    **LETTURA MAGISTRALE**  
**Sviluppo preclinico e clinico di trabectedina, un esempio di ricerca traslazionale.**  
**Maurizio D'Incalci**, Istituto Mario Negri, Milano

**13.00 - 14.30** Pranzo - **Visione Poster**

**14.30 - 17.20** **SESSIONE 3: STUDI DI BASE PER INDIVIDUARE NUOVI BERSAGLI MOLECOLARI E STRATEGIE TERAPEUTICHE INNOVATIVE**

*Moderatori:*

**Oreste Segatto**, *Istituto Regina Elena, Roma*

**Giulia Piaggio**, *Istituto Regina Elena, Roma*

**14.30 - 14.50** **Zinco come adiuvante nelle chemioterapie per la riattivazione del pathway HIPK2/p53.**

**Gabriella D'Orazi**, *Università "G. d'Annunzio", Chieti*

**14.55 - 15.15** **Inibitori di istone deacetilasi e acido zoledronico: una inedita combinazione antitumorale.**

**Alfredo Budillon**, *INT-Fondazione Pascale, Napoli*

**15.20 - 15.40** **Ruoli epigenetici della poli(ADP-ribosilazione).**

**Paola Caiafa**, *Università "La Sapienza", Roma*

**15.45 - 16.05** **Il pathway ATM-Chk2 nella risposta allo stress genotossico.**

**Domenico Delia**, *Fondazione IRCCS Istituto Nazionale Tumori, Milano*

**16.10 - 16.30** **Ruolo di Che-1 nella risposta cellulare allo stress.**

**Maurizio Fanciulli**, *Istituto Regina Elena, Roma*

**16.35 - 16.50** Coffee break

**16.50 - 17.20** **Comunicazioni selezionate**

**16.50 - 17.00** **Role of DNA repair pathways in the control of common fragile site expression.**

**Patrizia Vernole**, *Università "Tor Vergata", Roma*

**17.00 - 17.10** **High sensitivity of BRCA1-defective breast cancer cells to cisplatin is mediated by impaired notch signaling.**

**Monica Ventura**, *Università "Magna Graecia" Catanzaro*

**17.10 - 17.20** **The DNA Damage Response induced by MYCN: potential targets for the treatment of MYCN amplified Neuroblastoma.**

**Marialaura Petroni**, *Università "La Sapienza", Roma*

**17.20 - 17.50** **OPENING LECTURE AICC-GISM SYMPOSIUM**

**The interaction between mesenchymal stem cells and breast cancer cells: implications for novel therapeutic approaches.**

**Nicola Normanno**, *INT-Fondazione Pascale, Napoli*

**WEDNESDAY, 23<sup>th</sup> NOVEMBER, 2011**

- 9.00 - 9.30**     **MAIN LECTURE**  
**Chromatin remodelling and senescence of mesenchymal stem cells.**  
*Umberto Galderisi, Seconda Università di Napoli*
- 9.30 - 11.05**   **SESSION A - THE DIFFERENT MAIN SOURCES OF MSCs**  
*Chairpersons:*  
*Rita Falcioni, Istituto Regina Elena, Roma*  
*Augusto Pessina, Università degli Studi di Milano*
- 9.30 - 9.50**     **Human placenta derived mesenchymal stromal cells: where do we stand?**  
*Ornella Parolini, Centro di Ricerca E. Menni, Brescia*
- 9.50 - 10.10**   **Amniotic fluid derived stem cells - A unique cell source for cardiovascular tissue engineering.**  
*Simon P. Hoerstrup, University of Zurich, Switzerland*
- 10.10 - 10.30**   **Mesenchymal stem cells from Wharton's jelly in domestic animals.**  
*Eleonora Iacono, Università degli Studi di Bologna*
- 10.30 - 10.50**   **Selected communications**
- 10.30 - 10.40**   **Forskolin promotes neuronal differentiation of human Wharton's jelly mesenchymal stem cells.**  
*Emanuela Paldino, Consiglio Nazionale delle Ricerche, Roma*
- 10.40-10.50**   **The development of a novel method for the isolation and cryopreservation of human umbilical cord Wharton's Jelly mononuclear cells that contain a sub-population of Mesenchymal Progenitor cells and umbilical cord vascular cells that contain a sub-population of endothelial progenitor cells for the production of 2 cellular therapy products.**  
*Zacharias G Kallis, C.B.B. Lifeline Biotech Ltd, Nicosia Cyprus*
- 10.50 - 11.05**   **General discussion**
- 11.05 - 11.20**   **Coffee break**

- 11.20 - 13.00** **SESSION B - THE POTENTIAL THERAPEUTIC USE OF MSCs**  
*Chairpersons:*  
**Ornella Parolini**, *Centro di Ricerca E. Menni, Brescia*  
**Umberto Galderisi**, *Seconda Università di Napoli*
- 11.20 - 11.40** **Mesenchymal stromal cells: a new actor in allogeneic transplantation and inflammatory bowel disease?**  
**Franco Locatelli**, *Ospedale Pediatrico Bambino Gesù, Roma*
- 11.40 - 12.00** **MSC and autoimmune disease therapy - beyond tissue engineering.**  
**Alan Tyndall**, *University of Basel, Switzerland*
- 12.00 - 12.20** **Bone marrow-derived mesenchymal stem cell therapy for neurodegenerative diseases.**  
**Franca Fagioli**, *AO O.I.R.M. - S. Anna di Torino*
- 12.20 - 12.50** **Selected communications**
- 12.20-12.30** **Use of human Adipose-derived Stem Cells in a mouse model of neuropathic pain.**  
**Stefania Niada**, *Università degli Studi di Milano*
- 12.30-12.40** **Effects of the hypoxic microenvironment on stem cells isolated from omental and subcutaneous adipose tissue from obese patients.**  
**Giuseppe Coroniti**, *Università "La Sapienza", Roma*
- 12.40-12.50** **Phenotypical and functional characterization of bone marrow-derived mesenchymal stromal cells in pediatric patients affected by Acute Lymphoblastic Leukemia.**  
**Conforti Antonella**, *Ospedale Pediatrico Bambino Gesù, Roma*
- 12.50 - 13.00** **General discussion**
- 13.00 - 14.00** **Lunch - Poster viewing**
- 14.00 - 16.00** **SESSION C - THE MAIN CHALLENGES WITH MANUFACTURING MSCS: THE CELL FACTORIES EXPERIENCE**  
*Chairpersons:*  
**Franco Locatelli**, *Ospedale Pediatrico Bambino Gesù, Roma*  
**Enrico Lucarelli**, *Istituto Ortopedico Rizzoli, Bologna*
- 14.00 - 14.20** **Translating research into clinical scale manufacturing of mesenchymal stromal cells.**  
**Karen Bieback**, *Heidelberg University, Germany*

- 14.20 - 14.40** Validation of analytical methods in GMP: a practical approach.  
*Ivana Ferrero, AO O.I.R.M. - S. Anna di Torino*
- 14.40 - 15.00** Stem cells in veterinary medicine: the italian regulatory.  
*Salvatore Macrì, Ministero della Salute, Roma*
- 15.00 - 15.40** Selected communications
- 15.00 - 15.10** Large animal model (swine) for cell-based cardiovascular repair in acute myocardial infarction: one year follow up.  
*Antonio Crovace, Università di Bari*
- 15.10 - 15.20** DNA profiling: GMP validation of an identity test for human cell lines.  
*Simone Sponza, Procelltech srl, Colletterto Giacosa (TO)*
- 15.20 - 15.30** DNA profiling for the monitoring of cross-contamination in mesenchymal stem cells for clinical application.  
*Marta Serra, Istituto Ortopedico Rizzoli, Bologna*
- 15.30 - 15.40** Ex *in vivo* visualization of transfected human mesenchymal stem cells after transplantation: a reliable cell-labelling protocol for optical imaging.  
*Ilaria V. Libani, Università degli Studi di Milano*
- 15.40 - 16.00** General discussion
- 16.00 - 16.15** Premiazione dei migliori poster e chiusura lavori

# ABSTRACTS

## Relazioni ad Invito



foto&grafica: Mauro Di Giovanni

## **DNA E SUE FUNZIONI QUALI BERSAGLI NELLA TERAPIA ANTITUMORALE**

Franco Zunino

*Fondazione IRCCS Istituto Nazionale Tumori, Milano*

Il DNA è il bersaglio molecolare di molti farmaci usati nella terapia citotossica dei tumori. Questi farmaci, in generale agenti che producono vari tipi di lesioni al DNA, sono considerati non selettivi poiché interferiscono con processi fondamentali della cellula determinando rilevanti effetti tossici. Una migliore conoscenza del loro meccanismo di azione ha tuttavia evidenziato una base di relativa selettività che ne giustifica l'utilità terapeutica. Nonostante l'elevata potenza citotossica degli agenti che danneggiano il DNA, la principale limitazione della loro efficacia terapeutica è rappresentata dalla resistenza delle cellule tumorali, un fenomeno comune alla maggior parte dei farmaci antitumorali. Sebbene le attuali conoscenze sui meccanismi della trasformazione neoplastica e sul comportamento maligno dei tumori abbiano suggerito nuovi basi alla moderna terapia farmacologica, gli approcci mirati ad inattivare funzioni tumore-specifiche a livello genico non hanno ancora applicazioni pratiche sia per la complessità degli interventi farmacologici sia per la discutibile efficacia di colpire una singola alterazione. Recentemente è emerso l'interesse terapeutico di altre molecole capaci di modulare specifiche funzioni DNA-dipendenti (per esempio, trascrizione) o di interagire con particolari strutture del DNA (per esempio, strutture G-quadruplex presenti nel DNA telomerico ed in regioni "promotori" che regolano la trascrizione di importanti oncogeni). Seppure agendo attraverso meccanismi più specifici, l'efficacia di queste nuove molecole, come di altri agenti target-specifici, appare tuttora limitata. E' presumibile che il potenziale terapeutico di nuove molecole capaci di modulare specifiche funzioni DNA-dipendenti attivate durante la risposta al trattamento citotossico (per esempio, inibitori dei processi di riparazione del DNA) possa essere ottimizzato in razionali terapie di combinazione.

## COMBINAZIONE DI GEMCITABINA E RADIOTERAPIA PER IL TRATTAMENTO DEL GLIOBLASTOMA MULTIFORME

Alessandra Fabi, Giulio Metro, Maria A. Mirri, Antonello Vidiri, Andrea Pace, Mariantonia Carosi, Michelangelo Russillo, Marta Maschio, Diana Giannarelli, Alfredo Pompili, Francesco Cognetti, Carmine M. Carapella

*Regina Elena Istituto Nazionale Tumori - Roma*

Il glioblastoma multiforme (GBM) rappresenta la forma più aggressive di neoplasia cerebrale primitiva e la sopravvivenza mediana dalla diagnosi è circa 12 mesi nonostante le nuove opzioni terapeutiche. La radioterapia (RT) rappresenta ad oggi il trattamento principale del GBM di nuova diagnosi e tra i farmaci a potenziale radiosensibilizzante la gemcitabina (Gem), antimetabolita ciclo-cellulare specifico, rappresenta tra gli agenti più interessanti. Risultati su tumori solidi (pancreas, polmone) suggeriscono che dosi a infusione prolungata di gemcitabine determina un'attività più alta rispetto a infusioni standard (30 min). In un precedente studio di fase I (*J Neurooncol. 2008;87(1):79-84*) in pazienti affetti da GBM di nuova diagnosi abbiamo identificato la Dose Massima Tollerata (DLT) della Gem settimanale di 10/mg/m<sup>2</sup>/min in associazione alla RT (2.0 Gy per frazione, dose totale 60 Gy). Il farmaco veniva somministrato 24-72 ore prima dell'inizio della RT. Il livello di dose iniziale pianificato di Gem corrispondeva a 200 mg/m<sup>2</sup>/settimanale (level 1), con dosi scalari di 25 mg/m<sup>2</sup>. Un totale di 10 pazienti sono entrati nello studio, al I livello 2 dei 3 pazienti hanno sviluppato un peggioramento neurologico, e tale livello è stato considerato come DLT. Su questa base la dose considerate "massima dose tollerata" è stata di 175 mg/mq/settimanale associata alla RT.

Nello studio di fase II (*Cancer Chemother Pharmacol. 2010;65(2):391-7*), pazienti con diagnosi di GBM furono trattati con RT concomitante a Gem a 175 mg/mq settimanale x 6 settimane. L'obiettivo dello studio era l'attività del farmaco in termine di risposte. Dopo 4 settimane del trattamento RT+Gem il paziente cominciava trattamento con Temozolomide 150-200 mg/mq per 5 giorni ogni 28. Un totale di 23 pazienti entrarono nello studio, età mediana 57 anni (range 43–72) e Karnofsky Performance Status mediano 90 (range 70–100). Diciassette pazienti avevano ricevuto una resezione parziale della neoplasia. Quattro pazienti risposero al trattamento (17.5%) e 14 (61%) ebbero una stabilità di malattia, per un tasso di controllo globale di malattia del 78.5%. La mediana del tempo di progressione di malattia furono di 6.8 mesi e quello di sopravvivenza globale di 10.1 months. Il trattamento RT + Gem è stato ben tollerato e le uniche tossicità di grado 3 sono state neutropenia e ipertransaminasemia. Un totale di 20 pazienti furono valutati per il promotore della metilguanina metiltransferasi (MGMT), e in 11 la MGMT risultò metilata. Nei pazienti con MGMT metilata e non, il controllo di malattia fu ottenuto in 10/11 (91%) e in 7/9 pazienti (77.5%), rispettivamente.

Il trattamento concomitante RT + Gem in pazienti affetti da GBM risulta attivo e ben tollerato e l'attività è stata osservata sia nei pazienti con MGMT che senza MGMT metilata. Ulteriori studi sono necessari per confermare ulteriormente i risultati.

## **FOTEMUSTINA E GLIOBLASTOMA RICORRENTE: UNA NUOVA OPPORTUNITÀ PER UN VECCHIO FARMACO**

Raffaele Addeo, Liliana Montella, Patrizia Iodice, Salvatore Del Prete

*U. O. C. di Oncologia Frattamaggiore (NA); ASLNA2NORD Monterusciello (NA)*

I glioblastomi di alto grado rappresentano il tumore primitivo cerebrale maligno più frequente negli adulti e continua ad essere associato ad una prognosi infausta. Tuttavia, a dispetto delle opzioni terapeutiche oggi disponibili, e dall'approccio aggressivo adottato, spesso comprendente sia la chirurgia che la combinazione della radioterapia che la chemioterapia adiuvante, la pressoché totalità dei pazienti ha una recidiva. La maggior parte dei dati a nostra disposizione sulle opzioni di trattamento nei GMB recidivati derivano da studi di fase due non comparativi, a causa spesso della limitata efficacia di molti agenti ed all'assenza di un trattamento standard. La chemioterapia con agenti somministrati simultaneamente o sequenzialmente può essere utilizzata per aumentare l'efficacia della terapia. I principali prerequisiti del successo di un trattamento chemioterapico sono la sensibilità delle cellule tumorali al meccanismo d'azione del farmaco ed una esposizione sufficiente. La Fotemustine è un agente chemioterapico appartenente alle colroetilnotrosouree liofile di terza generazione, disegnata con l'ambizioso obiettivo di realizzare una notrosourea provvista di attività antitumorale, migliore sotto il profilo farmacocinetico rispetto ai composti esistenti, capace sia di attraversare la barriera emato-encefalica, che di raggiungere elevate concentrazioni nelle cellule tumorali. Le sue proprietà farmacocinetiche, la rendono un farmaco liposolubile, in grado di superare facilmente la barriera emato-encefalica e raggiungere nel distretto cerebrale concentrazioni biologicamente attive. A dosi attive il farmaco non scervo da tossicità considerevoli, soprattutto ematologiche, che in passato ne hanno frenato lo sviluppo atteso, sulla scorta dei dati preclinici a nostra disposizione. La nuova strategia terapeutica "cronica" di somministrazione della FTM, d'altro canto, se garantisce l'efficacia clinica mantenendo la dose complessiva, sempre 400 mg/mq, tuttavia la frazione in più somministrazioni, nello stesso periodo di otto settimane. Questa modalità di somministrazione diversa fa sì che il paziente non incorra più in nessuna tossicità acuta o irreversibile che comprometta non solo il piano terapeutico ma stessa qualità di vita. Il rispetto della dose biologicamente e terapeuticamente attiva, garantisce nel contempo un tasso di risposte cliniche sovrapponibili a quelle ottenute con la schedala tradizionale. La validità di questa opzione è testimoniata anche dai risultati della sopravvivenza libera da malattia e da quella globale, certamente superiori a quelle ottenute da altre opzioni come con la Temozolamide o la polichemioterapia contenente la procarbazine. La consistente attività clinica e la sostanziale assenza di tossicità di questa nuova schedala apre una nuova strada di ricerca sul possibile uso della FTM in combinazione con agenti della terapia biologica. I risultati a nostra disposizione suggeriscono di disegnare nuovi studi randomizzati con l'obiettivo di ottimizzare i trattamenti basati sulla combinazione della FTM con altri agenti chemioterapici o con agenti della terapia biologica o anche con la radioterapia. Molto attraente risulta la prospettiva di sviluppare associazioni con terapie biologiche, come quella antiangiogenica.

## **RUOLO PROGNOSTICO/PREDITTIVO DELL'ESPRESSIONE DI GENI DEL RIPARO NELLA RISPOSTA AL TRATTAMENTO CHEMIOTERAPICO**

Giovanna Damia

*Istituto di Ricerche Farmacologiche Mario Negri, Milano*

Negli ultimi anni la caratterizzazione molecolare dei tumori umani, la delucidazione del meccanismo d'azione dei farmaci antitumorali e delle vie coinvolte nelle riparazioni delle lesioni indotte dagli stessi ha aperto la possibilità di personalizzare la terapia in campo oncologico. L'identificazione di markers molecolari in grado di predire la risposta al trattamento chemioterapico potrebbe aiutare ad identificare quei pazienti che hanno più probabilità di rispondere e al contempo identificare quei pazienti con meno probabilità di rispondere, che potrebbero quindi venire già da subito indirizzati ad altre terapie e al tempo stesso non essere sottoposti ad un trattamento tossico inefficace.

Il cisplatino è un farmaco largamente usato nella pratica clinica oncologica in diverse neoplasie. Agisce causando danni al DNA che vengono processati e riparati da diversi sistemi: Il nucleotide excision repair (NER), la via di Fanconi anemia (FA), il mismatch repair (MMR), il sistema di riparazione della ricombinazione omologa (HR) e translesion repair (TR). Evidenze sperimentali suggeriscono che la capacità cellulare di riparare il danno al DNA condiziona la sensibilità al trattamento con cisplatino. Infatti cellule che mancano di NER o HR sono estremamente sensibili al cisplatino, mentre cellule con una overespressione delle proteine coinvolte in questi pathways presentano una aumentata capacità di riparazione e quindi una certa resistenza al trattamento con lo stesso. Recentemente si è cercato di traslare queste conoscenze andando a studiare retrospettivamente e, anche se più raramente, prospetticamente il ruolo delle proteine e/o dell'espressione dei mRNA di geni coinvolti nei sopracitati sistemi di riparazione come markers predittivi di risposta ad una terapia contenente cisplatino in diverse neoplasie (carcinoma polmonare, ovarico, tumore capo-collo, etc), spesso con risultati contrastanti. Verranno presentati i dati da noi ottenuti sull'espressione di geni importanti per la sensibilità/resistenza al cisplatino in una coorte retrospettiva di pazienti con carcinoma dell'ovaio e le loro correlazioni coi diversi parametri clinico-patologici, incluso la risposta al trattamento. Il nostro studio ha infatti valutato l'espressione di geni codificanti per proteine coinvolte nei pathway di risposta cellulare al danno indotto dal cisplatino (*PARP1*, *ERCC1*, *XPA*, *XPF*, *XPG*, *BRCA1*, *FANCA*, *FANCC*, *FANCD2*, *FANCF*, *PolEta*, *Chk1* and *Claspin*) mediante RT-PCR in una coorte di pazienti con tumore ovarico (13 con tumore borderline e 155 con carcinoma -77 pazienti di stadio I and 88 pazienti di stadio III). L'analisi univariata ha rilevato che elevati livelli di *ERCC1*, *XPA*, *FANCC*, *XPG* and *PolEta* correlavano con una aumentata sopravvivenza (OS) e un maggior intervallo libero da malattia (PFS), mentre elevati livelli di *BRCA1* correlavano con PFS. Tali correlazioni venivano perse nell'analisi multivariata.

## VARIANTI GERMINALI E SOMATICHE DEI GENI DI RIPARO DEL DNA: IMPLICAZIONI PROGNOSTICHE PER I LINFOMI AGGRESSIVI

Davide Rossi

*Divisione di Ematologia, Dipartimento di Medicina Clinica e Sperimentale, Università degli Studi del Piemonte Orientale "Amedeo Avogadro", Novara*

Il linfoma diffuso a grandi cellule B è il tipo di linfoma più frequente nei paesi occidentali. Nonostante la introduzione di nuovi e più efficaci regimi terapeutici, ~40% dei pazienti con linfoma diffuso a grandi cellule B fallisce la terapia. Ad oggi, la stratificazione prognostica dei pazienti affetti da linfoma diffuso a grandi cellule B è basata principalmente sulle caratteristiche cliniche e biologiche del tumore. Tuttavia, la eterogeneità nella risposta interindividuale alla chemioterapia nei tumori è influenzata, oltre che dalle caratteristiche del tumore, è anche influenzata dal background genetico dell'ospite. Numerosi chemioterapici utilizzati per il trattamento del linfoma diffuso a grandi cellule B agiscono provocando un danno genotossico al DNA in grado di indurre la cellula tumorale in apoptosi tramite la via di *TP53*. Il gene *MLH1* codifica per una proteina coinvolta nella riparazione delle lesioni al DNA prodotte dalla doxorubicina e dai derivati del platino. *MLH1* funziona come molecola di collegamento tra il complesso proteico responsabile della riparazione del DNA e il meccanismo di induzione della morte cellulare regolato da *TP53*. Quando il danno al DNA prodotto dai chemioterapici risulta eccessivo e non riparabile, *MLH1* attiva *TP53* e inducendo la cellula tumorale in apoptosi. Il genotipo del gene *MLH1* condiziona la prognosi dei pazienti affetti da linfoma diffuso a grandi cellule B. I pazienti che portano l'allele variante del polimorfismo *MLH1* rs1799977 presentano un rischio di morte di incrementato di ~3.2 volte. L'incremento del rischio di morte è legato ad una maggiore probabilità di fallire sia la terapia di prima linea con schema rituximab-CHOP sia la terapia di seconda linea con schema rituximab-DHAP seguito da trapianto. L'effetto prognostico sfavorevole del polimorfismo *MLH1* rs1799977 nel linfoma diffuso a grandi cellule B è indipendente dal profilo di rischio IPI del paziente e dalla cellula di origine del tumore, i due sistemi di classificazione prognostica del linfoma diffuso a grandi cellule B ad oggi più largamente accettati. Il significato prognostico sfavorevole del polimorfismo *MLH1* rs1799977 nel linfoma diffuso a grandi cellule B è generalizzabile e riproducibile in coorti di pazienti trattate in istituzioni indipendenti. Poiché l'allele variante del polimorfismo *MLH1* rs1799977 si associa ad una ridotta espressione della proteina *MLH1*, è verosimile che nei pazienti portano l'allele variante di *MLH1* rs1799977 le cellule linfomatose siano meno sensibili alla apoptosi innescata dal danno al DNA prodotto dalla chemioterapia.

## NEUROTOSSICITÀ DEI TRATTAMENTI ANTITUMORALI E STRATEGIE DI NEURO PROTEZIONE

Andrea Pace

*Istituto Tumori Regina Elena, Roma*

La tossicità sul sistema nervoso periferico di alcuni farmaci antitumorali è nota da tempo ma i meccanismi di azione sul nervo periferico dei diversi farmaci non sono ancora ben determinati. Il profilo clinico della neurotossicità indotta dai derivati della vinca (Vincristina, Vindesina, Vinblastina) e dai taxani (Taxolo e Taxotere) è quello di una polineuropatia sensori-motoria assonale distale, prevalente agli arti inferiori, dose dipendente e solitamente reversibile dopo la sospensione del trattamento. L'attività antitumorale di questi farmaci si esercita su una proteina del citoscheletro, la tubulina, che orienta il fuso mitotico nella divisione cellulare, inibendone la polimerizzazione o la de-polimerizzazione. La neurotossicità periferica di questa classe di farmaci è legata all'importante ruolo che la tubulina svolge nell'orientare il flusso assonale nel nervo. Un diverso meccanismo sembra invece essere responsabile della neurotossicità indotta dal cisplatino e da altri composti del platino (carboplatino, oxaliplatino). I derivati del platino si concentrano nel ganglio delle radici dorsali inducendo una neuronopatia con sofferenza secondaria delle fibre sensitive di grosso calibro e un quadro di polineuropatia sensitiva cronica che insorge tardivamente, dopo una dose cumulativa totale superiore ai 300mg/mq. La neuronopatia indotta dai derivati del platino è considerata non completamente reversibile. Meno chiaro è l'inquadramento della neuropatia indotta dall'oxaliplatino che mostra un andamento bifasico con precoce sofferenza acuta delle fibre sensitive di piccolo calibro associata a una sofferenza cronica tardiva analoga a quella indotta dagli altri composti del platino anche se meno severa. Tra i nuovi farmaci antitumorali va segnalata la neurotossicità periferica evidenziata in corso di terapia con bortezomib in pazienti affetti da mieloma multiplo in cui si riscontra una neuropatia sensitiva dose-correlata e dose limitante. Attualmente la neurotossicità è considerata l'effetto collaterale dose-limitante di molti trattamenti antitumorali, mentre altre tossicità sistemiche come la mielotossicità e la gastrotossicità sono controllate da fattori di crescita e terapie di supporto. Numerose sostanze sono state testate come possibili agenti neuroprotettori, tra queste vanno segnalate i fattori di crescita del nervo periferico (Nerve Growth Factor, analoghi dell'ACTH, Org 2776), gli agenti antiossidanti (Amifostina, Glutazione ridotto, vitamina E) e recentemente l'acetilcarnitina. Recentemente sono stati pubblicati risultati incoraggianti sulla possibile efficacia della supplementazione di vitamina E nella prevenzione della neurotossicità periferica e della ototossicità indotte dal cisplatino. Studi sull'animale hanno dimostrato la non interferenza della vitamina E sull'attività antitumorale del cisplatino e si è recentemente concluso uno studio italiano multicentrico randomizzato controllato con placebo i cui risultati confermano il ruolo neuroprotettivo della supplementazione di vitamina E.

## **BASI MOLECOLARI E NUOVE OPZIONI PER TRATTAMENTI EPIGENETICI ALTERNATIVI CONTRO IL CANCRO**

Lucia Altucci

*Seconda Università degli Studi di Napoli, Dipartimento di Patologia generale, Napoli*

Un numero crescente di aberrazioni genetiche, come le traslocazioni cromosomiche, è stata identificata come causa di leucemia mieloide acuta e dimostrata agire sulla de-regolazione genica sia a livello genetico che epigenetico. Mentre le aberrazioni genetiche che si verificano nella leucemia mieloide acuta sono abbastanza chiare, solo recentemente abbiamo preso coscienza della de-regolazione epigenetica associata con la leucemia, in particolare con le leucemie mieloidi acute. La deposizione di "*epigenetic marks*" sulla cromatina - modificazioni post-traduzionali delle proteine nucleosomali e la metilazione del DNA - è compiuta da enzimi, che sono spesso inseriti in multi-complessi di regolazione che hanno acquisito funzionalità aberranti nella leucemogenesi. Questi enzimi sono i "*targets*" per i cosiddetti "farmaci epigenetici". Infatti, recenti risultati indicano che i "farmaci epigenetici" possono costituire un tipo completamente nuovo di sostanze anti-cancro con un potenziale terapeutico interessante. Prova di principio viene dagli studi con inibitori delle istone deacetilasi.

L'utilizzo di approcci epigenetici come trattamento contro il cancro sarà discusso in dettaglio.

## TELOMERES AS DRUG TARGET

Annamaria Biroccio

*Regina Elena Cancer Institute, Rome*

The burgeoning knowledge about the structure of telomeres and the roles of various factors involved in telomere maintenance provides several possible targets for pharmacological intervention. To date the area that has received major drug discovery attention is the targeting the telomeric G-quadruplex (G4) structure. G4 ligands were initially designed to counteract telomerase action at telomeres. Surprisingly, their antiproliferative effects can occur in telomerase-negative cells and follow kinetics, which cannot be merely explained by telomere shortening.

In this context, our study began with an attempt to understand the mechanisms by which G4 ligands can rapidly inhibit cell growth. Impressively enough, we found that RHPS4, one of the most selective G4 ligand, triggers a strong DNA damage response at telomeres with the formation of several foci containing authentic telomeric factors like TRF1 and markers of an early response to DNA breaks. This is typical of the telomere deprotection occurring during cellular senescence or upon telomere injury. Therefore, we characterized the telomeric changes induced by RHPS4 in details using various biochemical and imaging techniques. Our findings showed that RHPS4 specifically delocalizes the terminal protein Pot1 from the telomeres. The functional relevance of this observation was directly assessed by showing that Pot1 overexpression leads to RHPS4 cellular resistance and to the absence of RHPS4-induced telomere damage foci. This by itself constitutes an important step forward in our understanding of the mechanisms of telomere capping and of G4 ligand action. However, our work goes one important step further, by demonstrating, in mouse xenograft models, that during RHPS4 treatment there is a strong correlation between the apparition of telomeric DNA damage foci and the antitumor effect. In particular, we show that Pot1-overexpressing tumors are completely unresponsive to the antitumoral activity of RHPS4 and are unable to induce damage foci. This clearly links pharmacological deprotection of telomeres to cancer treatment, for which no direct evidence previously existed.

The validation of the telomere as pertinent drug target at the preclinical level, encouraging us to the evaluation of therapeutic combined option in future clinical protocols. In this context, we have recently reported a specific link between the telomere-targeting agent RHPS4 and chemosensitivity toward camptothecins in colorectal tumors. More interestingly, the integration of a PARP inhibitor on the combination treatment proved to have a high therapeutic efficacy ensuring a complete regression of the tumor as well as a significant increase in overall survival and cure of mice.

The impact of these findings is broad for basic and biomedical research as well as for clinical applications: they reveal the telomere specific effects of a G4 ligand and the importance of the 3' tail and of their bound-factors for the response to anti-telomere strategy; they validate G4 ligands as very promising antitumor molecules.

## **POLY(ADP-RIBOSE) POLYMERASE (PARP) INHIBITORS AS MODULATORS OF TUMOR DRUG RESISTANCE TO CHEMOTHERAPY**

Lucio Tentori<sup>1</sup>, Annalisa S. Dorio<sup>1</sup>, Alessia Muzi<sup>1</sup>, Patrizia Vernole<sup>2</sup>, Grazia Graziani<sup>1</sup>  
<sup>1</sup>*Department of Neuroscience* - <sup>2</sup>*Department of Public Health and Cell Biology, University "Tor Vergata", Rome, Italy*

Poly(ADP-ribose) polymerase (PARP) inhibitors are a promising class of anticancer agents currently in clinical trials either in combination with chemotherapy [e.g., the methylating compound temozolomide (TMZ), the topoisomerase I (TOPO I) poisons irinotecan (CPT-11) or topotecan] and radiotherapy or as monotherapy. In fact, PARP inhibitors exert cytotoxic effects as single agents in BRCA mutated tumors, which are defective in the homologous recombination repair (HR) of DNA double strand breaks (DSBs). In preclinical models we have demonstrated that PARP inhibitors enhance the antitumor activity of TMZ or of TOPO I inhibitors especially in mismatch repair (MMR) deficient tumors, including colorectal cancer that is frequently characterized by MMR dysfunction. Moreover, the PARP inhibitor GPI 15427 increases the efficacy also of CPT-11 and TMZ combination against MMR deficient colon cancer *in vitro* and in tumor xenografts. hMLH1 is mainly involved in the processing of O6-methylguanine:C/T mismatches responsible for the cytotoxic effects of TMZ. Recently, it has been suggested that MSH3 might be involved in the repair of DSBs induced by intra-strand cross-links provoked by platinum derivatives through the intervention of HR. The role of the different MMR components in the susceptibility of colon cancer cells to TOPO I poisons have not been clarified, yet.

The human colon cancer cell lines HCT116 (known to have a homozygous mutation in the MMR hMLH1 gene on chromosome 3 and homozygous frameshift mutations in the MMR hMSH3 gene on chromosome 5), the HCT116 derived cell lines in which only the wild-type hMLH1 (HCT116+3) or both the wild-type hMLH1 and hMSH3 genes (HCT116+3+5) have been replaced, via chromosome transfer, were used to test their susceptibility to anticancer drugs with different mechanisms of action. The hMLH1 and hMSH3 deficient HCT116 cells and the HCT116+3+5 cells were more sensitive to SN38 (the active metabolite of CPT-11) than HCT116+3 cells that, instead, were highly sensitive to TMZ. Interestingly, the hMLH1 and hMSH3 proficient HCT116+3+5 cells were more resistant to oxaliplatin than the other cell lines. HCT116, characterized by a higher PARP-1 expression with respect to the other cell lines, were the most sensitive to the PARP inhibitor GPI 15427 as single agent. Stable silencing of PARP-1 expression resulted in increased chemosensitivity. The results suggest that that hMLH1, hMSH3 or PARP-1 status may predict differential sensitivity to chemotherapeutic agents.

## **NUOVI INIBITORI DI PARP IN SVILUPPO: RAZIONALE PER LA LORO ASSOCIAZIONE CON CHEMIOTERAPICI**

Claudio Pisano

*Centro di Ricerca, Area di Oncologia, sigma-tau Industrie Farmaceutiche Riunite S.p.A. Pomezia, Roma*

Nel 2006 Benson et al. , Collins e Workman hanno stabilito che modificazioni genetiche, sinergia, origine e ospite sono quattro elementi cardine dipendenti l'uno dall'altro, che costituiscono una potenziale debolezza specifica delle cellule tumorali e se considerati opportunamente possono rappresentare il tallone di Achille delle cellule tumorali. In particolare, la sinergia si riferisce alla generazione di interazioni genetiche sintetiche letali o letalità sintetica, definita da Kaelin come la condizione in cui due (o più) mutazioni non alleliche e non essenziali, di per sé non letali, lo diventano se presenti all'interno della stessa cellula.

Si ritiene che utilizzare un approccio farmacologico che induca una letalità sintetica presenti numerosi vantaggi, tra cui fornire finestre terapeutiche più ampie. Infatti, se una data mutazione, correlata a un certo tumore, sensibilizza le cellule tumorali a un farmaco che inibisce il suo partner sintetico letale, le cellule normali che non possiedono quella particolare mutazione non saranno sensibili al farmaco.

Un esempio tipico di questo settore di ricerca è PARP. PARP-1 inibisce il processo di "base exchange-repair" (BER), che porta all'accumulo di rotture a singolo filamento che, a sua volta, porta alla formazione di rotture a doppio filamento "dannose" (DSBs), che non possono essere riparate in cellule non esprimenti BRCA1/2. Due studi pionieristici (Bryant et al. 2005; Farmer et al. 2005) sono alla base della dimostrazione preclinica dell'attività antitumorale selettiva di inibitori di PARP1 su cellule tumorali non esprimenti BRCA1/2. Vista l'efficacia a livello preclinico degli inibitori di PARP1 sono stati avviati studi clinici di Fase I-II in pazienti oncologici esprimenti sia BRCA1/2 mutato che altri tipi di mutazione.

In particolare, gli inibitori di PARP-1 in sviluppo sono: Olaparib, (AZD2281-AstraZeneca), Iniparib (BSI-201- Sanofi-Aventis), AG 014699(Pfizer), Veliparib (ABT-888-Abbott), MK-4827(Merck), CEP-9722 (Cephalon), INO1001 (Genentech) sono in clinica in

Di particolare interesse sono alcune evidenze precliniche, che suggeriscono un potenziale più ampio di utilizzo degli inibitori di PARP. E' stato infatti osservato che, oltre a BRCA1/2, la perdita di funzione di varie proteine coinvolte nei meccanismi di riparo del DNA generi sinteticamente un fenotipo letale quando PARP viene inibito. L'inattivazione di questi geni è stata riportata in un set di tumori umani e potrebbe quindi costituire un biomarker predittivo per selezionare pazienti da trattare con gli inibitori di PARP.

Di estremo interesse a questo riguardo, sono le associazioni con altri farmaci e/o chemioterapici, che inducendo alterazioni di pathways correlate a PARP inducono quella che viene definita come letalità sintetica condizionata.

## SVILUPPO PRECLINICO E CLINICO DI TRABECTEDINA, UN ESEMPIO DI RICERCA TRASLAZIONALE

Maurizio D'Incalci

*Dipartimento di Oncologia, Istituto di Ricerche Farmacologiche "Mario Negri", Milano*

La presentazione sarà focalizzata sui principali studi sul meccanismo d'azione e l'attività antitumorale preclinica e clinica di trabectedina (Yondelis, ET-743), un composto di origine marina registrato in Europa per la terapia di seconda linea dei sarcomi delle parti molli e per il carcinoma dell'ovaio. Il farmaco è stato selezionato per lo sviluppo clinico sia per il suo meccanismo d'azione originale che per la sua attività antitumorale *in vivo* in numerosi tumori sperimentali e xenografts fra cui sarcomi e carcinomi dell'ovaio.

Trabectedina si lega nel solco minore del DNA ed i suoi effetti biologici sono dipendenti sia dai meccanismi di riparazione del DNA che dai meccanismi di regolazione della trascrizione genica. Per alcuni tipi di sarcomi, la cui patogenesi è legata all'espressione di geni di fusione, con deregolazione di alcuni fattori trascrizionali trabectedina sembra avere un effetto altamente specifico. Il farmaco blocca la capacità transattivante del fattore trascrizionale deregolato. Questo è stato osservato sia in modelli cellulari *in vitro* che in modelli tumorali *in vivo* e sono in corso studi per verificarlo a livello clinico.

Recentemente si è osservato che trabectedina è anche un modulatore della produzione di citochine, chemochine e fattori angiogenici da parte dei macrofagi. Questi effetti sul microambiente tumorale sono probabilmente alla base di osservazioni cliniche di risposte antitumorali che avvengono dopo numerosi cicli di terapia e con lunghe fasi di controllo della malattia con una lenta riduzione della massa tumorale, poco compatibile con un effetto citotossico diretto.

L'insieme dei dati supporta l'idea che trabectedina agisca sia inibendo la crescita tumorale attraverso la modulazione di geni specifici che hanno un ruolo nella proliferazione, differenziamento e morte cellulare, sia modificando il microambiente tumorale ed inibendo l'angiogenesi.

Utilizzando l'esempio della trabectedina si discuterà quanto importante sia l'interazione interdisciplinare tra ricercatori sperimentali e clinici per lo sviluppo razionale di un farmaco antitumorale.

## ZINCO COME ADIUVANTE NELLE CHEMIOTERAPIE PER LA RIATTIVAZIONE DEL PATHWAY HIPK2/p53

Gabriella D'Orazi

*Universita' "G. d'Annunzio", Chieti*

L'oncosoppressore p53 è spesso inattivato nei tumori da mutazioni o da deregolazione della proteina. Uno degli attivatori di p53 è la proteina chinasi HIPK2 che fosforila p53 in serina 46 (Ser46) per la sua specifica attività apoptotica. HIPK2 contribuisce anche al mantenimento della conformazione wild-type di p53. HIPK2 è coinvolto nella risposta all'ipossia agendo come co-repressore di HIF-1 $\alpha$ ; di contro, l'ipossia induce la degradazione proteasomica di HIPK2 instaurando quindi un circuito regolatorio delle molecole HIF-1/HIPK2/p53 con vantaggio proliferativo del tumore. Noi abbiamo trovato che l'inattivazione di HIPK2 in ipossia non è irreversibile, infatti, l'inibizione di HIF-1 $\alpha$  in cellule tumorali e in tumori *in vivo*, con supplementazione di zinco, riattiva HIPK2 e di conseguenza p53, con ripristino della chemiosensibilità e dell'inibizione tumorale. Sebbene l'utilizzo dello zinco in combinazione con farmaci anti-tumorali non sia stato ancora esplorato sistematicamente, l'assenza di biotossicità può rendere questa molecola un interessante adiuvante da sfruttare in combinazione con le terapie antitumorali classiche per modulare pathway importanti per la sopravvivenza del tumore e per la risposta ai farmaci come quello di HIF-1 e di HIPK2/p53.

## **INIBITORI DI ISTONE DEACETILASI E ACIDO ZOLEDRONICO: UNA INEDITA COMBINAZIONE ANTITUMORALE**

Francesca Bruzzese, Alessandra Leone, Biagio Pucci, Maria Rita Milone, Chiara Ciardiello, Monia Rocco, Daniele Santini, Elena Di Gennaro, Imma Chianese, Carmine Carbone, Michele Caraglia, Alfredo Budiillon

*Unità Farmacologia Sperimentale Oncologica, INT "G. Pascale" Napoli*

Nonostante i continui sforzi per sviluppare terapie efficaci e sicure, i pazienti con tumore localmente avanzato, recidivante o metastatico della prostata (PCa) e con mieloma multiplo (MM) non migliorano in maniera significativa la sopravvivenza globale. L'esistenza di vie multiple di sopravvivenza nei tumori solidi può essere la causa dell'attività limitata delle strategie anti-cancro convenzionali e di quelle basate sull'uso di farmaci target specifici. L'utilizzo di farmaci ad effetto pleiotropico, come gli inibitori dell'istone deacetilasi (HDACI) e i bifosfonati (BPs) possono far superare tale resistenza. L'HDACI, LBH589 (Panobinostat™), ha dimostrato una significativa attività antitumorale preclinica e clinica, sia in neoplasie ematologiche che nei tumori solidi, ed è attualmente in studi di fase II-III clinica. L'acido zoledronico (ZOL) è un aminobifosfonato utilizzato nel trattamento delle metastasi ossee poiché è in grado di inibire la formazione di osteoclasti da cellule precursori immaturi o direttamente inducendo apoptosi in osteoclasti maturi. I bisfosfonati ZOL hanno anche una potente attività antitumorale dimostrata in modelli preclinici e recentemente anche in alcuni studi clinici. In questo studio è stata valutata come nuova strategia terapeutica antitumorale la combinazione di LBH589 e ZOL in modelli di PCa e di MM.. La simultanea o sequenziale esposizione delle cellule all'LBH589 e allo ZOL misurata attraverso l'utilizzo del metodo di Chou e Talalay, induce un potente effetto antiproliferativo sinergico *in vitro* in entrambi i modelli cellulari. L'effetto antitumorale osservato, è parallelo all'aumento dell'apoptosi, misurata attraverso analisi citofluorimetrica del contenuto di Annessina V e attraverso western blot con il clivaggio della PARP ed inoltre ad un aumento del contenuto delle specie reattive dell'ossigeno (ROS). L'effetto pro-apoptotico osservato è parzialmente revertito dall' isoprenol geranylgeraniolo (GGOH) indicando che l'interazione tra i due farmaci può avvenire, in parte, attraverso la via del mevalonato. Il sinergismo osservato è stato inoltre confermato mediante esperimenti in soft agar ed *in vivo* in modelli di xenotrapianto di cellule tumorali prostatiche umane in topi immunodeficienti. In seguito al trattamento con i due farmaci in combinazione, dopo 24 ore, si ha un'evidente riduzione dell'espressione della forma fosforilata di p38MAPK, misurata utilizzando la tecnologia Bioplex™, che si contrappone all'aumento di espressione della proteina fosforilata dopo trattamento con il solo ZOL. Il coinvolgimento del pathway di p38MAPK nel sinergismo osservato è stato dimostrato anche avvalendoci di una particolare linea cellulare le DU145 resistenti allo ZOL (DUR80) sviluppata in laboratorio che presenta un'alta espressione della proteina p38MAPK fosforilata. Questi risultati suggeriscono la possibilità dell'utilizzo di nuove strategie terapeutiche, in particolare della combinazione tra l'HDACI, LBH589 e lo ZOL, in tumori con metastasi ossee e negli stadi avanzati di malattia, là dove gli approcci terapeutici convenzionali hanno fallito.

## **RUOLI EPIGENETICI DELLA POLI(ADP-RIBOSILAZIONE)**

Paola Caiafa

*Dipartimento di Biotecnologie Cellulari ed Ematologia, Facoltà di Farmacia e Medicina, Università "La Sapienza", Roma*

L'ipermetilazione aberrante di promotori di geni costitutivi e l'ipometilazione globale sono eventi tipici che si verificano nelle cellule tumorali. I meccanismi molecolari alla base di questi cambiamenti dei quadri di metilazione, legati al cancro, non sono ancora del tutto chiari. E' stato suggerito un ruolo per la poli(ADP-ribosil)azione nel mantenere i quadri di metilazione genomica ed il meccanismo proposto prevede che i polimeri presenti su PARP-1 interagiscano in modo non covalente con Dnmt1 inibendo la sua attività enzimatica mentre in assenza di PARP-1 PARilata la Dnmt1 sarebbe libera di metilare il DNA (1). Il meccanismo prevede in alcuni casi un ruolo per il fattore trascrizionale CTCF (2). Dati recenti hanno confermato che PARP-1 sola o in associazione con CTCF è coinvolta nel mantenimento dello stato non metilato di alcune isole CpG localizzate su promotori di geni costitutivi (3-5). Questi dati possono spiegare non solo come avvenga la deregolazione del quadro di metilazione in cellule tumorali ma fanno supporre che PARP-1 PARilata sia presente su promotori di altri geni costitutivi e/o su sequenze genomiche che devono rimanere non metilate, svolgendo quindi un ruolo di regolazione epigenetica dell'espressione genica

## **IL PATHWAY ATM-CHK2 NELLA RISPOSTA ALLO STRESS GENOTOSSICO**

Domenico Delia

*Fondazione IRCCS Istituto Nazionale Tumori, Milano*

Le lesioni al DNA, e in particolare le rotture alla doppia elica (DSBs) scatenano una serie di risposte (DNA damage checkpoint responses, DDR) che regolano e coordinano la progressione del ciclo cellulare, la riparazione del DNA e, nel caso di danno irriparabile, l'apoptosi.

Il principale attivatore del DDR è ATM, una Ser/Thr chinasi deficiente nell'Ataxia Telangectasia, una sindrome neurodegenerativa caratterizzata da radiosensibilità e predisposizione tumorale. Studi di fosfoproteomica hanno identificato circa 1000 proteine fosforilate da ATM in cellule trattate con radiazione ionizzante. Una di queste è Chk2, una Ser/Thr chinasi la cui attivazione da parte di ATM, induce la fosforilazione di diverse proteine prevalentemente implicate nei checkpoints del ciclo cellulare, riparazione del DNA, apoptosi e senescenza.

Attualmente, sono circa 20 le proteine che vengono fosforilate da Chk2 o che interagiscono con essa, tra cui Cdc25a e Cdc25c (G1/S e G2/M checkpoints control), p53 e MDMX (apoptosi), pRb e E2F1 (G1/S checkpoint e apoptosi), Che1 (apoptosi e G2/M checkpoint), Per1 (apoptosi?), Brca1 e XRCC1 (riparazione del danno al DNA), TRF2 (stabilità del telomeri), HuR (stabilità dell'RNA) e FoxM1 (trascrizione di enzimi della riparazione al DNA), PSME3/REGγ (stabilità dei centrosomi e regolazione di PML nuclear bodies).

Recentemente, il pathway ATM-Chk2 è stato implicato nella senescenza indotta da lesioni del DNA derivanti da accorciamento dei telomeri, dall'attivazione di oncogeni e da agenti genotossici. La senescenza rappresenta una fondamentale barriera imposta alle cellule precancerose e che se inattivata consente la progressione tumorale. Chk2 promuove la senescenza, e questa attività è repressa da TRF2, una subunità del complesso telomerico detto "shelterin". Poiché i telomeri normali impediscono l'inappropriata attivazione del DDR sia mascherando l'estremità del cromosoma dal complesso di riparazione del DNA, sia attraverso una repressione locale di ATM/ATR da parte di TRF2, sembra quindi che esista un'analogia tra ATM e Chk2 nell'interplay con TRF2 sui telomeri.

## **ROLE OF CHE-1 IN DNA DAMAGE AND CELLULAR STRESS RESPONSE**

Maurizio Fanciulli

*Regina Elena Cancer Institute, Rome*

In response to diverse cellular stresses, cells activate DNA damage checkpoint pathway to protect genomic integrity and promote survival of the organism. Depending on DNA lesions and context, damaged cells with activated checkpoint can be eliminated by apoptosis or silenced by cellular senescence, or can survive and resume cell cycle progression and cell metabolism upon checkpoint termination. DNA damage response machinery (DDR) is constitutively activated in early, premalignant lesions of major types of human solid tumors and defects in DDR components probably contribute to the pathogenesis of all types of human cancer. In the past few years we could show, that DNA damage and other types of cellular stress lead to stabilization and accumulation of Che-1, an RNA polymerase II-binding protein that plays an important role in transcription activation of p53 and in maintenance of the G2/M checkpoint. Furthermore, our recent studies indicate that Che-1 sustains mutant p53 transcription and controls DDR in cancer cells. Here, we will discuss new results from our lab on the involvement of Che-1 in the regulation of mTOR pathway and cell metabolism in response to cellular stress.

## THE INTERACTION BETWEEN MESENCHYMAL STEM CELLS AND BREAST CANCER CELLS: IMPLICATIONS FOR NOVEL THERAPEUTIC APPROACHES

Nicola Normanno

*Cell Biology and Biotherapy Unit, INT-Fondazione "G. Pascale", Naples, Italy*

Bone marrow-derived mesenchymal stem cells (MSCs) play an important role in breast cancer progression. MSCs are recruited to developing tumors where they can enhance the metastatic potential of weakly tumorigenic cancer cells. In addition, MSCs and other bone marrow derived cells can form a "pre-metastatic niche" within specific tissues to which tumor cells metastasize. MSCs may also sustain the growth and survival of cancer cells within the bone microenvironment where they can contribute to form "niches" for dormant micrometastases that may later seed distant metastases.

Early studies from our group suggested that epidermal growth factor receptor (EGFR) signaling might be involved in the MSC-mediated pathogenesis of bone metastasis. In fact, we showed that the EGFR regulates in MSCs the secretion of osteotropic factors such as M-CSF and RANKL, and their ability to induce osteoclast activation (Normanno et al. *Endocr Relat Cancer* 2005). More recently, we demonstrated that transforming growth factor  $\alpha$  (TGF- $\alpha$ ) significantly increased in MSCs the secretion of several angiogenic factors such as vascular endothelial growth factor-A (VEGF), angiopoietin-2 (ANG-2), granulocyte-colony stimulating factor (G-CSF), hepatocyte growth factor (HGF), interleukin (IL)-6, IL-8, and platelet-derived growth factor-BB (PDGF-BB). Interestingly, we found that treatment with TGF- $\alpha$  also increased the ability of MSCs to sustain the migration of breast cancer cells, and that this phenomenon was mediated by VEGF and IL-6 (De Luca et al. *J Cell Physiol* 2011). Finally, we recently investigated the effects of zoledronic acid (ZA) on the interaction between MSCs and breast cancer cells (Gallo et al. *Ann Oncol* 2011). We found that treatment with ZA significantly reduced the ability of MSCs to migrate and to sustain the migration of breast cancer cells. This latter phenomenon was due to the significant reduction in the secretion of RANTES/CCL5 and IL-6 that was observed in ZA-treated MSCs. Interestingly, we also found that the combination of IL-6 and RANTES showed a cooperative effect on cell migration and on the activation of ERK, AKT and STAT3 signaling in breast cancer cells. These data let us to hypothesize that the effects of ZA on MSCs within the bone marrow microenvironment significantly contribute to its anti-tumor activity by affecting the ability of MSCs to migrate to developing tumors and/or pre-metastatic niches; by reducing, in MSCs that migrate to metastatic sites, the secretion of factors that sustain breast cancer cell metastatization; and by disturbing the interaction between MSCs and breast cancer cells within the bone marrow microenvironment.

Taken together, our findings suggest that different mechanisms are involved in the interaction between MSCs and breast cancer cells, and that they offer several opportunities for novel therapeutic strategies.

## CHROMATIN REMODELLING AND SENESCENCE OF MESENCHYMAL STEM CELLS

Tiziana Squillaro<sup>3,5</sup>, Nicola Alessio<sup>1,6</sup>, Marilena Cipollaro<sup>3</sup>, Mariarosa Anna Beatrice Melone<sup>7</sup>, Giuseppe Hayek<sup>8</sup>, Alessandra Renieri<sup>2</sup>, Antonio Giordano<sup>1,4,5</sup> and Umberto Galderisi<sup>1,3,5</sup>

<sup>1</sup>*Sbarro Institute for Cancer Research and Molecular Medicine, Center for Biotechnology, Temple University, Philadelphia, PA, USA.* - <sup>2</sup>*Medical Genetics, University of Siena, Italy* - <sup>3</sup>*Department of Experimental Medicine, Biotechnology and Molecular Biology Section, Second University of Naples, Naples, Italy* - <sup>4</sup>*Human Pathology and Oncology Dept., University of Siena, Italy* - <sup>5</sup>*Human Health Foundation, Spoleto, Italy* - <sup>6</sup>*Department of Biomedical Sciences, University of Sassari, Italy* - <sup>7</sup>*Department of Neurological Sciences, Second University of Naples, Naples, Italy* - <sup>8</sup>*Childhood Neuropsychiatry, Azienda Ospedaliera Senese, Siena, Italy*

Methyl cytosine protein binding 2 (MECP2) binds preferentially to methylated CpGs and regulates gene expression by causing changes in chromatin structure. The mechanism by which impaired MECP2 activity can induce pathological abnormalities in the nervous system of patients with Rett syndrome (RTT) remains unknown. Studies in different animal models have produced conflicting results. In a mouse model of RTT syndrome, the results indicate that MECP2 is involved in the maturation and maintenance of neurons, whereas in *Xenopus* embryos, MECP2 mutations seem to affect neural cell fate decisions. To gain further insight into the role of MECP2 in human neurogenesis, we compared the neural differentiation process in mesenchymal stem cells (MSCs) obtained from a RTT patient and from healthy donors. We further analyzed neural differentiation in a human neuroblastoma cell line carrying a partially silenced *MECP2* gene.

Senescence and reduced expression of neural markers were observed in proliferating and differentiating MSCs from the RTT patient, which suggests that impaired activity of MECP2 protein may impair neural differentiation, as observed in RTT patients.

Next, we used an inducible expression system to silence *MECP2* in neuroblastoma cells before and after the induction of neural differentiation via retinoic acid treatment. This approach was used to test whether MECP2 inactivation affected the cell fate of neural progenitors and/or neuronal differentiation and maintenance.

Overall, our data suggest that neural cell fate and neuronal maintenance may be perturbed by senescence triggered by impaired MECP2 activity either before or after neural differentiation.

## HUMAN PLACENTA-DERIVED MESENCHYMAL STROMAL CELLS: WHERE DO WE STAND?

Ornella Parolini

*Centro di Ricerca E. Menni, Fondazione Poliambulanza-Istituto Ospedaliero, Brescia*

Multipotent mesenchymal stromal cells (MSCs) have been isolated from bone marrow and other stromal tissues of the body. Nevertheless, the need remains to identify appropriate MSC-rich sources which are ethically acceptable and do not require invasive procedures for cell recovery. Considering that term placenta is normally discarded as medical waste, the fact that placental tissues also represent a rich and easily accessible source of MSCs has inspired great hope regarding the clinical potential of placenta-derived MSCs, with encouraging results obtained to date.

In our research center, we are particularly interested in cells (hAMSC) isolated from the mesenchymal region of the human amniotic membrane (AM). Three main characteristics of hAMSC make them viable candidates for cell-based therapeutic approaches: i) their absent or *low immunogenicity and their immunomodulatory properties*; ii) their *multi-lineage differentiation capacity in vitro*, suggesting their utility in tissue regeneration approaches; and iii) their ability to *successfully engraft long-term in various organ/tissues* after transplantation into neonatal animals, without evidence of inflammation or rejection, therefore indicating active tolerance of these cells.

In particular, we have shown that hAMSC fail *in vitro* to induce an allogeneic T-cell response and actively suppress T-cell proliferation induced by alloantigens or by a mitogenic stimulus. Moreover, hAMSC can block differentiation and maturation of monocytes into dendritic cells, by arresting the cells in the G0 phase and abolishing the production of some inflammatory cytokines. Recently, we have also demonstrated that hAMSC exert a significant anti-proliferative action on different tumor cell lines, arresting these cells in the G0/G1 phase of the cell cycle.

Considering these immunomodulatory/inhibitory properties of hAMSC, and given that AM has a long history as a surgical material due to its anti-inflammatory, anti-scarring and wound healing properties, our group is currently investigating the effects of hAMSC and fragments of the AM *in toto* in pre-clinical models of diseases involving inflammatory and fibrotic mechanisms.

We demonstrated that transplantation of either allogeneic or xenogeneic fetal membrane-derived cells reduces lung fibrosis in bleomycin-challenged mice. Successful outcomes were also obtained when AM fragments were applied as patches onto rat hearts with cardiac ischemia and onto the liver surface of rats with fibrosis induced by bile duct ligation. In all of these pre-clinical settings, we found that donor cells in host tissues were rare or absent, suggesting that placental cells might exert reparative effects through yet unknown paracrine-acting factors. Meanwhile, the fact that injection of conditioned medium generated by AM derived cells into bleomycin-challenged mice reduced lung fibrosis supports the important role of soluble factors in AM-mediated therapeutic effects.

On these bases, human AM can be considered as a source of derivatives (AM-fragments, cells and soluble effectors) with multi-faceted properties that may be exploited for novel regenerative/reparative medicine approaches.

## **AMNIOTIC FLUID DERIVED STEM CELLS - A UNIQUE CELL SOURCE FOR CARDIOVASCULAR TISSUE ENGINEERING**

Simon P Hoerstrup  
*University of Zürich*

In today's cardiovascular clinical scenario, the highest medical need for a growing tissue engineered replacement is in the field of pediatric applications treating congenital heart disease. In these patients, the introduction of autologous living, growing replacement structures would significantly reduce today's severe therapeutic limitations, which are mainly due to the need for repeat reoperations to adapt the current artificial prostheses to the somatic growth. Based on advanced imaging techniques, an increasing number of defects are diagnosed already prior to birth around week 20. Ideally, the cells to be used for patients with congenital heart disease can be obtained already during pregnancy to provide the time for the tissue engineering process prior to birth. In recent *in vitro* studies we have demonstrated the feasibility of using freshly isolated as well as cryopreserved amniotic fluid-derived cells for tissue engineering of heart valves. The deriving heart valves demonstrated adequate functionality in a biomimetic system. Based on these results, future research must aim at a systematic preclinical evaluation of these prenatally harvested progenitors with regard to their potential clinical implementation.

## MESENCHYMAL STEM CELLS FROM WHARTON'S JELLY IN DOMESTIC ANIMALS

Eleonora Iacono, Barbara Merlo

*Department of Veterinary Medical Sciences, University of Bologna*

Wharton's jelly (WJ) derives from extra embryonic mesoderm, and is largely made up of mucopolisaccharides serving for the developing fetus. Indeed, its cells contain gelatin-like mucus that encase fiber: these properties give it an elastic and cushion effect, which can tolerate the vibration, bending, stretching and twisting of an active fetus. In addition, WJ holds vessels together, may regulate blood flow, plays a role in providing nutrition to the fetus, stores chemistry for the onset of the labor and protects the supply line. Since 1990, stromal cells, which basically resemble mesenchymal fibroblasts found elsewhere during in utero development, were identified in human WJ. Stemness of these cells has been demonstrated by *in vitro* differentiation and by the identification of membrane markers specific for MSCs. In veterinary medicine, Mitchell et al. (2003) reported that cells isolated from swine umbilical cord matrix could differentiate *in vitro* in neuronal-like cells, capable of expressing glial specific proteins; more recently, it has been demonstrated that porcine umbilical cord MSCs resembled pluripotent cells, since they express early transcription factors Oct-4, Sox-2 and Nanog (Carlin et al 2006). Instead, Nanog expression by caprine WJ contradicts data previously obtained in swine: caprine WJ cells showed short doubling time, ability to generate clones and *in vitro* differentiation capacity, but did not express Nanog gene (Babaei et al 2008). However, in this study, cryopreserved cells were used after thawing for RNA extraction, which may affect Nanog gene expression. In 2007, Hoyonowski et al. demonstrated the presence of MSCs in equine umbilical cord matrix: isolated cells underwent *in vitro* differentiation and showed expression of embryonic and mesenchymal markers. In our laboratory, fibroblast like cells were observed after culture of equine WJ digested sample; compared with cells isolated from cord blood or amniotic fluid, cultured under the same conditions, cells from WJ expanded more rapidly. After culture in differentiation media, cells stained positive with Alizarin Red, Alcian Blue e Oil Red O staining protocols. Furthermore, the same cells expressed mesenchymal markers such as CD90, CD44, CD105 and were negative for hematopoietic markers. Recently, MSCs were also isolated from canine WJ (Filioli Uranio et al 2011), and Azari et al (2011) demonstrated that MSCs isolated from caprine WJ could be used for regenerative therapy of induced skin wound. In our experience, MSCs isolated from equine WJ can be successfully used in foals with septicemic or decubitus skin wounds. These results demonstrate that equine MSCs from these samples can be induced to form multiple cell types that underlie their value for regenerative medicine in injured horses. Results reported in literature demonstrate that MSCs from domestic animals WJ could be used as a model to study cell biology and have an application in therapeutic programs.

## **MESENCHYMAL STROMAL CELLS: A NEW ACTOR IN ALLOGENEIC TRANSPLANTATION AND INFLAMMATORY BOWEL DISEASE?**

Franco Locatelli

*Dipartimento di Oncoematologia Pediatrica, IRCCS Ospedale Pediatrico Bambino Gesù, Roma, Università di Pavia*

Mesenchymal stromal cells (MSCs) are multipotent cells that can be isolated from several human tissues, including bone marrow, adipose tissue and cord blood, and that can be expanded *ex vivo* for clinical use. They comprise a heterogeneous population of cells, which, through production of growth factors, cell-to-cell interactions and secretion of matrix proteins, has a role in the regulation of hematopoiesis. In recent years, several experimental *in vitro* studies have shown that MSCs are endowed with immunomodulatory properties and with the capacity to promote graft survival in animal models. In view of these properties, MSCs have been tested in pilot studies aimed at preventing/treating graft rejection and at accelerating recovery after hematopoietic cell transplantation (HCST). The available clinical evidence deriving from these studies indicates that MSC infusion is safe and promising in terms of capacity of preventing graft failure in patients given a T-cell depleted hematopoietic stem cell transplantation from an HLA-haploidentical relative. More debated is the effect of MSCs for what concerns their capacity of accelerating hematopoietic reconstitution after cord blood transplantation. Whether the favorable effect of MSCs largely depends on the type of transplantation thus remains a field of future investigation. MSCs have been shown to be also an effective treatment for many patients who experience steroid-resistant acute graft-versus-host disease (GvHD). The probability of survival of patients responding to MSC infusion has been demonstrated to be significantly better than that of patients who did not respond to the treatment. The optimal dosage, number of administrations and drugs (if any) with which MSCs can be associated for treatment of patients experiencing GvHD remain to be defined. Experimental data suggested also that immune modulatory functions of MSCs contribute to tissue repair and immune tolerance. Crohn's disease is a prototype example of inflammatory, immune-mediated disease in which MSCs can be considered to promote tissue repair. It is characterized by an uncontrolled immune response to intestinal bacteria causing tissue damage which, in the penetrating phenotype, involves the entire gut wall up to formation of fistula tracks. Our group has preliminarily shown that intra-fistula injection of autologous, bone marrow-derived MSCs is a feasible and safe therapeutic approach for fistulizing Crohn's disease. Indeed, we conducted a phase I-II trial in which 7 of the 10 patients with fistulizing Crohn's disease given autologous MSCs benefit from the complete closure of the fistula tracks. The sustained complete closure (7 cases) or incomplete closure (3 cases) of fistula tracks with a parallel reduction of Crohn's disease and perianal disease activity indexes. The percentage of mucosal and circulating regulatory T cells significantly increased during the treatment and remained stable until the end of follow up. These data suggest that MSCs could open new scenarios in the treatment of immune-mediated disorders.

## **MSC AND AUTOIMMUNE DISEASE THERAPY - BEYOND TISSUE ENGINEERING**

Alan Tyndall

*University of Basel, Switzerland*

Over 1,500 patients world wide have received a hematopoietic stem cell transplant (HSCT) as treatment for a severe autoimmune disease (AD). Most of these have been autologous and mostly have occurred in the past 15 years. Over 1,000 of these have been registered in the European Group for Bone Marrow Transplantation (EBMT) and European League Against Rheumatism (EULAR) combined data base. A recent retrospective analysis of 900 patients<sup>1</sup> showed that the majority had multiple sclerosis (MS; n=345) followed by systemic sclerosis (SSc; n=175), systemic lupus erythematosus (SLE; n=85), rheumatoid arthritis (RA; n=89), juvenile idiopathic arthritis (JIA; n=65) and idiopathic cytopenic purpura (ITP; n=37). An overall 85% 5-year survival and 43% progression free survival was seen, with 100-day transplant related mortality (TRM) ranging between 1% (RA) and 11% (SLE and JIA). Around 30% of patients in all disease subgroups had a complete response, often durable despite full immune reconstitution. In many, e.g. systemic sclerosis, morphological improvement such as reduction of skin collagen and normalisation of microvasculature was documented, beyond any predicted known effects of intense immunosuppression alone. The high TRM was in part related to conditioning intensity, comorbidity and age, and the final risk/ benefit assessment will be made after the results of the three randomised prospective clinical trials are known.

Recently, multipotent mesenchymal stromal cells (MSC) have are being tested in various AD, exploiting their immune modulating properties and apparent low acute toxicity. Despite encouraging small phase I/II studies, no positive data from randomised, prospective studies are as yet available in the peer reviewed literature.

## **BONE MARROW-DERIVED MESENCHYMAL STEM CELL THERAPY FOR NEURODEGENERATIVE DISEASES**

Franca Fagioli

*AO O.I.R.M. - S. Anna di Torino*

Amyotrophic Lateral Sclerosis (ALS), Alzheimer's disease (AD) and Parkinson's disease (PD) are the most common neurodegenerative disorders. The aetiology is unknown and despite different pathological hallmarks and classical clinical symptoms they share a common characteristic being neuronal loss. The available treatments provide symptomatic relief, none of them change the course of the disease. Stem-cell-based therapies represent a new possible scenario for neurodegenerative diseases.

Mesenchymal Stem Cells (MSCs) are multipotent stem cells that are very attractive in view of a possible cell therapy approach in neurodegenerative diseases because of their great plasticity and their ability to provide the host tissue with growth factors or modulate the host immune system. The administration of bone marrow-derived MSCs has led to beneficial effects in animal models for several neurodegenerative diseases. Expanded MSCs can survive and migrate after transplantation in the lumbar spinal cord of SOD1G93A mice, where they prevent astrogliosis and microglial activation and delay ALS-related decrease in the number of motoneurons.

Encouraging data obtained with stem cells in animal models of neurodegenerative diseases led recently to the first clinical trials transplanting MSCs. We performed two Phase I trials in ALS for the assessment of the feasibility and toxicity of transplantation of autologous MSCs into the spinal cord. The trials were approved and monitored by the National Institute of Health and by the Ethics Committees. 19 patients (11 M and 8 F) with ALS were enrolled in two consecutively phase 1 clinical trials. Nine subjects participated in the first trial that started in November 2001 and 10 patients participated in the second study that started in September, 2003.

Patients were followed up for 6-9 months and then treated with autologous mesenchymal stem cells isolated from bone marrow and implanted into the dorsal spinal cord with a surgical procedure. In addition to the clinical measures, we also took particular care to analyze behavioral and quality of life changes.

Eight patients died after a mean survival time of 31.6 (+21 SD) months from surgery (Range: 9-74). All deaths were deemed to be unassociated with the experimental treatment.

The most important result of our study is the neuroradiological demonstration of the lack of tumor formations or abnormal cell growth. All patients well tolerated the procedure and the side effects related to the surgery were mild and transient. Also, from the psychological point of view there was no deterioration in psychosocial status and all patients coped well. Only one patient reported a negative impact of the trial on their health and quality of life.

Our results show the safety of MSC transplantation in the central nervous system during a follow-up of nearly 9 years and is in support of applying MSC-based cellular clinical trials to neurodegenerative disorders.

## TRANSLATING RESEARCH INTO CLINICAL SCALE MANUFACTURING OF MESENCHYMAL STROMAL CELL

Karen Bieback

*Institute of Transfusion Medicine and Immunology, Medical Faculty Mannheim, Heidelberg University; German Red Cross Blood Service Baden-Württemberg – Hessen, Germany*

The idea of stem cell therapy sound so simple: obtain sufficient numbers of cells from human tissues, isolate and expand the stem cells and then transplant the appropriate number of cells at the correct location to the patient. However it is a complex, multistep process to translate research findings into routine therapies, because the latter have to comply with regulatory guidelines. Here the challenge relates to carefully balance the expected therapeutic benefits with potential risks. Mesenchymal stromal cells (MSC) are interesting examples in this context as these cells were already therapeutically applied in a very early phase of research. And up to now research and clinical trials run in parallel pointing out the importance of a “Bench to Bedside and Back”- process. Initially the therapeutic hope based on the multilineage differentiation potential. Clinical studies, however, indicated that the level of engraftment is much too low to explain the therapeutic benefits. Thus back in the research lab, data indicated that MSC have strong immune modulatory and pro-regenerative capacities which translated into clinical studies evaluating therapeutic efficacy in these contexts.

The therapeutic aim is to repair cell or tissue damage but without the risk of inducing tumors, severe immune reactions, or unwanted tissue development. Thus both safety and efficacy measures shall be considered in the establishment of the manufacturing process. Scarcity of

MSC often requires *ex vivo* expansion; extensive expansion in consequence may lead to ineffective or degenerated cells. Thus it is important to understand and carefully control the production process and accordingly to define measures that reliably predict safety and efficacy of cell therapeutics. To ensure that the product is safe, pure and potent, this approval by the regulatory bodies requires manufacturing, processing, and testing of cellular products according to current regulations including Good Tissue Practice, Good Manufacturing Practice and Good Clinical Practice. For MSC, general considerations in the translation process concern the procurement and potential of different cellular sources, therapeutic potency of MSC e.g. differentiation or immune modulation, and the manufacturing process including devices and material and quality control assays.

## VALIDATION OF ANALYTICAL METHODS IN GMP: A PRACTICAL APPROACH

Ivana Ferrero

*AO O.I.R.M. - S. Anna di Torino*

The quality and safety of advanced therapy products (ATP) must be maintained throughout their production and quality control (QC) cycle, ensuring their final use in the patient.

QC methods of ATP must be validated, complying with ICH Q2 Guidelines, considering the tests' accuracy, precision, repeatability, specificity, detection limit, linearity and range.

We validated three QC methods according to GMP and ICHQ2 rules, to evaluate the cell count, the LAL test and the immunophenotype analysis.

Materials and methods.

The cell count was performed by using the disposable count chamber Fast read 102® (a plastic slide divided into 10 grid wells [Volume: 10 µl]), already validated in our laboratory as an alternative count method to the Burkler chamber. The cell/ml concentration value is determined by the following calculation:  $(\sum \text{cells in 5 squares}/5) \times \text{dilution factor} \times 10^4$ . As the cell count is a potency test, during the validation we checked the following parameters: accuracy, precision, linearity. The evaluation of cell viability was performed by using Trypan Blue vital dye.

To validate the endotoxin test we used a kinetic Chromogenic LAL test. This is a limit test for the control of impurities, so in compliance with ICHQ and EU Pharmacopoeia we evaluated precision, specificity and detection limit. The test was performed on supernatant, on cell therapy products at different dilutions, and on pyrogen-free water as negative control.

The immunophenotype validation required performance qualification of the FACS Canto II using two types of standard beads, used daily to check cytometer reproducibly set up. The results obtained with both beads were compared together. As an identity test, we evaluated specificity by using the fluorescence minus one method (FMO): each cell population was stained with all kinds of fluorochrome except the one of interest. All experiments were repeated thrice to test precision.

Collected data were statistically analyzed by calculating means (M), standard deviation (DS) and coefficient of variation percentage (CV%) inter and intra operator.

Results:

All the tests performed met the established acceptance criteria: CV% <10%. For the cell count the precision reached by each operator had a CV% <10%; reproducibility: the CV% of the cell viability calculated in three different counts performed by the same operator was <5%.

The LAL test performed thrice on the same samples by a qualified operator is repeatable. The test was considered specific, because the percentage of spike recovery of each sample was between 0.25 UE/ml and 1 UE/ml with a CV% less than 10%. Correlation coefficient ( $\leq 0.980$ ) and CV% (<10%) of standard curve tested in duplicate showed the test's linearity and the minimum detectable concentration value of 0.005 EU/ml.

The results obtained during immunophenotype validation confirmed again the reproducibility and the specificity of the test.

## **STEM CELLS IN VETERINARY MEDICINE: THE ITALIAN REGULATORY**

Salvatore Macri

*Ministero della Salute, Roma*

The Decree No. 193/2006 implementing Directive 2004/28/EC amending the Community code for medicinal products is the rule which regulates the production and use of heterologous stem cells in veterinary medicine.

Currently a regulatory gap remains regarding the use of autologous stem cells in veterinary medicine, therefore, the Ministry of Health has embarked on a process for the preparation of specific guidelines.

The purpose of the guidelines is to define the general conditions and procedural guidelines for operators involved in the collection, handling, storage and clinical use of stem cells, following calls CSM in Veterinary Medicine.

In the presentation will illustrate the possible future directions related aspects are listed below: collection of biological material and manipulation of stem cells, processing, transportation, conservation, distribution and administration of multipotent stromal cells (MSC), traceability and recording data, labeling; features processing laboratory of the CSM.

# ABSTRACTS

## Posters



foto&grafica: Mauro Di Giovanni

**P1. HEPATITIS C VIRUS INDIRECTLY PROMOTES VIA FOCAL ADHESION KINASE THE CLEAVAGE OF POLY ADP-RIBOSE POLYMERASE AND THE ACQUISITION OF PRO-FIBROGENIC PHENOTYPE IN HEPATIC STELLATE CELLS**

Anna Alisi<sup>1</sup>, Mario Arciello<sup>2</sup>, Stefania Petrini<sup>4</sup>, Beatrice Conti<sup>2</sup>, and Clara Balsano,<sup>2</sup>  
<sup>1</sup>*Liver Research Unit of "Bambino Gesù" Children's Hospital and Research Institute, Rome, Italy;* <sup>2</sup>*Laboratory of Molecular Virology and Oncology, Fondazione A. Cesalpino, University "La Sapienza", Rome, Italy*

Liver fibrosis is a wound healing response, resulting from continuous destruction of liver parenchyma and its replacement by scar tissue. Hepatitis C virus (HCV) infection is one of the most common etiological factors in the development of fibrosis and its progression to hepatocellular carcinoma (HCC). The pivotal role of the epithelial-mesenchymal transition (EMT) and activation of hepatic stellate cells (HSCs) in liver fibrogenesis is now certain, while the network of molecular interactions connecting the HCV infection of hepatocytes with the HSC activation remains unclear. Focal adhesion kinase (FAK) is emerging as direct master regulator of HSCs activation. Therefore, firstly in this study we explored the indirect effects whether HCV may indirectly induce EMT and activate HSCs.

**Methods:** As model of HCV infection and replication, we used permissive Huh7.5.1 hepatoma cells infected with JFH1. Conditioned medium from these cells were used to stimulate human HSC line LX-2. We performed both functional assays (cytotoxicity, proliferation, DNA damage and adhesion), and molecular analysis (western blotting, immunoprecipitation assays and immunocytochemistry).

**Results:** HCV infection decreased adhesion of Huh7.5.1 cells and caused the removal of alpha-actinin from plasma membrane. These effects were accompanied by an increased FAK activation, that physically interact and phosphorylated alpha-actinin. The treatment of LX-2 cells, with conditioned medium from HCV-infected Huh7.5.1 cells, caused an increase in DNA damage measured as cleavage of poly ADP-ribose polymerase (PARP). Moreover, we observed an increased cell proliferation, expression of alpha-smooth muscle actin and hyaluronic acid release. All these findings suggest an indirect role of HCV hepatocyte infection on EMT and activation of HSCs. Silencing of FAK by siRNA revert the direct effects of HCV infection on Huh7.5.1 cells, and its indirect effects on the activation LX-2 cells. Interestingly, the conditioned medium from HCV-infected Huh7.5.1 cells were enriched in tumor necrosis factor-alpha, another effect that we found reverted under FAK silencing conditions.

**Conclusions:** Our findings demonstrate that HCV, through FAK activation is able to promote cytoskeletal reorganization in hepatocyte-like cells and DNA damage and pro-fibrogenic phenotype in HSCs. These data not only add relevant pieces to the puzzle of cellular/molecular interactions that characterize HCV-dependent fibrogenesis, but also provide novel potential clues to improve anti-fibrotic therapies.

## **P2. LIGANDI SPECIFICI PER IL DNA G-QUADRUPLIX: VALUTAZIONE DI AFFINITÀ MEDIANTE ESI-MS E STUDI DI ATTIVITÀ ANTITUMORALE SU COLTURE CELLULARI**

Alessandro Altieri<sup>a</sup>, Antonello Alvino<sup>a</sup>, Armandodoriano Bianco<sup>a</sup>, Annamaria Biroccio<sup>b</sup>, Silvia Borioni<sup>a</sup>, Valentina Casagrande<sup>a</sup>, Luca Ginnari-Satriani<sup>a</sup>, Marco Franceschin<sup>a</sup>, Carlo Leonetti<sup>b</sup>, Maria Savino<sup>a</sup>.

<sup>a</sup>*Dipartimento di Chimica, "La Sapienza" Università di Roma;* <sup>b</sup>*Laboratorio Chemioterapica Sperimentale Preclinica, Istituto Regina Elena, Roma*

Nella progettazione di nuovi farmaci antitumorali, concepiti come inibitori della telomerasi, una strategia largamente seguita consiste nell'indurre il suo substrato, il DNA telomerico, ad assumere una conformazione che non permetta il legame con l'enzima. I risultati migliori in questo campo si sono ottenuti con molecole in grado di indurre e stabilizzare strutture G-quadruplex, dove con questo termine si intendono particolari strutture a quadrupla elica assunte da sequenze di DNA ricche in guanine (quali quelle telomeriche) caratterizzate dallo specifico accoppiamento di quattro guanine basato su legami idrogeno di tipo Hoogsten. A questa classe di inibitori appartengono molecole aventi come caratteristica comune un esteso nucleo aromatico da cui si dipartono catene laterali basiche, cosicché tali molecole interagiscono col DNA in maniera sia idrofobica (interazioni di *stacking*), che elettrostatica.

In quest'ottica, sono state sintetizzate numerose molecole a scheletro perilenico, coronenico, truxenico e xantonico; inoltre sono stati derivatizzati analoghi di molecole naturali aventi proprietà antitumorali quali la taspina e la berberina.

In questa poster verranno illustrati i risultati dello studio delle interazioni tra tali molecole ed una serie di oligonucleotidi-modello, mediante spettrometria di massa ESI. La tecnica di Ionizzazione *Electro-Spray* permette infatti di osservare e quantificare le interazioni non covalenti che si possono avere tra il DNA e le piccole molecole.

I dati così ottenuti sono confrontati con quelli provenienti da saggi biofisici come il FRET (*Fluorescence resonance energy transfer*) per lo studio della stabilizzazione del G-quadruplex e studi di biologia molecolare mediante saggio TRAP (*Telomerase Repeat Amplification Protocol*) per lo studio dell'inibizione della telomerasi. Sui composti più interessanti sono stati condotti studi di attività antitumorale su colture cellulari.

### **P3. TEMOZOLOMIDE INDUCED c-MYC-MEDIATED APOPTOSIS VIA AKT SIGNALING IN MGMT EXPRESSING GLIOBLASTOMA CELLS**

Donatella Amendola<sup>1</sup>, Igea D'Agnano<sup>2</sup>, Rodolfo Marchese<sup>1</sup>, Antonio Stigliano<sup>1,3</sup>, Ugo De Paula<sup>4</sup>, Barbara Bucci<sup>1</sup>

<sup>1</sup>Centro Ricerca S. Pietro, Fatebenefratelli Hospital, <sup>2</sup>Istituto di Biologia Cellulare e Neurobiologia CNR, <sup>3</sup>Cattedra di Endocrinologia, II Facoltà di Medicina, Università "La Sapienza", <sup>4</sup>U.O. di Radioterapia Oncologica S. Pietro, Fatebenefratelli Hospital, Rome, Italy

**Purpose:** We investigated the molecular mechanisms underlining the cytotoxic effect of Temozolomide (TMZ) in human O6-Methylguanine-DNA Methyl transferase (MGMT) depleted or not glioblastoma cell lines. Since TMZ is used in clinics in combination with Radiotherapy, we also studied the effects of TMZ in combination with Irradiation (IR).

**Methods:** Cell colony-forming ability was performed by using clonogenic assay. Cell cycle analysis and apoptosis were evaluated by Flow Cytometry (FCM). Cell cycle molecules were detected by Western blot and co-immunoprecipitation assay.

**Results:** Our data showed that TMZ, independent of MGMT expression, inhibited glioblastoma cell growth via an irreversible G2 block in MGMT depleted or the induction of apoptosis in MGMT expressing cells. When TMZ was administered in combination with IR, the apoptosis was greater than the one observed with the two agents used separately. This TMZ-induced apoptosis in the MGMT expressing cells occurred through Akt/Glycogen-Synthase-Kinase-3 $\beta$  (GSK3 $\beta$ ) signaling and was mediated by Myelocytomatosis (c-Myc) oncoprotein. Indeed, TMZ phosphorylated/activated Akt led to phosphorylation/inactivation of GSK3 $\beta$  which resulted in the stabilization of c-Myc protein and subsequent modulation of the c-Myc target genes involved in the apoptotic processes.

**Conclusion:** c-Myc expression could be considered a good indicator of TMZ effectiveness.

#### **P4. A TRANSGENIC MOUSE MODEL TO IMAGE NF-Y TRANSCRIPTIONAL ACTIVITY IN PHYSIOLOGICAL PROCESSES OF LIVING ORGANISMS**

S. Artuso, I. Manni F. Goeman, B. Ramachandran, G. Bossi, C. Cencioni, F. Spallotta, G. Toietta, A. Maggi, M. Capogrossi, C. Gaetano, A. Sacchi, P. Ciana and G. Piaggio  
*Istituto Tumori Regina Elena, Roma*

The transcription factor NF-Y plays a fundamental role in key cellular processes like cell cycle, proliferation and differentiation. In agreement with this, it was shown to be essential for mouse embryonal development and perturbations of the NF-Y are involved in the pathogenesis of many human diseases including cancer. To study the temporal and spatial patterns of NF-Y-dependent transcription in living animals, we developed a new tool based on a NF-Y-responsive luciferase reporter (MITO-luc mice). We engineered transgenic mice expressing luciferase under the control of a promoter fragment whose activity is strictly NF-Y-dependent enabling real-time *in vivo* imaging of NF-Y activity in intact animals under true physiological condition. By *in vivo* and *ex vivo* bioluminescence imaging (BLI) and *in vitro* luciferase activity assays, we show that our reporter system is sensitive to monitor changes in cellular NF-Y activity and we demonstrate that NF-Y is active in highly proliferating tissues.

The MITO-luc reporter mice should facilitate the development of new anti/pro-proliferative drugs as well as investigations of the involvement of proliferation in disease pathogenesis.

## P5. LIVE CONFOCAL IMAGING OF MELANOMA AND ITS APPLICATION

Barbieri A<sup>1</sup>; Giudice A.<sup>1</sup>; Palma G.<sup>1</sup>; Rea D<sup>1</sup>.; Luciano A<sup>1</sup>.; Arra C<sup>1</sup>; Vannucci L<sup>2</sup>.

<sup>1</sup>Istituto Nazionale Tumori Fondazione G.Pascale, Napoli; <sup>2</sup>Accademia delle Scienze di Praga

Multiple biomedical imaging techniques are used in all phases of cancer management and represent an essential part of cancer clinical protocols. Imaging techniques are able to give morphological, structural, metabolic and functional information of cancer. Integration with other diagnostic tools, such as *in vitro* tissue and fluids analysis, assists in clinical decision-making. Early detection of cancer through screening based on imaging, is probably the major contributor to a reduction in mortality for certain cancers. Molecular imaging (MI) has made a significant impact on the field of oncology and it is playing an important role in the diagnosis and treatment of cancer. Thanks to molecular imaging, is possible to elucidate the basic mechanisms underlying cancer biology and to identify the most promising strategies for human clinical trials. In this way is possible to characterize many tumors and their response to therapy . In order to dissect the morph structural and functional properties of melanoma from the capsule to the inner of mass, we applied live confocal imaging techniques in mice C57BL/6 injected subcutaneously with 5.10 5 B16F10 murine melanoma cell line in the hind right leg. We find that this kind of tumor consists of a large proportion of collagen ( in green) in which melanoma cells (in red) are immersed in the inner part of tumor capsule, showing that melanoma cells are autofluorescent . Going deeper in the tumor mass, we showed that the initial ordinate organization of collagen disappeared, becoming less dense. All together these informations let us to know the biology and structural organization of tumor and microenvironment and its changes during treatments (i.e angiogenesis, necrosis, apoptosis and drug delivery). These findings will shed light on new strategies to improve drug delivery and the outcome of the treatments.

## **P6. RESVERATROL ACTS AS A TOPOISOMERASE II $\alpha$ POISON: INDUCTION OF DNA DAMAGE AND INHIBITION OF CHROMOSOME SEGREGATION**

E.Basso<sup>1</sup>, M.Fiore<sup>2</sup>, S.Leone<sup>1</sup>, F.Degrassi<sup>2</sup> and R.Cozzi<sup>1</sup>

<sup>1</sup> *Dipartimento di Biologia, Università "Roma TRE", Roma, Italia;* <sup>2</sup> *Istituto di Biologia Molecolare e Patologia CNR, Roma, Italia*

Resveratrol (3,4',5-trihydroxystilbene) is a well known polyphenol synthesized by a wide variety of plant species in response to injury, UV irradiation and fungal attack. Since in 1997 Jang and co-workers(1) showed the antitumoral potential of resveratrol (RSV) *in vivo*, many studies have revealed a variety of resveratrol intracellular targets whose modulation gives rise to overlapping responses that lead to growth arrest and death. One of the most interesting issue is the involvement of RSV in the maintenance of genomic stability through physical and chemical interactions with DNA, but also influencing the redox state of cells. On the other hand, RSV could also affect some aspects of DNA metabolism such as DNA repair, recombination and chromatin structure maintenance thereby indirectly modulating the integrity of genomic DNA.

Recently we demonstrated that RSV is able to induce a delay in S progression with a concomitant increase in  $\gamma$ H2AX expression in U87 glioma cells(2). Furthermore we showed it interferes on Topoisomerase II $\alpha$  (TOPO II) activity inducing micronuclei as a consequence of the conversion of TOPOII/DNA cleavable complexes to permanent DNA damage(3).

Here we present data about the activity of resveratrol on chromosome stability and segregation in CHO cells *in vitro*. RSV causes an increase of both structural and numerical chromosome aberrations as measured by the Cytokinesis-Block Micronucleus Assay implemented with the detection of kinetochores in micronuclei. RSV is also found to be able to affect the correct chromosome segregation as showed by the increase of polyploid and endoreduplicated cells. An effect on chromosome segregation, possibly mediated by the inhibition of TOPO II-mediated decatenating activity, is also suggested by the accumulation of early stage mitotic figures and the increase of anaphase bridges.

The confirmation of RSV as a TOPO II poison together with its ability in delay cell cycle progression, could render this molecule an important anti-tumour agent against high grade gliomas which are highly resistant to most therapies. The combination of RSV and other anti-glioma therapies may be a novel strategy for the treatment of glioma that deserve further investigation.

(1) M. Jang et al - Science 275 (1997) 218-220

(2) S.Leone et al - Cancer Lett 295 (2010) 167-72

(3) S.Leone et al – Int J Cancer (2011) in press

## **P7. ANTICANCER ACTIVITY OF THE NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITOR ABACAVIR ON PC3 PROSTATE CANCER CELL LINE**

G. Bozzuto<sup>1</sup>, L. Toccaceli<sup>1</sup>, G. Formisano<sup>1</sup>, F. Carlini<sup>2</sup>, B. Ridolfi<sup>2</sup>, G. Rezza<sup>3</sup>, A. Molinari<sup>1</sup>, S. Gaudi<sup>3</sup>

<sup>1</sup>*Department of Technology and Health, Istituto Superiore di Sanità, Rome, Italy;*

<sup>2</sup>*Department of Therapeutic Research and Medicines Evaluation, Istituto Superiore di Sanità, Rome, Italy;* <sup>3</sup> *Department of Infectious, Parasitic and Immune-Mediated Diseases, Istituto Superiore di Sanità, Rome, Italy*

Transposable Elements (TEs) comprise nearly 45% of the entire genome and are part of sophisticated regulatory network systems that control developmental processes in normal and pathological conditions. The retroviral/retrotransposon gene machinery consists mainly of Long Interspersed Nuclear Elements (LINEs-1) and Human Endogenous Retroviruses (HERVs) that code for their own endogenous reverse transcriptase (RT). Interestingly, RT is typically expressed at high levels in cancer cells. Recent studies report that RT inhibition by non-nucleoside reverse transcriptase inhibitors (NNRTIs) induces growth arrest and cell differentiation in vitro and antagonizes growth of human tumors in animal model. In the present study we analyze the anticancer activity of Abacavir (ABC), a nucleoside reverse transcription inhibitor (NRTI), on PC3 prostate cancer cell lines. Here we demonstrate that ABC induces a significant decrease in cell growth rate and a delay of the cell cycle in S phase. A high percentage of senescent cells were observed and many of them were committed to death. Few hours of ABC exposure significantly reduced the potential of migration and invasion in prostate cancer cells. Consistent with these observations, microarray analysis on PC3 cells shows that ABC induces specific and dose-dependent changes in gene expression, involving multiple cellular pathways. Notably, by quantitative Real-Time PCR we found that LINE-1 ORF1 and ORF2 mRNA levels were significantly up-regulated by ABC treatment. Our results demonstrate the potential of ABC as anticancer agent able to induce antiproliferative activity and trigger senescence in prostate cancer cells. Noteworthy, we show that ABC elicits up-regulation of LINE-1 expression, suggesting the involvement of these elements in the observed cellular modifications. These findings strongly support the emerging concept of endogenous RT as an attractive target in cancer therapy, providing an innovative strategy to circumvent the difficulties related to the genetic heterogeneity of cancer phenotypes.

## **P8. CHE-1 REGULATES THE MTOR PATHWAY IN RESPONSE TO GENOTOXIC AND METABOLIC STRESS**

<sup>1</sup>Tiziana Bruno, <sup>1</sup>Agata Desantis, <sup>1</sup>Valeria Catena, <sup>1</sup>Francesca De Nicola, <sup>1</sup>Frauke Goeman, <sup>1</sup>Simona Iezzi, <sup>1,5</sup>Cristina Sorino, <sup>2</sup>Maria Cristina Valerio, <sup>4</sup>Paolo D'Onorio De Meo, <sup>4</sup>Tiziana Castrignanò, <sup>2</sup>Cesare Manetti, <sup>5</sup>Aristide Floridi, <sup>1</sup>Giovanni Blandino, <sup>3</sup>Claudio Passananti and <sup>1</sup>Maurizio Fanciulli

<sup>1</sup>*Regina Elena Cancer Institute, Rome*; <sup>2</sup>*University of Rome "La Sapienza"*; <sup>3</sup>*CNR-IBPM Rome*; <sup>4</sup>*Caspur*; <sup>5</sup>*University of L'Aquila*

In all eukaryotes, mTOR is a master regulator that integrates the signals from nutrients and energy sensors with cell growth and proliferation. Nevertheless, mTOR not only controls the rate of protein synthesis, but also regulates transcriptional changes in response to a variety of conditions. In recent years, numerous stimuli have been shown to cause changes in the activity of mTOR cascade, and in addition, decreased mTOR signaling activity has found associated with many types of stress, suggesting that this pathway plays an important role in the adaptation to different stress conditions. Since Che-1 has been found involved in cellular response to several stress conditions, we hypothesized an involvement of Che-1 in the regulation of mTOR signaling that controls cell growth, proliferation and survival. Che-1 was found specifically phosphorylated by Chk2 in response to hypoxic and metabolic stress. Moreover, from an Affimetrix microarray analysis we observed that Che-1 depletion strongly inhibits the transcription of several genes involved in the regulation of mTOR activity. In particular, Che-1 depletion strongly down-regulates Sestrin3, Redd1, Redd2 and Deptor mRNA levels. Consistent with these findings, Che-1 depletion strongly affected mTOR inhibition in response to several cellular stresses, but the rescue of Redd1 or Deptor expression completely abrogated this effect. In addition, we found that Che-1 depletion strongly decreases the Warburg effect observed in hypoxic cells, and combined metabolomic and RNA-seq analyses revealed that the expression of several enzymes involved in metabolic pathways is regulated by Che-1. Finally, we demonstrated that Redd1 and Deptor expression mediate these effects.

### **P9. POLY (ADP-RIBOSE) POLYMERASE (PARP) INVOLVEMENT IN THE APOPTOSIS OF A PROSTATE CANCER CELL LINE TREATED WITH TRANSFORMING GROWTH FACTOR-BETA 3**

Caggia S, Frasca G, Graziano A. C. E., Cardile V

*Dipartimento di Scienze Bio-mediche, Sezione Fisiologia, Università degli Studi di Catania*

The poly (ADP-ribose) polymerase (PARP) family of proteins, of which there may be as many as 18 members, is characterized by the enzymatic property of poly (ADP-ribosylation). In the cell's nucleus, it is involved in a number of cellular processes relating mainly DNA repair and programmed cell death. PARP-1 is responsible for at least 80% of total cellular PARP activity, and together with its nearest relative PARP-2, constitutes the DNA damage response arm of the PARP family. PARP-1 and PARP-2 are DNA damage sensors, in that they bind rapidly to sites of DNA damage, and also DNA damage signalers, in that this binding activates their catalytic function which in turn modulates a wide range of proteins involved in the DNA damage response. Although PARP-1 binds to both single- and double-stranded DNA breaks, its role in single-strand break (SSB) repair via the base excision repair (BER) pathway has been most clearly defined. Once PARP detects a SSB it binds to the DNA, and, after a structural change, begins the synthesis of a poly (ADP-ribose) chain (PAR) as a signal for the other DNA repairing enzymes. PARP is inactivated by caspase cleavage. It is believed that normal inactivation occurs in systems where DNA damage is extensive. In these cases, more energy would be invested in repairing damage than is feasible, so that energy is instead retrieved for other cells in the tissue through programmed cell death. Several forms of cancer are more dependent on PARP than regular cells, making PARP an attractive target for chemotherapeutic cancer therapy. Transforming growth factor-beta (TGF- $\beta$ ) is the prototype of a family of secreted polypeptide growth factors. These cytokines play very important roles during development, as well as in normal physiological and disease processes, by regulating a wide array of cellular processes, such as cell growth, differentiation, migration, apoptosis, and extracellular matrix production. The aim of this research was to verify in DU-145, an androgen-non responsive prostate cancer cell line, the effects of the dose-dependent TGF- $\beta$ 3-treatment on the expression of some pro-apoptotic proteins. We have determined the expression of YY1, p53, PI3K, AKT, pAKT, PTEN, Bcl-2, Bax, iNOS, and PARP through Western blot analysis in DU-145 cultures treated with 10 and 50 ng/ml of TGF- $\beta$ 3 for 24 h. The results showed that in DU-145 cells TGF- $\beta$ 3 decreases YY1 and increases p53; hyper-physiological levels of p53 decrease expression of PI3K, Akt, pAkt, and Bcl-2, increase PTEN, and Bax, activating pro-oxidant genes to produce iNOS. Furthermore, TGF- $\beta$ 3 downregulates PARP expression. We believe that the findings could be important because of the clinical relevance that they may assume and the therapeutic implications for TGF- $\beta$  treatment of prostate cancer.

**P10. METHYLATION OF GSTP1, ERA, RASSF1A, CDKN2 (P16 INK4A) AND MGMT GENES: POTENTIAL BIOMARKERS IN PROSTATE CANCER**

Carosi M<sup>1</sup>, Francesconi A<sup>1</sup>, Sentinelli S<sup>1</sup>, Gallucci M<sup>2</sup>, Canalini P<sup>1</sup>, Feola A<sup>3</sup>, Di Domenico M<sup>3</sup>, Russo A<sup>1</sup>, Pescarmona E<sup>1</sup>

<sup>1</sup>Pathology Regina Elena National Cancer Institute of Rome, <sup>2</sup>Urology Regina Elena National Cancer Institute of Rome, <sup>3</sup>Department of General Pathology, Second University of Naples

Prostate cancer is the most common malignancy of the urogenital tract. To date, prostate-specific antigen (PSA) testing is widely used for screening and follow-up of prostate cancer. We currently lack the necessary tools to differentiate between latent disease with little likelihood of clinical manifestation and aggressive tumours that are likely to metastasize and lead to potentially lethal disease. Recent investigations revealed the aberrant DNA promoter hypermethylation as promising biomarker. DNA methylation is an important epigenetic mechanism of gene regulation and plays essential roles in tumour initiation and progression. The simultaneous analysis of the methylation status of many genes as tumour-suppressor genes, proto oncogenes, genes involved in cell adhesion, and genes involved in cell-cycle regulation can determine the fingerprints of prostate cancer. Glutathione S-transferase P1 (GSTP1) has been shown to be a biomarker for prostate cancer. In our study we analyzed the methylation promoter status of GSTP1, ER $\alpha$ , RASSF1A, CDKN2 (p16 INK4A) and MGMT genes in 25 patient specimens collected at the Urology Department of Regina Elena National Cancer Institute of Rome. DNA was isolated from the paraffin-embedded tissue macrodissected from histologically marked slides. DNA was modified by sodium-bisulfite treatment. Purified DNA was subjected to PCR amplification with specific primers designated to distinguish methylated from unmethylated DNA. The PCR products were analyzed on 3% agarose gels and visualized by UV transilluminator. Our preliminary results showed that the promoter methylation of MGMT was present in 70% of cases, p16INK4A in 22%, RASSF1A 86% and ER $\alpha$  89%, respectively. Our preliminary studies confirmed that the hypermethylation of either GSTP1, RASSF1A and ER $\alpha$  has been shown to be both sensitive and specific for detecting prostate cancer. Thus, preliminary results on the use of the methylation status of specific genes as potential tumour biomarkers and the risk stratification of patients with prostate cancer are promising. Future studies should focus on developing drug that can target specific genes.

**P11. O<sup>6</sup>-METHYLGUANINE DNA METHYLTRANSFERASE (MGMT): EXPRESSION IN LUNG NEUROENDOCRINE CANCER**

Carosi M<sup>1</sup>, Baldelli R<sup>2</sup>, Francesconi A<sup>1</sup>, Visca P<sup>1</sup>, Covello R<sup>1</sup>, Palmarelli P<sup>1</sup>, Barnabei A<sup>2</sup>, Appetecchia M<sup>2</sup>, Facciolo F<sup>3</sup>, Di Domenico M<sup>4</sup>, Pescarmona E<sup>1</sup>

<sup>1</sup>Pathology, <sup>2</sup>Endocrinology, <sup>3</sup>Thoracic Surgery, Regina Elena National Cancer Institute of Rome, <sup>4</sup>Department of General Pathology Second University of Naples

Neuroendocrine carcinomas (NECs) represent relatively rare and heterogeneous malignancies. New somatostatin analogs, covering a higher number of SSTR subtypes, were developed, including pasireotide (SOM230), which controls 25% of carcinoid syndromes resistant to full dose of somatostatin analogs. Chimeric analogs, which bind SSTR2/SSTR5 and dopamine-2 receptor subtype (D2), are in preclinical phase of development. Among the numerous molecular targeted agents investigated in NETs, mTOR inhibitors and VEGF/VEGFR/PDGFR inhibitors are in most advanced clinical phase of investigation. In particular, everolimus, sunitinib, and bevacizumab are all studied in phase III trials. Both everolimus and sunitinib produced significant survival benefit versus placebo in advanced progressing well-differentiated pancreatic NECs. In recent years, the oral alkylating agent temozolomide (TMZ) has emerged as an active agent in PNETs. Like dacarbazine, TMZ is converted to the active alkylator MTIC that induces DNA methylation at the O6 position of guanine. A phase II study investigating the combination of TMZ and thalidomide demonstrated an objective response rate of 45% in the PNET subset of patients. A recent retrospective study of TMZ combined with capecitabine in 30 chemo-naive PNET patients reported an objective radiographic response rate of 70% and median progression free survival of 18 months. Side effects were relatively tolerable, with a grade 3/4 adverse event rate of only 12%. The aim of this study was to evaluate the expression of MGMT methylation in neuroendocrine tumors of the lung (NET-lung); the method to determine the hypermethylation status of MGMT, namely methylation-specific PCR, allowing the selection of patients most likely to benefit from TMZ treatment. DNA was isolated from the paraffin-embedded tissue macrodissected from histologically marked slides. DNA was modified by sodium-bisulfite treatment. Purified DNA was subjected to PCR amplification with specific primers designated to distinguish methylated from unmethylated DNA. The PCR products were analyzed on 3% agarose gels and visualized by UV transilluminator. Previous results seem to indicate that the MGMT methylation of the promoter is present in 4 of 7 samples evaluated (57%). Considering the significant effect and the few adverse effects, there might be a wider indication for TMZ treatment of aggressive NET-lung. However, more data are necessary to decide whether MGMT methylation should be used as a surrogate marker for predicting tumour TMZ sensitivity.

## **P12. ANTICANCER ACTIVITY OF LIPOSOMAL CISPLATIN (LIPOPLATIN) IN CISPLATIN-SENSITIVE AND -RESISTANT OVARIAN CANCER CELLS**

Naike Casagrande, Monica De Paoli, Marta Celegato, Cinzia Borghese, Maurizio Mongiat, Alfonso Colombatti, Donatella Aldinucci

*Centro di Riferimento Oncologico, Division of Experimental Oncology 2, Aviano (PN), Italy*

Cisplatin is one of the most widely used and effective chemotherapeutic agents for the treatment of several human malignancies; unfortunately its continued use is greatly limited by severe dose limiting side effects and intrinsic or acquired drug resistance. Recently a novel liposomal formulation of cisplatin, lipoplatin (Regulon, Inc., Mt. View, U.S.A) was developed in order to reduce the systemic toxicity of cisplatin while simultaneously improving the targeting of drugs to primary tumour and metastasis.

The aim of this preclinical study was to evaluate lipoplatin activity in the cisplatin (CDDP)-sensitive human ovarian adenocarcinoma cell line 2008, and its -resistant variant, C13\*. Lipoplatin demonstrated to inhibit cell proliferation in a dose-dependent manner and to be more active than the reference drug cisplatin in the cisplatin-resistant C13\* cell line. It proved to be cytotoxic against cisplatin-resistant C13\* cells, with activity levels comparable to those induced on the parent cisplatin-sensitive 2008 cells. Treatment of both 2008 and C13\* cells with lipoplatin induced apoptosis, as evaluated by Annexin-V staining and DNA fragmentation, activation of caspases 8, 9 and 3, Bcl-2, Bcl-XL down-regulation and Bax up-regulation. Lipoplatin induced a blockade at the G2M phase of the cell cycle in 2008 cells but only slightly affected the cell cycle of C13\* cells. In addition, lipoplatin inhibited the activity of thioredoxin reductase (TrxR), a selenoenzyme which is over-expressed in many tumor cells contributing to drug resistance, and increased Reactive Oxygen Species (ROS) accumulation, also in the presence of the ROS scavenger N-Acetylcysteine (NAC). Furthermore, lipoplatin reduced the expression of EGFR and its phosphorylated form, and inhibited both 2008 and C13\* cells migration. Cisplatin-resistant C13\* cells, but not 2008 cells, were able to generate multicellular spheroid. Treatment with Lipoplatin, but not cisplatin, was able to inhibit spheroid formation and to induce apoptosis. In formed multicellular spheroid lipoplatin increased apoptosis, as evaluated by Annexin-V staining after disruption by Trypsin-EDTA. Finally lipoplatin treatment of nude mice carrying C13\* ovarian tumor significantly inhibited tumor growth *in vivo*. Altogether, our results confirmed that lipoplatin has potential for the treatment of ovarian cancer.

### **P13. RESVERATROL SYNERGIZE WITH THE RAF KINASE INHIBITOR SORAFENIB ON THE GROWTH INHIBITION OF HUMAN CANCER CELL**

Castellano M, Lombardi A, Misso G, Zappavigna S, Luce A, Porto S, Marra M, Giuberti G, Murolo M, De Rosa G, Stiuso P, Caraglia M, Abbruzzese A

*Dipartimento di Biochimica e Biofisica "F. Cedrangolo", Seconda università degli studi di Napoli*

We have studied the effects of resveratrol on the inhibition of the cellular growth and on the apoptosis of the cells of pancreatic carcinoma . We have demonstrated that the resveratrol has an important effect of inhibition dose- manner dependent on all cellular lines studied (BxPc3, MiaPaca, Panc-1).

Nonetheless, despite all of the impressive health benefits being attributed to resveratrol, considerable controversy exists as to whether resveratrol is the active molecule in vivo. The controversy stems from the fact that resveratrol is rapidly metabolized to its 3- and 4'-O-sulfate, and 3-O-glucuronide conjugates. In the light of these considerations, a new formulation able to change resveratrol pharmacokinetic and pharmacodistribution, inducing a longer half-life into the blood, would be of great potential usefulness to take advantage of the resveratrol anti-apoptotic and anti-proliferative effect in peripheral tumours. The use of nanotechnologies can be used to change pharmacokinetic profile of drugs. In particular, stealth liposomes, have been largely investigated to enhance efficacy as well as to reduce the toxic side-effects of anticancer drugs.

In this way, the stealth liposomes can freely circulate without being destroyed by the immune system because dressed again with a film of polietilenglicole (PEG), an inactive substance that not alert the immune system and they stay in the blood for 2-3 weeks without opening, then they gradually enter the cancerous cells passing from the gating of the capillary ones of the tumor. The new capillaries to bedew the cancer areas, in fact, are more permeable in comparison to those of the healthy fabrics, therefore they make a sort of accumulation of the liposomes in the neoplastic fabric. Just in the microenvironment of tumour the liposome releases the active principle, the resveratrol, that so it can develop its toxic action on the cancerous cells. We have so drawn a new pharmacological formulation of resveratrol that foresees its insertion inside liposomes and we evaluated the inhibitory effects of these agents (liposome encapsulated with resveratrol) against pancreatic cancer growth and progression.

The effects of anti-tumor activity of resveratrol encapsulating liposomes were evaluated in vitro on BxpC3, MiaPaca and Panc-1 and on breast carcinoma cells MDA MB 231, MDA MB 436, CG5.

In all cell lines both formulations of liposomes encapsulating RESV potentiated growth inhibition induced by free Resveratrol.

#### **P14. SYNERGISTIC ACTIVITY OF “VERTICAL” COMBINATIONS OF AGENTS TARGETING THE RAF/MEK/ERK CASCADE AS A THERAPEUTIC STRATEGY IN HUMAN TUMORS**

Cesta Incani U, Ciuffreda L, Di Sanza C, Falcone I, Del Curatolo A, Cognetti F, Milella M  
*Medical Oncology A, Regina Elena National Cancer Institute, Rome, Italy*

Background: ATP-competitive, BRAF-selective, kinase inhibitors have potent antitumor effects in mutant BRAF(V600E) tumors and are clinically effective in malignant melanoma; however, under certain conditions they paradoxically activate the MEK/ERK kinase module downstream. In addition, different tumor models exhibit variable responses to MEK inhibition and MEK blockade may induce compensatory signaling through both upstream pathway elements (RAF) and parallel pathways (PI3K/AKT/mTOR).

Methods: We set out to define molecular and functional effects of single and combined BRAF (GSK2118436A, BRAF-I) and MEK (GSK1120212B, MEK-I) inhibition, using WB analysis to dissect signaling and fixed dose-ratio experimental design (1000:1) to assess functional synergism by conservative isobologram analysis.

Results: In A549 lung adenocarcinoma (KRAS G12S), BRAF-I (10 nM) induces hyperphosphorylation of MEK, ERK and p90RSK, while MEK-I (10 nM), alone or in combination with BRAF-I, potently offsets MAPK activation. Combined BRAF-I and MEK-I suppress malignant growth and survival at 72 h with highly synergistic effects in the A549 lung adenocarcinoma (KRAS G12S), HCT116 colon carcinoma (KRAS G13D), and MIAPACA pancreatic adenocarcinoma (KRAS G12V) models (combination indexes – CI – 0.077, 0.001, and 0.047, respectively). Conversely, in other lung cancer models with Q61H and G12C KRAS mutations (H460 and Calu-1, respectively) or wt-KRAS (Calu-3) the combination of BRAF-I and MEK-I produced modestly additive (H460, CI 0.8) to highly antagonistic antitumor effects (Calu-1 and Calu-3, CI 2x10<sup>4</sup> and 4.4, respectively). Similar results were obtained in melanoma models: in the M14 model (mut-BRAF/wt! -NRAS), both BRAF-I and MEK-I had pronounced growth inhibitory effects as single agents, but were frankly antagonistic in combination; in the ME1007 model (wt-BRAF/mut-NRAS), MEK-I, but not BRAF-I, effectively inhibited cell growth but there was no synergistic effect with the combination, despite the fact that BRAF-I induced MEK/ERK hyperactivation.

Conclusions: Overall, our data indicate that combined inhibition of multiple signaling elements along the RAF/MEK/ERK pathway results in strongly synergistic growth inhibition, particularly in tumors with specific KRAS mutations. Additional studies to better define genetic determinants of sensitivity/resistance and molecular mechanisms of therapeutic synergism of combined BRAF-I and MEK-I are currently ongoing.

## **P15. LA REGOLAZIONE DEI GENI ATG MODULA LA RISPOSTA DELLA CELLULA TUMORALE AI CHEMIOTERAPICI**

Condello M<sup>1</sup>, Lista P<sup>2</sup>, Arancia G<sup>1</sup>, Meschini S<sup>1</sup>

<sup>1</sup>Dipartimento Tecnologie e Salute, <sup>2</sup>Dipartimento Farmaco - Istituto Superiore di Sanità, Roma

Nello studio di nuovi farmaci ad azione antitumorale bisogna tener conto delle principali caratteristiche delle cellule tumorali e del microambiente in cui esse si sviluppano. Esse sono in grado di sviluppare meccanismi subcellulari di sopravvivenza all'azione citotossica dei chemioterapici. Alcuni tumori, infatti, sviluppano forme di resistenza all'apoptosi, principale meccanismo di morte cellulare indotto dai farmaci convenzionali. L'esistenza di meccanismi di morte alternativi, come l'autofagia, consente di superare, in taluni casi, tale problema. È noto che man mano che le cellule tumorali crescono e si trasformano in una massa solida, nutrienti ed ossigeno iniziano a scarseggiare all'interno della massa stessa diventando così metabolicamente stressata. In queste regioni l'autofagia interviene come meccanismo di difesa cellulare, poichè rifornisce la cellula dei nutrienti necessari. Sia come meccanismo di morte che di difesa cellulare, l'autofagia inizia con l'inglobamento degli organuli citoplasmatici danneggiati o di altro materiale cellulare all'interno di strutture caratteristiche costituite da doppia membrana, chiamate autofagosomi, e prosegue con la fusione di questi con i lisosomi, formando gli autofagolisosomi, dove si ha la digestione o la modificazione del materiale da riutilizzare. Considerando il duplice ruolo svolto dall'autofagia, numerosi studi sono stati avviati allo scopo di comprendere i pathways molecolari coinvolti e le principali proteine deputate al controllo di questo complesso processo. I prodotti dei geni *ATG* (*AuTophagic*) controllano le fasi principali del processo autofagico per cui la comprensione di questi fattori costituisce un ottimo punto di partenza per poter modulare il processo autofagico a scopi terapeutici.

Scopo del nostro lavoro è stato quello di studiare il ruolo di alcuni geni *ATG* nella autofagia indotta su una linea di osteosarcoma umano in seguito al trattamento con la voacamina, un alcaloide indolico naturale estratto dalla pianta *Peschiera fuchsifoliae*. In prima analisi la microscopia elettronica a trasmissione ha confermato l'ipotesi che la voacamina induce autofagia, dimostrando la presenza di vacuoli autofagici. Mediante analisi con Western Blotting è stata verificata la conversione della proteina LC3 (omologa di Atg8) dalla forma LC3-I alla forma LC3-II; tale proteina è, infatti, associata agli autofagosomi maturi. Inoltre l'uso di inibitori di diverse fasi del processo autofagico (3-metiladenina, bafilomicina-A, E64d e pepstatina) e l'analisi dell'espressione di LC3 in Western Blotting e microscopia confocale hanno dato un notevole contributo per lo studio del flusso autofagico nel nostro modello sperimentale. I nostri studi sono proseguiti con esperimenti di silenziamento dei principali geni *ATG* (*ATG5*, *ATG6*, *ATG7* o *ATG12*) con la valutazione dell'effetto indotto da tale silenziamento sulla citotossicità della voacamina. Il silenziamento di ciascun gene, in particolare dell'*ATG5*, riduce la conversione della proteina LC3 dalla forma I a quella II e la morte cellulare indotta dalla voacamina, dimostrando l'esistenza di una relazione diretta tra l'induzione dell'autofagia come meccanismo di morte cellulare e l'espressione in particolare dei geni *ATG5* e *ATG8* (LC3). Gli studi futuri permetteranno di approfondire il coinvolgimento di altri geni, come l'*ATG6*, con lo scopo ultimo di modulare l'autofagia a scopo terapeutico.

## P16. THE FIRST SELECTIVE DNA G-QUADRUPLEX GROOVE BINDERS: EVIDENCE FOR TELOMERIC DNA DAMAGE AND TUMOR CELL DEATH

Sandro Cosconati<sup>‡</sup>, Angela Rizzo<sup>\*</sup>, Bruno Pagano<sup>¶</sup>, Sara Iachettini<sup>\*</sup>, Roberta Trotta<sup>¶</sup>, Stefano De Tito<sup>†</sup>, Ilaria Lauri<sup>‡</sup>, Luciana Marinelli<sup>†</sup>, Concetta Giancola<sup>°</sup>, Ettore Novellino<sup>†</sup>, Annamaria Biroccio<sup>\*</sup>, Antonio Randazzo<sup>¶</sup>

<sup>‡</sup>*Dipartimento di Scienze Ambientali, Seconda Università di Napoli*; <sup>\*</sup>*Centro Ricerche Sperimentali, Istituto Regina Elena, Roma*; <sup>¶</sup>*Dipartimento di Scienze Farmaceutiche, Università di Salerno*; <sup>†</sup>*Dipartimento di Chimica Farmaceutica e Tossicologica, Università di Napoli "Federico II"*; <sup>°</sup>*Dipartimento di Chimica delle Sostanze Naturali, Università di Napoli "Federico II"*; <sup>°</sup>*Dipartimento di Chimica "P. Corradini", Università di Napoli "Federico II"*

Targeting of DNA secondary structures such as G-quadruplexes is now considered an appealing opportunity for drug intervention in anticancer therapy. So far, efforts made in the discovery of chemotypes able to target G-quadruplexes mainly succeeded in the identification of a number of polyaromatic compounds featuring end-stacking binding properties. Against this general trend, we were persuaded that the G-quadruplex grooves can recognize molecular entities with better drug-like and selectivity properties. Our efforts resulted in the discovery of six compounds that were able to bind the [d(TGGGGT)]<sub>4</sub> G-quadruplex groove. In the present study, the most promising derivative was used as a seed for searching similar entities in several commercially available databases and NMR experiments allowed to identify a small focused library of structural analogues with G-quadruplex groove binding properties. By a back and forth approach, the structural features responsible for G-quadruplex groove recognition were delineated, while isothermal titration calorimetry (ITC) measurements allowed for the identification of chemotypes featuring a tighter binding than distamycin A, which is the most potent G-quadruplex groove binder known so far. Differently from distamycin A, the best binders also proved to be G-quadruplex selective over duplex. These results propelled the biological characterization of the new ligands demonstrating their ability to induce selective DNA damage at telomeric level and induction of apoptosis and senescence on tumor cells. These results finally demonstrate that induction of tumor cell death can be efficiently achieved by compounds able to selectively target the G-quadruplex grooves.

## **P17. MOLECULAR ALTERATIONS OF SURVIVAL PATHWAYS IN OVARIAN CARCINOMA CELLS RESISTANT TO PLATINUM COMPOUNDS**

Cossa G, Lanzi C, Gatti L, Cassinelli G, Carenini N, Zunino F, Zaffaroni N and Perego P  
*Fondazione IRCCS Istituto Nazionale Tumori, Milano*

Resistance of cancer cells to antitumor agents is a multifactorial event and may involve the deregulation of pro-survival signaling pathways. Previous evidence from our laboratory indicates that ovarian carcinoma cells selected from the IGROV-1 cell line for resistance to cisplatin (IGROV-1/Pt1) and oxaliplatin (IGROV-1/OHP) display increased phospho-ERK1/2 levels. Thus, signalling occurring through the EGFR-Ras-Raf-MEK1/2-ERK1/2 axis, by mediating activation of downstream events, may play a role in the resistance of ovarian carcinoma cells to platinum compounds. The aims of the present study were to explore the molecular mechanism leading to activation of survival pathways in these cell systems and to design drug combinations to improve cell sensitivity to platinum compounds. Because ERK1/2 kinases are known to be dephosphorylated by dual specificity phosphatases (DUSPs), we examined the expression of DUSPs in the studied cell lines both at the transcriptional (i.e., using qPCR analyses) and protein level (i.e., Western blot). Using these approaches, we found that the expression of several members of the DUSP family (i.e., DUSP5, DUSP6, DUSP23) were reduced in IGROV-1/Pt1 and IGROV-1/OHP and in other cisplatin-resistant ovarian carcinoma cell lines as compared to their parental counterparts. Such findings suggest that DUSP downmodulation may play a role in the hyperactivated status of ERK1/2. As MEK1/2 kinases play a central role in ERK1/2 pathway activation, we tested whether their pharmacological inhibition could increase sensitivity to platinum compounds in IGROV-1 cells and restore sensitivity in IGROV-1/Pt1 and IGROV-1/OHP platinum resistant cells. Treatment of IGROV-1 cells with cisplatin/oxaliplatin in combination with the MEK1/2 inhibitor CI-1040 resulted in a synergistic effect, as evaluated by the combination index method. Treatment of IGROV-1/OHP cells evidenced a slight synergism for the cisplatin/CI1040 combination, whereas an additive effect was obtained for the oxaliplatin/CI1040 combination. In the IGROV-1/Pt1 variant, an additive effect was obtained with both platinum drugs. The mechanism underlying synergism and cell death induction was further analyzed. IGROV-1 cells underwent apoptotic cell death through the intrinsic pathway (e.g., caspase 9 cleavage), after the upregulation of BH3-only proteins PUMA and Bad. In detail, Bad phosphorylation – a known inactivation mechanism mediated by survival kinases – was relieved after cell treatment with cisplatin/oxaliplatin in combination with CI-1040. This may explain at least in part the synergism, as confirmed by a higher caspase 3/9 cleavage after combination treatment. In contrast, IGROV-1/OHP cells displayed a lower caspase 3/9 cleavage after combination treatment, as compared with singledrug treatments. Overall, our results support that targeting survival pathways activated by EGFR may increase cell sensitivity to platinum compounds. However, the efficacy of this approach appears related to the molecular alterations of tumor cells including the down-regulation of specific DUSPs and may be reduced in cell systems with acquired drug resistance.

**P18. EPIGENETIC FINGERPRINT IN ENDOMETRIAL CARCINOGENESIS: THE HYPOTHESIS OF AN UTERINE FIELD CANCERIZATION**

Antonia Feola, Daniele Conti, Valentina Tomei, Maria, Antonia Carosi, Angela Santoro, Maria Ludovica Genna, Arianna Francescani, Gaetano De Rosa, Michele Caraglia, Giuseppe Pannone and Marina Di Domenico

*Department of Human Pathology and Oncology University of Siena; Department of Pathology, National Cancer Institute Regina Elena, Rome; Department of General Pathology, Second University of Naples*

Transcriptional silencing by CpG island hypermethylation plays a critical role in endometrial carcinogenesis. In a collection of benign, premalignant and malignant endometrial lesions, a methylation profile of a complete gene panel, such steroid receptors (*ER $\alpha$* , *PR*), DNA mismatch repair (*hMLH1*), tumour-suppressor genes (*CDKN2A/P16* and *CDH1/E-CADHERIN*) and WNT pathway inhibitors (*SFRP1*, *SFRP2*, *SFRP4*, *SFRP5*) was investigated in order to demonstrate their pathogenetic role in endometrial lesions.

Our results indicate that gene hypermethylation may be an early event in endometrial endometrioid tumorigenesis. Particularly, *ER $\alpha$* , *PR*, *hMLH1*, *CDKN2A/P16*, *SFRP1*, *SFRP2* and *SFRP5* revealed a promoter methylation status in endometrioid carcinoma, whereas *SFRP4* showed demethylation in cancer. P53 immunostaining showed weak-focal protein expression level both in hyperplasic lesions and in endometrioid cancer. Non endometrioid cancers showed very low levels of epigenetic methylations, but strong P53 protein positivity. Fisher exact test revealed a statistically significant association between *hMLH1*, *CDKN2A/P16* and *SFRP1* genes methylation and endometrioid carcinomas and between *hMLH1* gene methylation and peritumoral endometrium ( $p < 0.05$ ).

Our data confirm that the methylation profile of the peritumoral endometrium is different from the altered molecular background of benign endometrial polyps and hyperplasias. Therefore, our findings suggest that the methylation of *hMLH1*, *CDKN2A/P16* and *SFRP1* may clearly distinguish between benign and malignant lesions. Finally, this study assessed that the employment of an epigenetic fingerprint may improve the current diagnostic tools for a better clinical management of endometrial lesions.

Methylation-specific PCR (MSP) was performed to assess gene inactivation. P53 and steroid receptors expression were evaluated by LSAB/HRP immunohistochemistry.

**P19. cAMP-PKA SIGNALS REGULATE THE INTERACTION OF KDM1/LSD1 WITH ESTROGEN RECEPTOR AND PROTEINS OF THE TRANSCRIPTION INITIATION COMPLEX**

A. Feola, A. Bertoni, A. Morano, F. Aceto, P. Giovannelli, C. Garbi, A. Migliaccio, A. Porcellini, Avvedimento V.E. and M. Di Domenico.

*Dip. Patologia Generale, Seconda Università di Napoli, Italia*

Lysine specific demethylase, LSD1 or KDM1, binds the active estrogen receptor alpha and demethylates histone H3 lysine 9 (me2K9) at the promoters and ERE sites of several estrogen-induced genes. Its activity is essential for estrogen-induced transcription. The binding of the receptor to the enzyme is dependent on LSD1 phosphorylation by cAMP-PKA and mutagenesis of a threonine at the NH2 terminal segment of LSD1 (T112) severely impairs estrogen-induced transcription and local H3K9 demethylation induced by estrogen, without compromising the enzymatic activity. PKA phosphorylation of this site is essential for releasing LSD1 from the nuclear co-repressor complex N-CoR and for the binding of LSD1 to the estrogen receptor and several transcription factors, including the large subunit of RNA polymerase II, the single strand binding protein, RPA, and the catalytic subunit of PKA. Conversely, inhibition of PKA-induced phosphorylation of LSD1 dissociates the enzyme from the receptor complex and from chromatin of estrogen-induced genes. PKA phosphorylation of the enzyme is secondary to estrogen receptor activation, because inhibition of estrogen receptor by ICI prevents LSD1 phosphorylation by PKA. Unexpectedly, we find that the LSD1 mutant that cannot be phosphorylated by PKA does not bind the co-repressor complex N-CoR and stimulates LSD1-silenced genes and the basal transcription of estrogen-induced genes.

These data illustrate the mechanism of activation of LSD1 by estrogens and point to the hierarchy of multiple signals elicited by estrogens during the transit from the membrane to the nucleus.

## **P20. RUOLO DEL GENE ONCOSOPPRESSORE KCTD11<sup>REN</sup>(KCTD11) NEL CARCINOMA PROSTATICO UMANO**

M Fischietti, D Nicosia, R Gallo, D Capece, D Verzella, F Zazzeroni, A Gulino, E Alesse  
*Università degli Studi de L'Aquila*

Il carcinoma prostatico è associato a numerose alterazioni del patrimonio genetico. La perdita di geni oncosoppressori localizzati sui cromosomi 8p, 10q, 16q, 17p, 18q e 21q sembra essere un evento importante nell'insorgenza del carcinoma prostatico (CaP) e della neoplasia intraepiteliale prostatica (PIN). In particolare, l'analisi dei microsatelliti ha rivelato un aumento della frequenza della perdita di eterozigosi (LOH) della regione cromosomica 17p13 (microsatellite D17S960) sia nella PIN che nel CaP, suggerendo che i geni localizzati in questo *locus* genico possano avere un ruolo importante nella progressione tumorale. Il gene KCTD11<sup>REN</sup>(KCTD11) mappa nel *locus* 17p13.2, in una regione in parte coincidente con quella del marcatore microsatellite D17S960. KCTD11 è un gene oncosoppressore la cui funzione è perduta nei medulloblastomi, sia in seguito a delezione allelica sia per l'intervento di meccanismi epigenetici che ne silenziano l'espressione. Inoltre, KCTD11 è un inibitore del signaling di Sonic-Hedgehog/Patched/Gli-1, una delle principali pathway responsabili della proliferazione cellulare nel carcinoma prostatico. In questo lavoro abbiamo dimostrato che KCTD11 è un gene oncosoppressore frequentemente mutato nel CaP. Oltre alla perdita di eterozigosi di KCTD11, nei campioni prostatici tumorali analizzati si osserva una riduzione dell'espressione della proteina, probabilmente dovuta ad eventi di regolazione epigenetica. In questo contesto tumorale, l'inattivazione di KCTD11 è associata ad una deregolazione della pathway di Sonic-Hedgehog e della proliferazione cellulare in quanto l'espressione forzata di KCTD11 in linee cellulari prostatiche determina un blocco della proliferazione, dovuto, almeno in parte, all'induzione degli inibitori del ciclo cellulare p27/Kip1 e p21/Waf1. La caratterizzazione di nuovi geni coinvolti nella tumorigenesi prostatica è di fondamentale importanza per l'identificazione di potenziali markers diagnostici e/o prognostici per questa neoplasia.

**P21. DELIVERY OF A SPECIFIC PHOSPHOTYROSYL-PEPTIDE AFFECTING ERBB-3/p85 INTERACTION OVERCOMES TRASTUZUMAB RESISTANCE OF HUMAN BREAST CANCERS**

Folgiero V, Di Carlo SE, Bossi G, Spugnini EP, Bon G, Di Benedetto A, Germoni S, Accardo A, Milella M, Morelli G, Mottolese M, Falcioni R  
*Regina Elena Cancer Institute, Rome*

Amplification of ErbB-2 oncogene occurs in almost 25% of breast cancers and is associated with poor patient outcome. Trastuzumab (Herceptin), a humanized monoclonal antibody directed to the ectodomain of ErbB-2, induces clinical responses in ErbB-2-overexpressing tumors prolonging patient survival. Nevertheless, mostly metastatic breast cancers escape Herceptin treatment suggesting that these tumors possess different mechanisms that can induce therapeutic resistance. Among the EGFR family, the ErbB-2/ErbB-3 heterodimer is the strongest stimulator of the PI3K/Akt pathway, which has been reported as an oncogenic unit acting to counteract the effects of Herceptin treatment. By the use of a phosphopeptide mimicking the binding site of ErbB-3 for the N-SH2 domain of p85, we demonstrate that depletion of ErbB-3/p85 interaction strongly induces apoptosis and sensitizes responsiveness to Herceptin treatment in ErbB-2 overexpressing breast cancer cells. The administration of phosphopeptide *in vivo* inhibits 80% tumor take that is abolished in combination with Herceptin therapy. The lung metastases formation is abrogated by delivery of the phosphopeptide encapsulated into liposomes with Herceptin treatment. These results indicate that ErbB-3 receptor is clinically relevant in predicting the response to Herceptin therapy in metastatic ErbB-2 overexpressing breast cancers and that this specific phosphopeptide could represent a new therapeutic strategy to overcome Herceptin resistance.

## **P22. ANALISI DEL miRNoma NELL'EPATOCARCINOMA INDOTTO DA DIETILNITROSAMINA**

Gaggiano A, Tessitore A, Capece D, Fischietti M, Verzella D, Zazzeroni F, Gulino A, Alesse E

*Dipartimento di Medicina Sperimentale - Università degli Studi de L'Aquila*

Il carcinoma epatocellulare (HCC) costituisce una delle più frequenti cause di morte per cancro. Evidenze sempre maggiori indicano che sia alterazioni genetiche o epigenetiche di regioni codificanti sia una deregolazione dei microRNA (miRNA) giocano un ruolo rilevante nello sviluppo dell'HCC. I miRNA sono piccole molecole di RNA (18-22 nucleotidi) non codificante che agiscono come regolatori dell'espressione proteica tramite la degradazione o l'inibizione traduzionale di specifici RNA messaggeri. I miRNA sono coinvolti in svariati processi biologici e patologici e, in particolare, nell'insorgenza e progressione del cancro, influenzando la proliferazione cellulare, l'apoptosi, la migrazione e la capacità invasiva. Crescenti evidenze mostrano inoltre che i miRNA possono essere utilizzati come indici diagnostici, prognostici o bersagli terapeutici nel cancro.

In questo studio è stato testato l'effetto della dietilnitrosamina (DEN), un carcinogeno chimico in grado di indurre carcinomi epatici, in un modello murino. Gli animali sono stati trattati con DEN e sacrificati dopo 11 mesi. Il tessuto tumorale epatico è stato analizzato per la caratterizzazione del miRNoma in comparazione con il tessuto epatico normale, ottenuto da topi non trattati. Risultati preliminari hanno messo in luce diversi miRNA differenzialmente espressi nei tumori (i.e. mir-21, mir-31, mir-155, mir-574-3p, mir-145). Ulteriori analisi sono in corso per la conferma e la validazione dei dati.

Inoltre, sullo stesso modello murino, è iniziato uno studio di epatocarcinogenesi indotta da una dieta ad alto contenuto lipidico, capace di provocare steatosi epatica non alcolica (NAFLD) e successivamente evoluzione ad epatocarcinoma. Anche in questo modello l'analisi del miRNoma sarà effettuata in relazione alle diverse fasi di progressione del danno epatico.

### **P23. MicroRNA REGULATION BY MUTANT P53 ONCOPROTEIN IN COLON CANCER**

A. Gurtner, V. Ambrosino, E. Falcone, G. Bossi, A. Sacchi, G. Piaggio

*Dept. of Experimental Oncology, Laboratory of Molecular Oncogenesis, Regina Elena Cancer Institute, Rome*

miRNAs have emerged as key post-transcriptional regulators of gene expression (Lim LP et al Nature 2005) involved in diverse physiological and pathological processes.

Deregulated miRNA expression has been documented in diverse cancers (Lynam-Lennon N et al Biol Rev Camb Philos Soc 2009). Although miRNAs can function as both tumour suppressors and oncogenes in tumour development (Esquela-Kerscher A et al Nature Rev Cancer 2006), a widespread downregulation of miRNAs is commonly observed in human cancers and promotes cellular transformation and tumorigenesis (Kumar M. S et al Nature Genet 2007; Chang T C et al Nature Genet 2008; Zhang L et al PNAS U S A 2008 ; Lynam-Lennon N et al Biol Rev Camb Philos Soc 2009). Thus, miRNAs may be potential targets for cancer therapy (Manni I et al FASEB J 2009; Lynam-Lennon N et al Biol Rev Camb Philos Soc 2009 ; Fabbri M et al Expert Opin Biol Ther 2007); still, the mechanisms through which miRNAs are regulated in cancer remain unclear. Wtp53 is a key tumor suppressor mutated in approximately 50% of human cancers (Brosh R et al Nat Rev Cancer 2009). Mutp53 proteins can acquire GOF activities favoring tumor induction, maintenance, spreading (Brosh R et al. Nat Rev Cancer 2009; Di Agostino S et al Cancer Cell 2006; Gurtner A et al JBC 2010). miRNAs can be regulated by wtp53 at transcriptional level (Hermeking H Cancer Cell 2007; Shi M et al Biocim Biophys Acta 2010; Yamakuchi M et al PNAS U S A 2010) but data about mutp53 dependent miRNA expression are not available yet. wtp53 interaction with the Drosha processing complex facilitates the processing of pri-miRNAs to pre-miRNAs. Of note, an overexpressed mutp53 interacts with Drosha processing complex and impairs the pri-miRNAs processing of 2 miRNAs (Suzuki HI et al Nature 2009). Taken together, these data support the idea that mutp53 might exert its GOF activity being responsible for the miRNA downregulation present in cancer.

In order to identify new mechanisms underlying mutp53 GOF activity associated with dysregulation of microRNA in cancer, we have performed a genome wide analysis of miRNA expression (352 miRNAs, TebuBio) in colorectal adenocarcinoma SW480 before and after mutp53 depletion. Our preliminary results revealed that mutp53 depletion is associated with up-regulation of 31 mature miRNAs (corresponding to 41 miRNA genes) and down-regulation of only 3 miRNAs. Validation of genome wide miRNA expression profile by qRT-PCR analysis for mature forms and primary precursors (pri-miRNAs) shows that mutp53 plays a role both at transcriptional and posttranscriptional level.

All together these preliminary results suggest a main role for mutp53 in the down-regulation of miRNA expression in cancer cells.

#### **P24. ALTERATION OF CELL CYCLE CHECKPOINTS CONFERS SENSITIVITY TO THE G-QUADRUPLEX LIGAND RHPS4 IN NORMAL CELLS**

Iachettini S, Salvati E, Rizzo A, Marcellini V, Porru M, Mondello C<sup>1</sup>, D’Incalci M<sup>2</sup>, Leonetti C and Biroccio A

*Experimental Chemotherapy Laboratory, Regina Elena Cancer Institute, Rome;*  
*<sup>1</sup>Genetics Molecular Institute, CNR, Pavia; <sup>2</sup>“Mario Negri” Institute, Milano.*

RHPS4 induces a rapid activation of a DNA damage response pathway specifically at telomeres by interfering with telomere replication, and consequently inhibits proliferation of transformed and tumor cell lines while primary cells remains unaffected. In order to investigate the molecular basis of sensitivity to RHPS4, primary and transformed cells of different histotypes were treated and analyzed in terms of induction of a DNA damage response at telomeres, viability and proliferation. As a result, a response to RHPS4 was detected only in transformed cells while normal counterpart remained unaffected by the treatment. Cen3 tel is a fibroblast cell line immortalized with hTERT which undergoes spontaneous transformation *in vitro* with the development of a preneoplastic or neoplastic phenotype. Specific changes of cell cycle checkpoints have been traced at different stages of their life span in association with the acquisition of a transformed phenotype. Early Cen3tel cells, which do not show transformed morphology nor specific molecular changes, are relatively insensitive to RHPS4 treatment both in terms of cell growth and viability and of activation of DNA damage response at telomeres. Even the down regulation of p16INK4A and p14ARF, does not influence the sensitivity to RHPS4 treatment. Contrarily, p53 mutation, together with the up regulation of c-myc is determinant in the process of sensitization of cells to the G4 ligand. Lastly, the progression in the tumorigenicity of those cells increases the sensitivity to the molecule. A minimal and transient activation of a DNA damage response is detectable in normal cells exposed to RHPS4. Normal cells, in which p53 is interfered, show a sustained activation of this pathway and an induced sensitivity of cells in terms of reduction of viability, indicating that the presence of efficient repair checkpoint is determinant for the survival of treated cells. Anyway in a contest of tumorigenic cell lines like HeLa and RKO, the presence of active p53 does not confer resistance to RHPS4 treatment. Concluding, the alteration of cell cycle checkpoints is a required step for the acquisition of RHPS4 sensitive phenotype. Conversely, in a contest of tumorigenic cell line, restoring a single checkpoint is not sufficient to recover the RHPS4 sensitivity due to the presence of other mutations.

**P25. mTOR AND Bcl-2 COMBINED INHIBITION: SYNERGISTIC EFFECTS IN ACUTE LYMPHOBLASTIC LEUKEMIA CELLS ON PROLIFERATION AND APOPTOSIS, THROUGH Mcl-1 DOWN-REGULATION**

Iacovelli S<sup>1</sup>, Ricciardi MR<sup>1</sup>, Bergamo P<sup>2</sup>, Rinaldo C<sup>3</sup>, Licchetta R<sup>1</sup>, Allegretti M<sup>1</sup>, Mirabili S<sup>1</sup>, Vitale A<sup>1</sup>, Testi A<sup>1</sup>, Petrucci MT<sup>1</sup>, Milella M<sup>2</sup>, Foà R<sup>1</sup>, Tafuri A<sup>1</sup>

<sup>1</sup>*Division of Hematology, Department of Cellular Biotechnologies and Hematology, "La Sapienza" University of Rome;* <sup>2</sup>*Medical Oncology A and* <sup>3</sup>*Dpt. of Experimental Oncology, Regina Elena National Cancer Institute, Rome, Italy*

ALL cells are frequently characterized by the constitutive activation of the mTOR signaling cascades. Thus, molecular therapies targeting mTOR have been proposed and mTOR inhibitors proved effective on reducing cell proliferation with marginal activity on apoptosis. Therefore, a combined treatment with mTOR inhibitors and Bcl-2 inhibitors has been reported. In addition, since the Mcl-1 overexpression is reported as the major resistance factors to the Bcl-2 inhibitor, ABT-737, the combined use with mTOR inhibitors which control through the AKT/mTOR pathway, Mcl-1 levels, is conceivable. The aim of this study was to evaluate the combined effects of mTOR (CCI-779) and Bcl-2/Bcl-XL (ABT-737) inhibition in ALL cell lines and primary samples. In MOLT-4 cell line exposure to CCI-779 induced a flat dose-response curve (35-55% growth inhibition) at concentrations ranging between 1 and 5000 nM and apoptosis induction was not seen until 5000 nM. In CEM-S, CEM-R and JURKAT cell lines, only minor cytostatic effects were observed until 20000 nM. In MOLT-4 cells, ABT-737 induced dose and time-dependent growth inhibition (IC-50= 198nM) followed at higher concentrations (250-500nM) by induction of apoptosis. In contrast, the CEM-S, CEM-R and JURKAT cells, proved resistant (IC-50 >5 µM), displaying Mcl-1 overexpression. Therefore, we investigated the combined activity of ABT-737 and CCI-779, on the resistant phenotypes. The JURKAT cell line showed a significantly higher (p< 0.01) induction of apoptosis following exposure to ABT-737 and CCI-779 (both at 1000nM), as compared to the single agents. A similar activity was observed on the CEM-R cell line. In both cell lines, CCI-779 exposure down-regulate Mcl-1 protein levels by proteasome degradation. This effect was associated with AKT<sup>S473</sup> de-phosphorylation, following prolonged CCI-779 exposure. However, Mcl-1 down regulation was not uniformly induced in the resistant phenotypes, as demonstrated in the CEM-S cell line. Furthermore, in this case, RNA interference of Mcl-1, did not revert the resistant phenotype. In primary ALL blasts, CCI-779 exposure (5000 nM) induced in 4/21 samples only weakly apoptosis (sub-G1 peak< 20%) while ABT-737 (50nM) induced higher levels (> 40%) of apoptosis in the majority (15/21) of them. Among the remaining six samples, resistant to ABT-737, the combined CCI-779 and ABT-737 treatment overcame resistance, inducing Mcl-1 down-regulation and apoptosis (in 2/6). In summary, the Bcl-2/Bcl-XL (ABT-737) inhibition is an active proapoptotic treatment of ALL, in addition the combined use of mTOR (CCI-779) inhibition may revert ABT-737 resistant phenotypes in a proportion of ALL, via AKT inactivation and Mcl-1 degradation. However, different resistant mechanisms involved in ALL cells needs further investigation.

## **P26. INHIBITION OF CATHEPSIN B AS ANTIMETASTATIC STRATEGY FOR HUMAN MELANOMA: *IN VITRO* AND *IN VIVO* INVESTIGATIONS**

Matteo Marconi<sup>1</sup>, Barbara Ascione<sup>1</sup>, Laura Ciarlo<sup>1</sup>, Rosa Vona<sup>1</sup>, Lucrezia Gambardella<sup>1</sup>, Carlo Leonetti<sup>2</sup>, Anna M. Mileo<sup>3</sup>, Marco G. Paggi<sup>3</sup>, Caterina Catricalà<sup>4</sup>, Walter Malorni<sup>1</sup> and Paola Matarrese<sup>1</sup>

<sup>1</sup>*Dipartimento del Farmaco, Istituto Superiore di Sanità, Roma;* <sup>2</sup>*Dipartimento di Oncologia Sperimentale, Istituto Nazionale Tumori Regina Elena, Roma;* <sup>3</sup>*Dipartimento di Prevenzione e Diagnostica, Istituto Nazionale Tumori Regina Elena, Roma;* <sup>4</sup>*Dipartimento dermatologia oncologica, Istituto Dermatologico San Gallicano IRCCS, Roma*

Background: Cathepsins represent a group of proteases involved in determining the metastatic potential of cancer cells. Among these are cysteinyl- (e.g. cathepsin B and cathepsin L) and aspartyl-proteases (e.g. cathepsin D), normally present inside the lysosomes as inactive proenzymes. Once released in the extracellular space, cathepsins contribute to metastatic potential by facilitating cell migration and invasiveness.

Results: In the present study we first evaluated, by *in vitro* procedures, the role of cathepsins B, L and D, in the remodeling, spreading and invasiveness of eight different cell lines derived from four primary and four metastatic melanoma lesions. Among these, we considered two cell lines derived from a primary cutaneous melanoma and from a supraclavicular lymph node metastasis of the same patient. To this purpose, the effects of specific chemical inhibitors of these proteases, i.e. CA-074 and CA-074Me for cathepsin B, Cathepsin inhibitor II for cathepsin L, and Pepstatin A for cathepsin D, were evaluated. In addition, we also analyzed the effects of the biological inhibitors of these cathepsins, i.e. specific antibodies, on cell invasiveness. We found that i) cathepsin B, but not cathepsins L and D, was highly expressed at the surface of metastatic but not of primary melanoma cell lines and that ii) CA-074, or specific antibodies to cathepsin B, hindered metastatic cell spreading and dissemination, whereas neither chemical nor biological inhibitors of cathepsins D and L had significant effects. Accordingly, *in vivo* studies, i.e. in murine xenografts, demonstrated that CA-074 significantly reduced human melanoma growth and the number of artificial lung metastases.

Conclusion: These results suggest a reappraisal of the use of cathepsin B inhibitors (either chemical or biological) as innovative strategy in the management of metastatic melanoma disease.

## **P27. LA DOWN-REGOLAZIONE DI LAMINA A/C È ASSOCIATA ALL'ESPRESSIONE DI UN FENOTIPO PIÙ AGGRESSIVO NEL NEUROBLASTOMA UMANO**

Maresca G<sup>1</sup>, Natoli M<sup>1</sup>, Arisi I<sup>2</sup>, Trisciuglio D<sup>3</sup>, Brandi R<sup>2</sup>, D'Aguanno S<sup>4</sup>, D'Onofrio M<sup>2</sup>, Urbani A<sup>4</sup>, Del Bufalo D<sup>3</sup>, Felsani F<sup>1</sup>, e D'Agnano I<sup>1</sup>

<sup>1</sup>CNR-IBCN; <sup>2</sup>EBRI; <sup>3</sup>Istituto Nazionale dei Tumori Regina Elena; <sup>4</sup>Dipartimento di Medicina Interna, Università di Tor Vergata, Laboratorio di Proteomica, Fondazione Santa Lucia-IRCCS; Roma

Il neuroblastoma è uno dei tumori solidi infantili più aggressivi. La maggior parte delle cellule di neuroblastoma mantiene la capacità di differenziare *in vitro* in presenza di diversi stimoli e ciò le rende un buon modello per studiare il potenziale differenziativo di tali tumori.

Le lamine nucleari (di tipo A e B) sono proteine che appartengono alla classe dei filamenti intermedi di tipo V, formando una struttura reticolare al di sotto della membrana nucleare interna, determinando in gran parte la forma complessiva del nucleo interfascico. Le lamine di tipo A sono espresse nei tessuti differenziati e la loro espressione risulta ridotta o assente in molti tumori umani.

Il nostro obiettivo è stato quello di indagare il ruolo della Lamina A/C, appartenente alle lamine di tipo A, nel differenziamento e nella tumorigenesi delle cellule di neuroblastoma. Come modello cellulare abbiamo utilizzato la linea di neuroblastoma SH-SY5Y, che esprime ad alti livelli la Lamina A/C ed è in grado di differenziare *in vitro*. Abbiamo scelto quale stimolo differenziativo l'acido retinoico (RA), poiché è il composto più efficace nell'indurre il differenziamento delle cellule di neuroblastoma ed è inoltre utilizzato in clinica per aumentare la sopravvivenza dei pazienti affetti da neuroblastoma.

Il silenziamento della Lamina A/C nella linea SH-SY5Y ha causato un blocco dei processi differenziativi attivati da RA, in quanto risultano inibite sia la formazione dei neuriti che l'espressione di marcatori specifici. Lo studio del profilo di espressione genica delle cellule SH-SY5Y silenziate per il gene *LMNA* (cellule LMNA-KD), in presenza o meno dello stimolo differenziativo, ha confermato una repressione di diversi geni necessari al differenziamento. Le cellule LMNA-KD mostrano inoltre un incremento nell'espressione di geni relati alla progressione tumorale associato ad una aumentata capacità di migrazione/invasione. L'acquisizione di un fenotipo più aggressivo da parte delle cellule LMNA-KD viene mantenuta *in vivo* in seguito ad inoculo delle cellule in topi nudi. I nostri dati dimostrano che la perdita d'espressione della Lamina A/C nel neuroblastoma rappresenta un marcatore significativo di progressione tumorale.

**P28. ARTICHOKE POLYPHENOLS INDUCE APOPTOSIS AND DECREASE THE INVASIVE POTENTIAL OF MDA-MB231 THE HUMAN BREAST CANCER CELL LINE**

Anna Maria Mileo<sup>1</sup>, Donato Di Venere<sup>2</sup>, Vito Linsalata<sup>2</sup>, Rocco Fraioli<sup>3</sup> And Stefania Miccadei<sup>4</sup>

<sup>1</sup>Laboratories "C" Department of Development of Therapeutic Programs, <sup>3</sup>Immunology Department of Experimental Oncology, <sup>4</sup>Molecular Pathology "A" Department of Development of Therapeutic Programs, Regina Elena National Cancer Institute, Rome, Italy; <sup>2</sup>CNR-Institute of Sciences of Food Production (ISPA), Bari Italy

The human breast cancer cell line, oestrogen receptor negative, MDA-MB231, was used to evaluate the antitumor effect of polyphenolic extracts from the edible part of artichokes (AEs). Treatment of cancer cells reduced cell viability and inhibited cell growth in a dose-dependent manner. Importantly, AEs did not have any effect on normal breast epithelial cells, MCF10A. Chlorogenic acid (ChA), the most representative component of the polyphenolic fraction of artichoke, had no prominent effects on the cell death rate of MDA-MB231 cells. The addition of AEs to the cells, rather than ChA, triggered apoptosis via a mitochondrial and a death-receptor pathway, as shown by the activation of caspase-9 and caspase-8 respectively. Furthermore, an increase of the Bax:Bcl2 ratio and up-regulation of cyclin-dependent kinase inhibitor, p21<sup>WAF1</sup>, crucial apoptotic players, were documented. According to our data on activation of caspase-9, a loss of mitochondrial transmembrane potential ( $\Psi_m$ ) was shown. Cell motility and invasion capabilities were remarkably inhibited by AEs-treatment in highly invasive MDA-MB231 cells. In addition, a significant decrease of proteolytic activity of metalloproteinase-2 protein (MMP-2), involved in degrading components of the extracellular matrix, was detected. Our findings indicate that AEs reduced cell viability, inhibited cell growth, triggered apoptotic mechanisms and showed inhibitory properties against the invasive behaviour of MDA-MB231, a human breast cancer cell line. Altogether, these data indicate the potential chemopreventive activity of artichoke polyphenolic extracts.

## **P29. ANTI-APOPTOTIC EFFECT OF THE HPV-16 E7 ONCOPROTEIN VIA ITS PHYSICAL INTERACTION WITH THE ACTIN-BINDING PROTEIN GELSOLIN**

Anna M. Mileo<sup>1</sup>, Carmen Vico<sup>1</sup>, Emanuele Bellacchio<sup>2</sup>, Paola Matarrese<sup>3</sup>, Claudia Abbruzzese<sup>1</sup>, Antonio Federico<sup>1</sup>, Stefano Della Bianca<sup>1</sup>, Stefano Mattarocci<sup>1</sup>, Walter Malorni<sup>3,4</sup> and Marco G. Paggi<sup>1</sup>

<sup>1</sup>*Department of Development of Therapeutic Programs, National Cancer Institute "Regina Elena", Rome;* <sup>2</sup>*Research Laboratories, "Bambino Gesù" Children's Hospital, Rome;* <sup>3</sup>*Department of Therapeutic Research and Medicines Evaluation, Istituto Superiore di Sanità, Rome;* <sup>4</sup>*Istituto San Raffaele Sulmona, (AQ)*

The oncoprotein E7 from Human Papillomavirus 16 (HPV-16 E7) plays a pivotal role in HPV post-infective carcinogenesis due to its ability to reprogram the host cell to favor viral replication and cell transformation. The physical interaction with targeted host components is essential to HPV-16 E7 activity. We used the yeast two-hybrid (Y2H) technology to recognize novel potential cellular partners for the viral oncoprotein of interest. This allowed the identification of the actin-binding protein gelsolin (GSN) as a target of HPV-16 E7. Following biochemical validation and generation of a 3D molecular model for the interaction, functional analysis showed that, in HaCaT human immortalized keratinocytes, the stable expression of HPV-16 E7 influenced the balance between F- and G-actin. We found evidence supporting a role of HPV-16 E7 in affecting GSN expression and function by a) hindering its susceptibility to caspase-3 cleavage and b) inhibiting the pro-apoptotic functions of the GSN N-terminal moiety, one of the two products deriving from the caspase-3 activity on the full-length GSN molecule. These events synergistically led to increased cell survival, as showed by the ability of HPV-16 E7 to impair the apoptosis induced in HaCaT cells throughout the overexpression of the GSN N-terminal moiety. Our results provide support to the hypotheses generated from the 3D molecular model and encourage design small molecules able to interfere with the physical interaction between HPV-16 E7 and GSN. This might help move important steps towards the identification of agents specifically targeting HPV-16 E7-expressing cells.

### **P30. RUOLO DELLA LAMINA A/C NEL DIFFERENZIAMENTO DI CELLULE DI ORIGINE NERVOSA**

Marta Nardella\*, Giovanna Maresca\*, Donatella Amendola°, Barbara Bucci°, Armando Felsani\* e Igea D'Agnano\*

\*CNR, Istituto di Biologia Cellulare e Neurobiologia; °Centro Ricerca S. Pietro, Ospedale Fatebenefratelli, Roma

Le lamine nucleari (di tipo A e B) sono proteine che rivestono la membrana nucleare interna formando i cosiddetti filamenti intermedi della lamina nucleare. La principale funzione delle lamine è quella di dare supporto all'involucro nucleare, determinando in gran parte la forma complessiva del nucleo interfascico. E' stato anche ipotizzato un ruolo delle lamine di tipo A nell'invecchiamento fisiologico delle cellule staminali adulte quali regolatori del loro differenziamento. In particolare, in un precedente lavoro del nostro laboratorio abbiamo dimostrato che le lamine di tipo A sono necessarie per il differenziamento di cellule di neuroblastoma umano.

Lo scopo del presente lavoro è stato di studiare se le lamine di tipo A (lamine A/C) potessero essere coinvolte nei processi di maturazione di cellule di origine nervosa. Abbiamo inizialmente studiato l'effetto del silenziamento del gene LMNA, codificante per la proteina lamina A/C, in cellule di neuroblastoma umano (SH-SY5Y) che rappresentano un utile modello cellulare per lo studio del differenziamento neuronale. Le cellule che hanno mostrato un silenziamento del gene LMNA del 70% (LMNA-KD) presentano un significativo aumento (circa 3 volte) dell'espressione del gene PROM-1, che codifica per la proteina CD133, e del gene CD34, considerati entrambi marcatori di staminalità. Le cellule LMNA-KD, a differenza della linea WT, mostrano anche la capacità di formare sfere a densità clonale, in condizioni di non-aderenza. L'acquisizione di un fenotipo con caratteristiche di staminalità correla con l'aumentata espressione della glicoproteina trans-membrana P-gp ed è associata ad un incremento della resistenza a farmaci antitumorali. Dati preliminari ottenuti in precursori neurali embrionali murini mostrano che l'espressione di lamina A/C aumenta significativamente nel corso del loro differenziamento, suggerendo un ruolo cruciale di questa proteina nella maturazione di cellule staminali di origine nervosa.

**P31. INORGANIC PHOSPHATE AS A SIGNALING MOLECULE: NOVEL MOLECULAR MECHANISMS IN HUMAN OSTEOSARCOMA U2OS CELLS**

Silvio Naviglio, Luca Sorvillo, Raffaella D'Auria, Francesca Di Maiolo, Antonietta Esposito, Carmen Starace, Emilio Chiosi, Annamaria Spina  
*Department of Biochemistry and Biophysics, Second University of Naples, Medical School, Naples, Italy*

Osteosarcoma is the most common malignant primary bone tumor in children and adolescents and is characterized by a high metastatic potential. Its clinical outcome remains discouraging despite aggressive treatments. Thus, novel therapeutic approaches are needed.

Recent results indicate that inorganic phosphate (Pi) is capable of affecting specific signal transduction pathways and of acting as an active regulator of cell behavior.

Previously, we found that Pi inhibits proliferation of human osteosarcoma U2OS cells via an adenylate cyclase/cAMP mediated mechanism. Here we report that upon Pi treatment, U2OS cells became extremely hard to dislodge with trypsin. The lack of sensitivity to the trypsin action was paralleled by relevant changes in integrin subunits expression and accompanied by an increase of cell adhesion in cell-matrix adhesion assays. Interestingly, exposure of U2OS cells to Pi results also in a strong activation and protein level up-regulation of Rap1 small GTPase and in an early increase followed by a sustained inhibition of Erk1/2 phosphorylation. Importantly, the Pi-induced increase of cell adhesion was enforced by a cAMP analogue which specifically activates Epac/Rap1 and insensitive to PKA and MEK1/2 inhibitors. Our results enforce the evidences of inorganic phosphate as a signalling molecule, identify  $\beta 3$  integrin, Rap1, ERK1/2 as proteins whose expression and function are relevantly affected by Pi in osteosarcoma U2OS cells. The clinical significance and potential therapeutic applications by our data will be discussed.

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3. Khoshniat S, Bourguine A, Julien M, Weiss P, Guicheux J, Beck L. The emergence of phosphate as a specific signaling molecule in bone and other cell types in mammals. *Cell Mol Life Sci.* 2011 Jan; 68(2):205-18

**P32. INHIBITION OF PHOSPHATIDYLCHOLINE-SPECIFIC PHOSPHOLIPASE C DOWNREGULATES HER2 OVEREXPRESSION ON PLASMA MEMBRANE OF BREAST AND OVARIAN CANCER CELLS**

Luisa Paris, Maria Elena Pisanu, Laura Abalsamo, Francesca Spadaro, Serena Cecchetti, Alessandro Ricci, Rossella Canese, Carlo Ramoni, Egidio Iorio, Franca Podo  
*Istituto Superiore di Sanità, Roma*

**BACKGROUND:** Overexpression of the human epidermal growth factor receptor 2 (HER2) in breast (BC) and epithelial ovarian cancer (EOC) is associated with aggressive tumor phenotypes and increased resistance to antitumoral therapies. HER2 overexpression in BC is today held as a useful therapeutic target for humanized monoclonal antibodies (e.g. Trastuzumab) or for tyrosine kinase inhibitors (e.g. Lapatinib) which are able to induce down-regulation of this receptor with consequent effects on cell proliferation, differentiation and responsiveness to chemotherapy. These targeted approaches may instead show only limited antitumoral effect in EOC. A recent study in our laboratory pointed to the inhibition of an enzyme, phosphatidylcholine-specific phospholipase C (PtdCho-PLC), as a potential means to counteract the tumorigenic effects of HER2 amplification in BC cells (Paris L et al Breast Cancer Res 2010). In the present study we investigated the capability of controlling HER2 overexpression on the membrane of EOC cells by modulating the PtdCho-PLC activity.

**MATERIALS AND METHODS:** Membrane localization and interaction of PtdCho-PLC with HER2 were investigated on HER2-overexpressing ovarian (SKOV3.ip) and breast (SKBr3) cancer cell lines, using confocal laser scanning microscopy (CLSM), flow cytometry, isolation of lipid rafts from membrane fractions and immunoprecipitation analyses.

**RESULTS:** CLSM and flow cytometry analyses showed that PtdCho-PLC is differently distributed on the plasma membrane surface of BC and EOC cells. In particular, fine analysis of HER2 and PtdCho-PLC subcellular distribution demonstrated that the enzyme partially accumulates and physically interact with the receptor in membrane lipid rafts in BC, and in membrane non-rafts domains in EOC cells. Inhibition of PtdCho-PLC with the selective inhibitor tricyclodecan-9-yl-potassium xanthate (D609) resulted into altered rates of HER2 internalization and lysosomal degradation, and induced down-modulation of HER2 expression on the plasma membrane of both BC and EOC cells. Besides, PtdCho-PLC inhibition led to a strong retardation of HER2 re-expression on membrane and to a substantial decrease in the overall cellular HER2 contents. We also found that cell exposure to D609 resulted in a substantial down-modulation of PtdCho-PLC expression on the plasma membrane of both BC and EOC, while the overall PtdCho-PLC content was strongly reduced only in EOC cells.

Exposure to D609 (50 µg/ml, 24-72 h) induced substantial decreases in cell proliferation (in absence of apoptosis) in both SKOV3.ip (to 40±10%) and SKBr3 (to 30±10%).

**CONCLUSIONS:** Altogether, these data suggest that the activity of the PtdCho-PLC enzyme plays an important role in regulating both the HER2 endocytic pathway and the effects of HER2 amplification in HER2-overexpressing BC and EOC cells. These results suggest the interest of investigating the role of PtdCho-PLC targeting in weakening the oncogenic HER2-mediated signal in preclinical *in vivo* models of breast and ovarian cancer, thus possibly enhancing the effects of current combined treatments.

**P33. Let -7c/PBX2 LOOP AT THE CROSS-ROAD BETWEEN MYELOID TRANSFORMATION AND DIFFERENTIATION**

A. Pelosi, S. Careccia, V. Lulli, F. Lo-Coco, G. Piaggio, U. Testa, M. Levrero, M.G. Rizzo  
*Istituto Tumori Regina Elena, Roma*

Acute promyelocytic leukaemia (APL), bearing the leukaemia promoting PML/RAR $\alpha$  fusion protein, is a peculiar subtype of acute myeloid leukaemia (AML). We have recently shown that a small subset of microRNAs (miRs) is differentially expressed *in vivo* in APL cells and modulated by all-trans-retinoic acid (ATRA)-based treatment. In particular, we found that PML/RAR $\alpha$ -positive blasts from APL patients display low levels of miR let-7c as compared with normal promyelocytes and its expression increases after all-trans-retinoic acid (ATRA) treatments.

Let-7c belongs to let-7 family of miRs, with important roles in many biological processes and frequently repressed in human cancers. Based on these data, we investigated the biological role of let-7c in AML. We found that let-7c forced expression promotes cell cycle arrest and myeloid differentiation, as evaluated by morphological and immunophenotypic analysis. Intriguingly, we observed that the let-7c over-expression in promyelocytic cell line NB4, specifically induces differentiation along granulocytic lineage. Moreover, we identified PBX2, a member of the TALE homeobox family with a reported role in leukemias, as a novel direct target of let-7c that may contribute to the AML phenotype. Interestingly, our preliminary data show that PBX2 could be involved in AML granulocytic differentiation induced by ATRA. Indeed, PBX2 silencing in AML cells stimulates myeloid differentiation. In contrast, PBX2 over-expression interferes with this process. Overall, this study suggests let-7c as a new player in the molecular network that controls leukemic differentiation, and could contribute to the identification of let-7c as a new potential molecular target for therapeutic intervention in AML.

**P34. THE DNA DAMAGE RESPONSE INDUCED BY MYCN: POTENTIAL TARGETS FOR THE TREATMENT OF MYCN AMPLIFIED NEUROBLASTOMA**

<sup>1</sup>Marialaura Petroni, <sup>1</sup>Veronica Veschi, <sup>2</sup>Andrea Prodosmo, <sup>2</sup>Cinzia Rinaldo, <sup>1</sup>Francesca Sardina, <sup>1</sup>Alberto Gulino, <sup>2</sup>Silvia Soddu and <sup>1</sup>Giuseppe Giannini.

<sup>1</sup>*Sapienza University of Rome;* <sup>2</sup>*Regina Elena Cancer Institute, Rome, Italy*

MYCN amplification (MNA) occurs in about 20% of neuroblastomas (NBs) and is associated with early tumor progression and poor outcome, despite intensive multimodal treatment. However, MNA NBs are not intrinsically resistant to chemo- and radiotherapy, and MYCN overexpression sensitizes NB cells to apoptosis. Thus, uncovering the molecular mechanisms linking MYCN to apoptosis might contribute to designing novel and more efficient therapies for MNA NBs.

Our data show that, much like other oncogenes, MYCN activates a DNA damage response (DDR) in neuroblastoma cells. Activation of this pathway has been recognized as an oncogene-provoked anticancer barrier in early human tumorigenesis leading to apoptosis or cellular senescence. In MNA NBs an active DDR exist, but obviously it fails to induce high levels of apoptosis, *per se*. However the p53<sup>S46</sup> kinase HIPK2 accumulates upon MYCN expression due to an ATM-dependent (and thus DDR-connected) pathway that inhibits its degradation. Enhancement of the HIPK2-p53 pathway is strictly related to the reduced threshold for apoptosis induced by MYCN. Indeed p53 or HIPK2 knock down impair MYCN induced sensitization to apoptosis. We further show that the HIPK2-p53 pathway is conserved in at least 50% of primary MNA NBs and can be targeted by the non-genotoxic p53-reactivating compound Nutlin-3 to induce massive death of MNA NBs. In addition to DDR activation, MYCN promotes the expression of the NBS1 by a direct transcriptional activation. NBS1 belongs to MRE11/RAD50/NBS1 complex, a major player of the DNA double strand break responses involved in the sensing, processing and repair of DSBs. Whether NBS1 might be required to appropriately process the DNA damage and the genetic instability eventually induced by MYCN and whether this pathway could be also exploited for therapeutic strategies is under investigation.

**P35. ANTI-APOPTOTIC ROLE OF CHE-1/HAX1 INTERACTION IN SEVERE CONGENITAL NEUTROPENIA (SCN), A PRELEUKEMIC CONDITION**

C. Pisani<sup>1</sup>, A. Onori<sup>1</sup>, M.G. Di Certo<sup>2</sup>, N. Corbi<sup>1</sup> and C. Passananti<sup>1</sup>

<sup>1</sup>*IBPM-CNR c/o Regina Elena Cancer Institute, Rome, Italy;* <sup>2</sup>*IBCN-CNR, IRCCS Fondazione S. Lucia, Rome, Italy*

Che-1/AATF is a multifunctional human protein involved in the control of: programmed cell death, cell cycle checkpoints and tumor-genes. In order to study Che-1 functions we have recently identified several novel Che-1-interacting human proteins, all involved in tumorigenesis and in control of cell-death programs. One of these proteins is the mitochondrial protein "Hax1". Hax1 is a multifunctional protein involved in signal transduction, cytoskeletal control, mRNA transport and programmed cell death. Hax1 deficiency causes autosomal recessive severe congenital neutropenia (SCN), or Kostmann disease. Kostmann disease constitutes a primary immunodeficiency syndrome associated with increased cell death (apoptosis) in myeloid cells. Importantly, SCN represents a preleukemic condition. Actually a high percentage of SCN affected individuals develop a clonal proliferative disease leading to myelodysplastic syndrome or acute leukaemia.

We are currently working on several human cell lines including immortalized B lymphocytes and fibroblasts deriving from healthy donor and Hax1 deficient patients. We found a down-regulation of Che-1 specifically in patient cell lines, mostly evident in EBV transformed B cells. The decrement of Che-1 concomitantly to the absence of WT Hax-1 protein in B cell line from SCN patient could be related to their increased susceptibility to apoptotic inducers treatment. Our idea is that some of the Hax1 functions, altered in Kostmann disease, may be played through Che-1/Hax1 direct contact and consequently through the cross-talk between Che-1 and Hax1 pathways. We will investigate a possible involvement of Che-1/Hax1 interaction in the major cancer related pathways.

**P36. ALTERATIONS OF PHOSPHATIDYLCHOLINE METABOLISM IN HER2-OVEREXPRESSING OVARIAN CANCER CELLS: COMPARATIVE EVALUATION OF THE EFFECTS OF CISPLATIN AND PHOSPHOLIPASE C INHIBITION**

Pisanu M, Paris L, Ricci A, Canese R, Spadaro F, Bagnoli M, Mezzanzanica D, Canevari S, Iorio E, Podo F

*Istituto Superiore di Sanità, Roma*

Epithelial ovarian cancer (EOC) is a genetically heterogeneous disease frequently presenting with the traits of poor prognosis. Due to its asymptomatic nature at early stages, low percentage of cases is in fact diagnosed when the malignancy is confined within the ovary. A better understanding of genomic and metabolomic alterations associated with EOC may lead to the identification of new biomarkers of tumor progression and therapeutic response, and to the design of specifically targeted therapies. Magnetic resonance spectroscopy (MRS) represents a powerful tool to detect changes in the metabolite profiles of cancer cells exposed to anticancer agents. We previously reported increase in the MRS-detected phosphocholine (PCho) content of EOC cells compared with non tumoral counterparts (Iorio E et al, *Cancer Res* 2005). Major contributions to PCho accumulation derived from the overexpression and activation of two enzymes involved in phosphatidylcholine (PtdCho) metabolism, choline kinase (Chok) and PC-specific phospholipase C (PtdCho-PLC) (Iorio E et al, *Cancer Res* 2010; Spadaro F et al *Cancer Res* 2008). Purpose of the present study was to compare the effects of a conventional antitumor agent (cisplatin) with those of a PtdCho-PLC inhibitor (D609) on the MRS profiles of choline derivatives and mobile lipids (ML) in a HER2-overexpressing EOC cell line, SKOV3.ip, isolated from the ascitic exudates of SCID mice following intraperitoneal injection of SKOV3 cells. Compared with the parental cell line, SKOV3.ip exhibited enhanced *in vivo* tumorigenicity and an about two-fold higher PCho content, associated with enhanced PtdCho-PLC and ChoK activity. We also found 1.7-fold higher HER2 and PtdCho-PLC expression on the plasma membrane where these enzymes physically interacted, as also previously observed on breast cancer cells (Paris L et al, *Breast Cancer Res* 2010). Cisplatin (5 $\mu$ M) induced in SKOV3.ip cell cycle arrest in G0/G1 and block of cell proliferation in absence of apoptosis, reduction in the overall HER2 content, cellular swelling, and a strong decrease in PtdCho-PLC activity with no significant change in the PCho level. Selective pharmacological inhibition of PtdCho-PLC (D609, 50  $\mu$ g/ml) induced a similar block in cell cycle and cell proliferation, along with cellular swelling, a down-modulation of HER2 on the plasma membrane and a long-lasting decrease in the overall HER2 content; these effects were associated with a significant decrease in PtdCho-PLC protein expression and PCho level. Both cisplatin and D609 induced alterations in the lipid biochemical machinery, as shown by significant increases in the MRS-detected ML signals in intact cells and formation of cytosolic lipid bodies. Overall, our results suggest the interest of using an integrated platform of MRS and cell biology approaches to evaluate, at the preclinical level, the effectiveness of therapies targeted against the PC metabolism, as possible approaches to reinforce and monitor the effects of antitumor strategies in ovarian cancer.

**P37. *IN VITRO* EVALUATION OF THE CARDIOTOXIC EFFECTS OF 5-FLUOROURACIL FOR THE PREVENTION OF CARDIOVASCULAR DAMAGE IN WORKERS OCCUPATIONALLY EXPOSED**

Porto S<sup>(1)</sup>, Zappavigna S<sup>(1)</sup>, Marra M<sup>(1)</sup>, Abbruzzese A<sup>(1)</sup>, Sannolo N<sup>(2)</sup>, Naviglio S<sup>(1)</sup>, Castellano M<sup>(1)</sup>, Luce A<sup>(1)</sup>, Caraglia M<sup>(1)</sup>, Lamberti M<sup>(2)</sup>

<sup>(1)</sup> *Dipartimento di Biochimica e Biofisica "F. Cedrangolo", Seconda Università degli Studi di Napoli;* <sup>(2)</sup> *Dipartimento di Medicina Sperimentale, Sezione di Medicina del Lavoro, Igiene e Tossicologia Industriale, Seconda Università degli Studi di Napoli*

Occupational exposure to antineoplastic drugs is of considerable importance because a large percentage of health workers will be in contact every day. Doxorubicin (DOXO) and 5-fluorouracil(5-FU) are cytotoxic drugs but their clinical use is often limited by their cardiotoxic side effects. In this study we have determined the mechanism through these drugs induce cardiotoxicity. As experimental model we have used the rat cardiomyocytes (H9c2) cell line and the human colon adenocarcinoma (HT-29) cell line. We have evaluated the effects of increasing concentrations of 5-FU and DOXO on the proliferation of H9c2 and HT-29 cells by the vitality MTT test. Cells were treated for 24h, 48h and 72h with two drugs alone or in combination with 10<sup>-4</sup>M of levofolene (LF). 5-FU induced a time- and dose-dependent growth inhibition in both cell lines. The 50% growth inhibition was reached at 72 h with concentrations of 4 µM and 0.4 mM on HT-29 and H9c2, respectively. The addition of LF to 5-FU enhanced this effect. These data suggested that the HT-29 cells were more sensitive to 5-FU compared with H9c2. The 50% growth inhibition induced by DOXO was reached at 72 h with concentrations of 0.118 µM on H9c2, compared with an IC50 of 0.31 µM for HT-29. We have evaluated the apoptotic effects of these drugs by FACS analysis after labelling with PI and annexin V. We have found that the treatment with 5-FU was able to induce apoptosis in 32% of H9c2 cells, an effect that was enhanced by treatment with LF (38% of apoptotic cells). However, only about 10% of apoptotic cells were detectable when HT-29 were treated with the two drugs alone. On the other hand, DOXO had very little effects on apoptosis of both H9c2 and HT-29 cells (5-7% apoptotic cells). We have evaluated the expression of proteins involved in apoptosis( caspase 3 and caspase 9) by Western Blotting. After 48h 5-FU induced an increase of caspase 3 fragmentation in H9c2 especially in combination with LF; 5-FU induced a decrease of pro-caspase 9 (marker of activation). After 24h DOXO induced an increase of caspase 3 fragmentation less evident than that induced by 5-FU and LF alone or in combination. After 24h DOXO induced an increase of expression of pro-caspase 9 that returned to baseline levels after 48h. There were no significant changes in the expression of pro-caspase 3 and 9 induced by pharmacological treatments on HT-29 cells .

The results obtained suggest that the cardiomyocytes are more susceptible to the apoptotic effects of 5-FU and LF respect to adenocarcinoma cells. This could be due to the fact that the cardiomyocytes are normal cells and therefore more sensitive to the stress induced by cytotoxic drugs.

### **P38. NF-KB INHIBITORS IN THE CONTROL OF THE PRO-INFLAMMATORY PHENOTYPE INDUCED BY HYPOXIA IN PROSTATE TUMOR CELLS**

<sup>1-3</sup>Lorenzo Principessa, <sup>1</sup>Giuseppe Coroniti, <sup>2-3</sup>Luisa Salvatori, <sup>3</sup>Alessandra Verdina, <sup>1-2-3</sup>Elisa Petrangeli, <sup>1-4</sup>Matteo Antonio Russo, <sup>2-3</sup>Linda Ravenna.

<sup>1</sup>Dpt Experimental Medicine, Sapienza University of Rome; <sup>2</sup>Inst Molecular Biology and Pathology, CNR; <sup>3</sup>Dept Therapeutic Program Development, Regina Elena Cancer Institute, IRCCS IFO; <sup>4</sup>Dpt of Cellular and Molecular Pathology, IRCCS San Raffaele Pisana, Rome

Hypoxia is a feature in prostate cancer and is associated with a poor prognosis. Besides, growing evidence indicates that the genotoxic metabolites and ROS released in inflammation are further contributing factors leading to prostate cancer progression. Recent reports have underlined the role of tumor cells in the endogenous synthesis of pro-inflammatory molecules in prostate cancer and showed that a coordinated system including inflammatory and reparative molecules is present in the prostate tissue, in the absence of detectable leukocyte infiltrate, and that is up-regulated in transformed cells. The present study aims at addressing the role of the key transcription factors controlling the cellular response to hypoxia (HIF1-2-3  $\alpha$ ) in the modulation of the inflammatory and metastasizing phenotype of the prostate cancer cells PC3, DU145 and LNCaP. We performed real-time and western blot analysis to evaluate gene expression and we created stably HIF1  $\alpha$  silenced DU145 cells to evaluate the role of HIF1 $\alpha$ . After oxygen withdrawal, we analyzed the activation kinetics of HIF-1 $\alpha$ , 2 $\alpha$  and 3 $\alpha$  and of NF-kB (p65 e p50) and evaluated the expression levels of VEGF, AGER, P2X7R, COX2, PTX3 and CXCR4 genes involved in angiogenesis, inflammation and metastasis. Nuclear translocation of HIF-1 $\alpha$  followed comparable kinetics in all prostate cells: early start, top after 4 hours and decline by 24. Expression of HIF1 $\alpha$  mRNA was stable for 4 hours, then abruptly decreased and stabilized under the base level. HIF-2 $\alpha$  did not seem to respond to reduced oxygen. HIF3 $\alpha$  was expressed only in DU145 and both mRNA and protein showed a late up-regulation. Hypoxia dependent increase in NF-kB nuclear translocation was observed in PC3 and DU145 cell lines. All wild type prostate cells expressed some or all of the analyzed pro-inflammatory molecules although at different levels. Hypoxic environment significantly up-regulated the transcription of all of these genes. In HIF1 $\alpha$  silenced DU145, HIF3 $\alpha$  and pro-inflammatory phenotype expression (except AGER) together with NF-kB nuclear translocation resulted up-regulated by hypoxia suggesting that HIF1 $\alpha$  is not an indispensable player for these effects in silenced cells. We therefore tested the effect of the NF-kB inhibitor Partenolide on the hypoxia induced pro-inflammatory phenotype both in wild type and in HIF1  $\alpha$  silenced DU145 cells. Partenolide was able to counteract COX-2, RAGE, PTX3 and CXCR4 expression in hypoxic wild type DU145 and VEGF, COX2, PTX3 and CXCR4 in silenced cells. Of note, Partenolide inhibited also hypoxia induced HIF1 $\alpha$  and HIF3 $\alpha$  nuclear translocation in non silenced DU145. Our data show that prostate tumor cells express molecules of the inflammatory response and that hypoxic microenvironment strongly up-modulates this phenotype. NF-kB which exerts a direct regulation of inflammatory genes and of HIF1 $\alpha$  nuclear translocation is a central mediator and a potential therapeutic target in prostate cancer hypoxic inflammation.

**P39. THE THIAZOLE-DERIVATIVE CPTH6, A NOVEL Gcn5 AND pCAF HAT INHIBITOR, INDUCES APOPTOSIS IN HUMAN LEUKEMIA CELLS**

Ylenia Ragazzoni<sup>1</sup>, Daniela Trisciuglio<sup>1</sup>, Andrea Pelosi<sup>3</sup>, Marianna Desideri<sup>1</sup>, Simone Carradori<sup>4</sup>, Chiara Gabellini<sup>1,2</sup>, Giovanna Maresca<sup>5</sup>, Riccardo Nescatelli<sup>7</sup>, Lucia Ricci Vitiani<sup>6</sup>, and Donatella Del Bufalo<sup>1</sup>

<sup>1</sup>Experimental Chemotherapy Laboratory and <sup>3</sup>Molecular Oncogenesis Laboratory, Regina Elena National Cancer Institute; <sup>2</sup>Department of Anatomy, Histology, Forensic medicine, Orthopedics, Section of Histology and Medical Embryology, <sup>4</sup>Department of Chemistry and Pharmaceutical Technologies, <sup>7</sup>Department of Chemistry, "La Sapienza" University; <sup>5</sup>Institute of Cell Biology and Neurobiology-CNR Santa Lucia Foundation-IRCCS; <sup>6</sup>Department of Hematology, Oncology, and Molecular Medicine, Istituto Superiore di Sanità; Rome, Italy

Recent studies strongly support the histone acetylation as a promising therapeutic target with a strong preclinical rationale in hematological and solid tumors. Although a growing body of evidence demonstrates a direct relationship between histone acetyltransferases (HAT) activity and development or progression of cancer disease, only limited progress has been made in the field of HAT inhibitors. Our study identifies the thiazole derivative 3-methyl-cyclopentylidene-[4-(4'-chlorophenyl)thiazol-2-yl]hydrazone (CPTH6) as a novel Gcn5 and pCAF HAT inhibitor. It inhibits H3/H4 histones and  $\alpha$ -tubulin acetylation of a panel of leukemia cell lines. Concentration- and time- dependent inhibition of cell viability, paralleled by accumulation of cells in the G0/G1 phase and depletion from the S/G2M phases, was observed. The role of mitochondrial pathway on CPTH6-induced apoptosis was demonstrated, being a decrease of mitochondrial membrane potential and the release of cytochrome c, from mitochondria to cytosol, induced by CPTH6. Also the involvement of Bcl-2 and Bcl-xL on CPTH6-induced apoptosis was found after overexpression of the two proteins in leukemia cells. Solid tumor cell lines from several origins were demonstrated to be differently sensitive to CPTH6 treatment in terms of cell viability, and a correlation between the inhibitory efficacy on H3/H4 histones acetylation and cytotoxicity was found. Differentiating effect on leukemia and neuroblastoma cell lines was also induced by CPTH6. These results make CPTH6 a suitable tool for discovery of molecular targets of HAT and, potentially, for the development of new anticancer therapies, which warrants further investigations.

#### **P40. ROLE OF TRF2 IN CAMPTOTHECIN RESISTANCE**

Rizzo A, Iachettini S, Salvati E, Marcellini V, Leonetti C and Biroccio A

*Experimental Chemotherapy laboratory, Regina Elena Cancer Institute, Rome, Italy*

Telomeres are bound by a six-protein complex known as shelterin; among these the Telomere Repeats binding Factor 2 (TRF2) plays a pivotal role in protecting chromosome ends against instability. More interestingly, growing evidence suggests a role for TRF2 in non-telomeric functions. Here, we report that the overexpression of TRF2 in tumor cells confers resistance to treatment with the topoisomerase I-inhibitor camptothecin (CPT). With the aim to investigate the mechanism involved in the TRF2-induced CPT resistance, we found that the percentage of cells positive for RAD51 was earlier increased in TRF2-overexpressing cells compared to parental one. Consistent with these results, the time course of signaling response triggered by the topoisomerase I inhibitor treatment revealed that the phosphorylated and activated forms of ATM, H2AX and Chk2 damage proteins were quickly shut down in TRF2-overexpressing compared to control cells. On the contrary, when TRF2 was depleted by RNA interference, the damage sensors were detected for a longer period after the end of CPT exposure, indicating the presence of unrepaired DNA. Finally, the RPA phosphorylation on Ser4 and Ser8, a marker of resected CPT-induced DSBs, was affected by the levels of TRF2, being persistent in TRF2-overexpressing compared to control or depleted cells after the end of CPT exposure. In conclusion, our results confers to TRF2 a crucial role on CPT resistance by facilitating the strand invasion of RAD51 to allow the DNA repairing by homologous recombination.

**P41. DISSECTING THE COMPLEX CROSSTALK NETWORK BETWEEN ENDOTHELIN-1 AXIS AND VASCULAR ENDOTHELIAL GROWTH FACTOR SYSTEM IN MELANOMA CELLS**

Francesca Spinella, Valentina Caprara, Roberta Cianfrocca, Laura Rosanò, Valeriana Di Castro, Pier Giorgio Natali<sup>§</sup>, and Anna Bagnato  
*Lab. of Molecular Pathology, and <sup>§</sup>Immunology, Regina Elena Cancer Institute, Rome, Italy*

Phenotypic and genotypic analyses of cutaneous melanoma have identified endothelin B receptor (ET<sub>B</sub>R) as tumor progression marker, thus representing a potential therapeutic target. We previously reported that the binding of ET-1 to ET<sub>B</sub>R stimulates angiogenesis and lymphangiogenesis directly on blood and lymphatic endothelial cells and by stimulating vascular endothelial growth factor (VEGF)-A and VEGF-C production. In this study we investigated as to whether in melanoma cells ET-1 axis may interact with VEGF-A and VEGF-C signaling pathways to heighten cellular responsiveness. We found that primary and metastatic melanoma cell lines expressed besides VEGF-A, also VEGF-C and its selective receptor VEGFR-3, at mRNA and protein level. Following ET-1 stimulation, VEGF-A and VEGF-C were upregulated, VEGFR-3 was rapidly phosphorylated and downstream signaling intermediates, such as p42/44 MAPK and Akt, were activated through ET<sub>B</sub>R. Inhibition of c-Src activity by PP2 reduced ET-1-induced VEGFR-3 phosphorylation, demonstrating that ET-1 transactivates VEGFR-3 through an intracellular mechanism mediated by c-Src to expand the signaling network. Stimulation with ET-1 in combination with VEGF-A or VEGF-C increased p42/44 MAPK and AKT phosphorylation, and resulted in a greater degree of melanoma cell migration compared to a single factor. Furthermore, this combination significantly enhanced the ET-1-induced vasculogenic differentiation of melanoma cell in tube-like structures, a phenomenon defined as *vasculogenic mimicry* and associated with high aggressive phenotype, indicating that crosstalk between ET-1/ET<sub>B</sub>R axis with VEGFR-3/VEGF-C system may enhance cancer cell motility and invasiveness and contributes to the promotion of cancer metastasis. Finally, in melanoma xenografts, ET<sub>B</sub>R antagonist suppressed tumor growth and neovascularization-related effectors, indicating that targeting ET<sub>B</sub>R related signalling cascade may represent a novel treatment of melanoma by impairing the crosstalk between ET axis and VEGF in melanoma. Supported by AIRC.

## **P42. ORGANOSULFUR DERIVATIVES OF THE HDAC INHIBITOR VALPROIC ACID SENSITIZES HUMAN LUNG CANCER CELL LINES TO CELL DEATH AND TO CISPLATIN CYTOTOXICITY**

Anna Tesei<sup>a,\*</sup>, Giovanni Brigliadori<sup>a</sup>, Silvia Carloni<sup>a</sup>, Paola Ulivi<sup>a</sup>, Chiara Arienti<sup>a</sup>, Anna Sparatore<sup>b</sup>, Piero Del Soldato<sup>c</sup>, Dino Amadori<sup>d</sup>, Rosella Silvestrini<sup>a</sup>, Wainer Zoli<sup>a</sup>

<sup>a</sup>*Biosciences Laboratory, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (I.R.S.T.), Meldola, Italy;* <sup>b</sup>*Dipartimento di Scienze Farmaceutiche "Pietro Pratesi," Università degli Studi di Milano, Milan, Italy;* <sup>c</sup>*CTG Pharma, Milan, Italy;* <sup>d</sup>*Department of Medical Oncology, I.R.S.T., Meldola (FC), Italy;*

**Background.** Lung cancer is the leading cause of cancer mortality worldwide and despite efforts made to improve clinical results, continuing poor survival rates indicate that novel therapeutic approaches are urgently needed. Valproic acid (VPA), a short-chain branched fatty acid used mainly for the treatment of epilepsy and bipolar disorder, has been shown to inhibit class I histone deacetylases (HDAC-I), a group of enzymes involved in chromatin remodelling and which are thought to play a role in tumor development. Although evidence of VPA's therapeutic efficacy has also been observed in patients with solid tumors, the very high concentration required to induce antitumor activity limits its clinical usefulness.

**Aims.** With the present study we proposed to analyse the activity of two VPA-derivatives, ACS2 and ACS33, *in vitro* experimental models of NSCLC, as single agent or in combination with cisplatin.

**Methods.** The *in vitro* experiments were performed on a panel of NSCLC cell lines (ChaGo-K1, CAEP, NCH1915). The cytotoxic activity was evaluated by SRB assay, protein expression level by W.blot and RT-Real Time PCR, invasive potential by matrigel invasion assay, TUNEL, cytochrome c release, mitochondrial membrane potential depolarization and acridine orange assay by cytofluorimetric techniques. To evaluate the interaction between drugs Kern's method, subsequently modified by Romanelli and co-workers, was used.

**Results.** In the present work, we used a panel of NSCLC cell lines to evaluate the activity and mechanisms of action of organosulfur valproic acid derivatives, a promising new class of compounds designed to improve the safety and efficacy of the valproic acid molecule and created by coupling it with a hydrogen sulphide moiety. Our results highlighted the increased cytotoxic activity of the novel organosulfur derivatives, ACS33 and ACS2, with respect to VPA, starting from low concentrations. In particular, ACS2 exhibited important pro-apoptotic activity triggered by the mitochondrial pathway and also showed anti-invasion potential. Furthermore, our *in vitro* results identified a highly effective combination schedule of ACS2 and cisplatin capable of inducing a synergistic interaction even when the two drugs were used at low concentrations, probably due to the modification induced by valproate derivative on chromatin condensation making easy the establishment of cisplatin-DNA adducts. **Conclusion.** Our *in vitro* data indicate the VPA derivative ACS2 in combination with platinum compounds could represent a promising alternative to traditional chemotherapeutic regimens used for advanced lung cancer. Further *in vivo* studies are now needed to confirm these results.

### **P43. HIGH SENSITIVITY OF BRCA1-DEFECTIVE BREAST CANCER CELLS TO CISPLATINUM IS MEDIATED BY IMPAIRED NOTCH SIGNALING**

Monica Ventura<sup>1,2,3</sup>, Lavinia Raimondi<sup>4</sup>, Maria Teresa Di Martino<sup>1,2,3</sup>, Pietro H. Guzzi<sup>5</sup>, Mario Cannataro<sup>5</sup>, Pierfrancesco Tassone<sup>2,3</sup>, Rossella Rota<sup>4</sup> and Pierosandro Tagliaferri<sup>1,3</sup>

<sup>1</sup>Medical Oncology Unit and <sup>2</sup>Referral Center for Innovative Treatments in Medical Oncology, "Campus Salvatore Venuta", "Magna Graecia" University and "Tommaso Campanella" Cancer Center, Catanzaro, Italy; <sup>3</sup>Department of Experimental and Clinical Medicine and Magna Graecia University, Catanzaro, Italy; <sup>4</sup>Laboratory of Angiogenesis, Department of Onco-hematology, Ospedale Pediatrico Bambino Gesù, IRCCS, Rome, Italy; <sup>5</sup>Department of Medical and Surgical Sciences, Magna Graecia University, Catanzaro, Italy

BRCA1 plays a crucial role in DNA repair mechanisms activated by exposure to anticancer agents with genotoxic potential. The HCC1937 is a BRCA1-defective breast cancer cell line which discloses a specific sensitivity to cisplatin (CDDP) as compared to its derivative clone, HCC1937/wtBRCA1, generated in our laboratories by transfection of BRCA1 full-length cDNA. In order to identify the molecular basis of BRCA1-related differential sensitivity to CDDP, we analyzed the whole gene expression profile of HCC1937 and HCC1937/wtBRCA1 which have been exposed *in vitro* to CDDP and genomic data underwent to Ingenuity® pathway analysis; by this approach we have been able to identify Notch signaling as the most affected pathway. We therefore hypothesized that the sensitivity of HCC1937 to CDDP may be related not only to BRCA1-related deficiency in DNA repair mechanisms, but also to changes in the Notch signaling pathway.

At this aim we evaluated, in HCC1937, the protein expression levels of Notch receptors, under basal conditions and after exposure to different doses of CDDP, demonstrating, following drug treatment, a significantly reduced expression of Notch1, Notch3 and the target gene Hes1. We have subsequently investigated by the use of MTT assay, the anti-proliferative activity of a pan-Notch inhibitor, gamma secretase inhibitor XII (GSIXII). The compound produced significant antitumor activity in HCC1937, with an IC<sub>50</sub> of 20 µM at 48 hours. By RT-PCR and Western Blotting we then identified the lower GSIXII dose able to induce Notch signaling modulation without producing a significant anti-proliferative effect. The selected dose (10 µM) was combined with different doses of CDDP in a cell viability assay. We observed a strong synergistic effect between GSIXII and CDDP after 24 to 72 hours exposure, with a 20-30 times reduction of the IC<sub>50</sub> for CDDP. Furthermore, we stably transfected HCC1937 with a dominant negative Notch3 (HCC1937/DNN3). HCC1937/DNN3, were more sensitive to CDDP when compared with the parental cell line (IC<sub>50</sub> 1-5 µM versus 20-30 µM respectively). Finally, Notch3 silencing by a Notch3 siRNA in HCC1937 cells produced a highly increased sensitivity to CDDP (30 fold IC<sub>50</sub> reduction).

In conclusion, our data suggest that down regulation of Notch3 signaling may have a critical role in the BRCA1-related differential sensitivity to CDDP and pharmacological inhibition of this pathway could be considered as an innovative anti-cancer strategy, alone or in combination with other compounds. (Supported by MIUR)

#### **P44. MALIGNANT MESOTHELIOMA. ROLE OF INFLAMMATION IN TUMOR PROGRESSION UNDER HYPOXIC CONDITIONS.**

Verdina A, Curzio G, Russo M.A., Ravenna L.  
*Istituto Regina Elena, Roma*

Hypoxia and inflammation are coincidental events in the pathogenesis and progression of many tumors including Malignant Mesothelioma (MM). They induce many changes in the cells including DNA-damage, oncogene activation, or impaired function of tumor suppressors. Hypoxia modulates gene expression through oxygen-sensitive transcription factors (HIF-1, HIF-2, HIF-3 alpha), but also through NF- $\kappa$ B which represents the master regulator of inflammatory response.

In this study we investigated the impact of hypoxia on the expression of pro-inflammatory molecules in two cell lines of MM (MSTO-211H and MPP-89) and the role of HIF-1 alpha and NF- $\kappa$ B in the up-regulation of pro-inflammatory genes.

The two cell lines responded similarly to the hypoxic stimulus: HIF-1 alpha protein translocated early into the nucleus, reaching the top after 4 hours of treatment, while the mRNA was down-regulated after 8 hours. HIF-2 alpha appeared not significantly modulated, showing a low nuclear translocation of the protein in MSTO. HIF-3 alpha responded with a late mRNA up-regulation in MSTO-211H and MPP-89 and nuclear translocation (48h) of the protein in MSTO-211H. In both cell lines an up-regulation of phospho(Ser276) p65 NF- $\kappa$ B subunit was observed in nuclear fraction after short times (30 min-2 hours) of hypoxic treatment.

Modulation of the pro-inflammatory phenotype following hypoxic stimulus was evident in both cell lines: we observed an induction of all studied pro-inflammatory genes, in particular of CXCR4 and COX2 in MSTO-211H. HMOX, a gene involved in oxidative stress showed the lowest level of induction and VEGF, crucial for systemic hypoxic response, was up-regulated in both cell lines. HIF-1 alpha silencing and the treatment with the NF- $\kappa$ B inhibitor Parthenolide, allowed us to distinguish the role of HIF-1 alpha and NF- $\kappa$ B in the cellular response to hypoxic stimulus. Our data showed that both factors are involved in the modulation of pro-inflammatory genes although at different levels. In particular, the up-regulation of CXCR4, a gene associated with increased proliferation, invasion and migration in several tumors and tumor cell lines, was strongly inhibited by HIF-1 alpha silencing. RAGE, an alarmin receptor up-regulated in metastatic tumors and associated with invasion and poor prognosis, was regulated by both HIF-1 alpha and NF- $\kappa$ B at lower level.

In conclusion, MM cells respond to hypoxic stimulation through the production of pro-inflammatory molecules, suggesting a cross-talk between hypoxia and inflammation in this tumor. The two master regulators of the hypoxic and inflammatory response, HIF-1 alpha and NF- $\kappa$ B, appear both required for the up-regulation of the hypoxia-dependent pro-inflammatory phenotype. This study represent a first step to identify factors involved in MM progression.

#### **P45. ROLE OF DNA REPAIR PATHWAYS IN THE CONTROL OF COMMON FRAGILE SITES EXPRESSION**

Patrizia Vernole<sup>1</sup>, Alessia Muzi<sup>2</sup>, Antonio Volpi<sup>1</sup>, Lucio Tentori<sup>2</sup>, Grazia Graziani<sup>2</sup>  
<sup>1</sup>*Department of Public Health and Cell Biology and* <sup>2</sup>*Department of Neuroscience,*  
*University of Rome "Tor Vergata", Rome, Italy*

Common fragile sites (CFS) are specific chromosomal areas, where gaps and breaks can occur when cells are exposed to stresses that mainly affect DNA synthesis, like incubation with aphidicolin (APC), an inhibitor of DNA polymerases. In the presence of APC, the replication fork can stall and slow down the duplicative process or collapse to generate DNA double strand breaks (DSB) when it encounters the fragile site regions. Homologous recombination (HR) is involved in the repair of DNA damage induced by APC, and RAD51, one of the key players in HR, is reported to ensure CFS stability. Since the mismatch repair system (MMR) is known to control HR, we investigated the influence of MMR and HR on chromosomal damage and CFS expression/distribution provoked by APC and/or by RAD51 silencing in two MMR-deficient human colon cancer cell lines (HCT15 and HCT116) and their MMR-proficient counterparts. Moreover, since poly(ADP-ribose) polymerase (PARP)-1 is involved in the regulation of replication fork progression by HR on damaged DNA, we also analyzed HCT116 cells in which PARP-1 was stably silenced.

MMR-deficient cells resulted more sensitive to chromosomal damage and CFS induction by APC compared to their MMR-proficient counterparts, suggesting the involvement of MMR in the control of CFS expression caused by APC. Differently from human lymphocytes in which the most expressed CFS is always FRA3B, in colon cancer cells the most expressed CFS was FRA16D in 16q23, an area containing the tumor suppressor gene *WWOX* often mutated in various cancers including colon carcinomas. These data confirm that the expression of different CFS may be associated with cell differentiation and probably also with specific chromosome rearrangements in tumor cells. Silencing of RAD51 provoked a higher number of breaks at CFS in MMR-proficient cells with respect to their MMR-deficient counterparts, likely as a consequence of the combined inhibitory effects on HR deriving from RAD51 silencing and the MMR-mediated suppression of HR. RAD51 silencing caused a broader distribution of breaks at CFS than that observed with APC. Treatment with APC of RAD51-silenced cells further increased CFS occurrence in MMR-proficient cells. On the contrary, silencing of PARP-1 did not cause chromosome breaks or affect the expression/distribution of CFS induced by APC both in MMR-proficient and MMR-efficient cells. Our results indicate that MMR modulates colon cancer sensitivity to chromosomal breaks and CFS induced by APC and RAD51 silencing.

#### **P46. REGOLAZIONE TRASCRIZIONALE ED EPIGENETICA DEL GENE ONCOSOPPRESSORE KCTD11<sup>REN</sup>**

D Verzella, MM Mancarelli, V Iansante, D Capece, M Fischietti, L Ciccocioppo, A Po, S Murgo, R Di Camillo, C Rinaldi, E Ferretti, F Zazzeroni, A Gulino, E Alesse  
*Università degli Studi de L'Aquila*

Il gene KCTD11<sup>REN</sup>(KCTD11), Potassium Channel Tetramerisation Domain-11 (Retinoic acid, EGF, NGF-responsive gene), deve il nome alla sua inducibilità da parte di fattori neurogenici ed alla presenza nella porzione N-terminale della proteina di un dominio di tetramerizzazione presente nei canali del potassio. Il gene KCTD11 codifica per un nuovo adattatore del complesso Cullina3/E3 ubiquitina-ligasi ed ha come target l'istone deacetilasi. KCTD11 è un gene finemente regolato durante lo sviluppo embrionale, la cui espressione nei tessuti adulti è confinata solo ad alcuni di essi. KCTD11 gioca un ruolo importante nella neurogenesi promuovendo il differenziamento di progenitori neuronali "committed" attraverso dei segnali inibitori della crescita cellulare. KCTD11 è un gene oncosoppressore capace di inibire la proliferazione cellulare attraverso la regolazione di p27<sup>Kip1</sup>. Recentemente abbiamo dimostrato che l'espressione di KCTD11 è frequentemente ridotta nel medulloblastoma umano (MB), in parte in seguito a perdita di eterozigosi (LOH) e in parte a causa di meccanismi epigenetici non ancora completamente caratterizzati.

Al fine di identificare gli eventi molecolari che regolano l'espressione di KCTD11 durante il differenziamento neuronale, nonché i meccanismi responsabili della sua deregolazione nei tumori, abbiamo caratterizzato la regione regolatoria del promotore di KCTD11. Sono stati così identificati un'isola CpG, diversi siti Sp1 e un sito κB. In questo studio, abbiamo dimostrato che il fattore di trascrizione NF-κB regola la trascrizione di KCTD11 durante il differenziamento cellulare in senso neuronale. Inoltre, Sp1 e la metilazione del DNA contribuiscono, almeno in parte, alla regolazione dell'espressione di KCTD11. Infine, alla luce del fatto che KCTD11 localizza sul braccio corto del cromosoma 17 in posizione 17p13, regione cromosomica frequentemente ipermetilata nei tumori, e che la metilazione è un meccanismo di regolazione dell'espressione di KCTD11, abbiamo analizzato un pannello di 117 campioni tumorali umani, rappresentanti 18 differenti tipi di cancro, dimostrando che KCTD11 risulta frequentemente down-regolato nei tumori.

#### **P47. THE APOPTOTIC ROLE OF Gal3 AND ITS IMPLICATIONS AS TARGET OF MOLECULAR THERAPY IN NEUROBLASTOMA**

<sup>1</sup>Veronica Veschi, <sup>1</sup>Marialaura Petroni, <sup>2</sup>Armando Bartolazzi, <sup>1</sup>Alberto Gulino and <sup>1</sup>Giuseppe Giannini

<sup>1</sup>*Department of Experimental Medicine, Sapienza University of Rome, 00161 Rome, Italy;* <sup>2</sup>*Department of Pathology, S. Andrea Hospital, 00189 Rome, Italy*

Neuroblastoma is the third most common extracranial solid tumor of childhood and remains a major therapeutic challenge in pediatric oncology despite decades of intensive research and therapeutic trials. The amplification of MYCN gene (MNA) is the most relevant adverse prognostic marker in human neuroblastoma but paradoxically its expression might also be associated to increased sensitivity to apoptosis. Neuroblastoma is characterized by the low frequency (<2%) of mutations in the p53 gene and as p53 isn't genetically inactivated but potentially functional in neuroblastoma, we began to study the p53 pathway as target of molecular therapy. An important component of this pathway is Galectin-3 (Gal-3), an anti-apoptotic factor that is biologically involved in a number of processes, largely depending on its particular localization. Furthermore, Gal-3 is repressed by p53 phosphorylated on S46 in response to DNA damage in order to commit cells to apoptosis; conversely, Gal-3 suppresses HIPK2-p53 induced apoptosis. In our work we evaluated the role and the expression of Gal-3 in neuroblastoma cells; we tested the effects of the molecule Nutlin-3, a well-known p53 pathway stimulator, in this context.

We recently demonstrated that MYCN can induce HIPK2, the proapoptotic p53 kinase, by the activation of DNA damage response (DDR) in MNA cells. We observed that surprisingly HIPK2 overexpression failed to induce apoptosis and sensitize MYCN single copy (MNSC) NB cells to DNA damaging drugs, suggesting that these cells might be protected from HIPK2-p53-induced apoptosis by Gal-3. We used the SHEP TET 21N cellular model to study the role of HIPK2-p53-Gal3 axis and we demonstrated that Gal-3 protects NB cells from apoptosis and that its depletion and HIPK2 overexpression cooperate in sensitizing these cells to apoptosis, suggesting the important role of Gal-3 in preventing apoptosis. Infact, Gal-3 is localized in the mitochondria of NB cells where it is able to prevent apoptosis inhibiting cytochrome c release and it is strongly expressed in MNSC cells at RNA and protein level, while, we detected very low levels of Gal-3 in MNA NB cells which can be made more resistant to apoptosis by transfection with a human Gal-3 construct. In an inducible cellular system, MYCN repressed Gal-3 expression, suggesting that its ability to sensitize NB cell to apoptosis might be closely dependent on the regulation of the HIPK2-p53-Gal-3 axis. We recently demonstrated that Nutlin-3 associated with other chemotherapy drugs determines an increase of HIPK2 expression levels and NMA NB cells apoptosis; in this work we observed that this molecule determines apoptosis also through Gal-3 repression.

**P48. ADP-RIBOSE POLYMERS LOCALIZED ON Ctf-Parp1-Dnmt1 COMPLEX PREVENT METHYLATION OF Ctf TARGET SITES**

Michele Zampieri, Tiziana Guastafierro, Roberta Calabrese, Fabio Ciccarone, Maria G. Bacalini, Anna Reale, Mariagrazia Perilli, Claudio Passananti, Paola Caiafa  
*Università "La Sapienza", Roma*

Poly(ADP-ribosyl)ation (PARylation) is involved in the maintenance of genomic methylation patterns through its control of DNA methyltransferase 1 (Dnmt1) activity. Our previous findings indicated that the CCCTC binding factor/Ctf may be an important player in key events whereby PARylation controls the unmethylated *status* of some CpG-rich regions. Ctf is able to activate Poly (ADP-ribose) polymerase 1 (Parp1) which ADP-ribosylates itself and, in turn, inhibits DNA methylation *via* non-covalent interaction between its ADP-ribose polymers and Dnmt1. By such a mechanism, Ctf may preserve the epigenetic pattern at promoters of important housekeeping genes. Data here reported evidence Dnmt1 as a new protein partner of Ctf. Moreover, we show that Ctf forms a complex with Dnmt1 and PARylated Parp1 at specific Ctf target sequences and that PARylation is responsible for the maintenance of the unmethylated *status* of some Ctf-bound CpGs. We suggest a mechanism by which Parp1, tethered and activated at specific DNA target sites by Ctf, preserves their methylation-free *status*.

**P49. SECRETED ADIPONECTIN IS AN EARLY MARKER OF ADIPOGENIC DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS**

Bellotti C, Perrone S, Martella E, Dozza B, Lucarelli E, Donati D  
*Osteoarticular Research Laboratory, Istituto Ortopedico Rizzoli, Bologna*

The pluripotency of mesenchymal stem cells (MSCs) is often assessed by the ability of MSC extracts to differentiate toward the osteogenic, chondrogenic, and adipogenic lineages *in vitro*. In particular, MSC adipogenesis is commonly evaluated by staining cell cultures with Oil Red O, which detects lipids that accumulate during adipogenic differentiation. While the Oil Red assay is simple and direct, it is problematic in that it requires approximately 3-4 weeks to detect adipogenic potential and necessitates the destruction of the specimen, inhibiting its use for further analysis and clinical application. Moreover, the oil red assay does not allow for the quantification of induction, preventing the determination of MSC potency. In this study, we investigated the possibility of assessing and quantifying MSC differentiation at earlier stages by monitoring adiponectin (ADPN) levels secreted into the induction medium. Adiponectin is a hormone secreted from adipose tissue that regulates several metabolic processes. MSCs were isolated from the iliac crests of patients and cultured. Cell supernatants were extracted at days 0, 3, 7, 10, 14, 17, and 21 after adipogenic induction and ADPN levels were determined by ELISA assay. Our results indicate that secreted ADPN levels steadily increase during adipogenesis and are definitively detectable at levels of  $5.986 \text{ ng/mL} \pm 2.545$  at day 10. Adipocyte-forming potential was confirmed by Oil Red staining. This study is relevant in that it demonstrates the ADPN assay is able to quantify MSC potency as well as shorten the time required to determine adipogenic potential. We therefore conclude that the ADPN assay is a fast, surrogate test that can be used to non-invasively qualify and quantify adipogenic differentiation and should be used to facilitate quality control in laboratory and clinical applications of MSCs in regenerative medicine.

## P50. PACLITAXEL PRIMED MESENCHYMAL STEM CELLS INHIBIT TUMOR GROWTH AND ANGIOGENESIS

Arianna Bonomi<sup>1</sup>, Valentina Coccè<sup>1</sup>, Loredana Cavicchini<sup>1</sup>, Francesca Sisto<sup>1</sup>, Maura Ferrari<sup>3</sup>, Stefania Navone<sup>2</sup>, Giovanni Marfia<sup>2</sup>, Eugenio Parati<sup>2</sup>, Giulio Alessandri<sup>2</sup> and Augusto Pessina<sup>1</sup>

<sup>1</sup>Department of Public Health-Microbiology-Virology, University of Milan, <sup>2</sup>Cellular Neurobiology Laboratory, Department of Cerebrovascular Disease; Fondazione IRCCS, Neurological Institute Carlo Besta, Milan; <sup>3</sup>Istituto Zooprofilattico Sperimentale della Lombardia e dell' Emilia Romagna, Brescia

Mesenchymal stem cells (MSCs) may represent an ideal candidate to deliver anti-cancer drugs, thanks to their easy adaptability to culture conditions and their homing to pathological tissues. In a previous study, we demonstrated that simple exposure of bone marrow (BM) derived MSCs to high doses of Doxorubicin, led them to acquire anti-proliferative potential towards co-cultured haematopoietic stem cells (HSCs), without showing any significant signs of toxicity. We concluded that MSCs may act as a reservoir for Doxorubicin subsequently released, as metabolite or in its original form, leading to HSCs-induced CFU inhibition. We thus hypothesized whether BM-MSCs, once primed *in vitro* with paclitaxel (PTX), an anticancer and antiangiogenic drug, can kill tumor cells (TCs) both *in vitro* and *in vivo*.

After priming for 24 h sub-confluent culture of human BM-MSCs with PTX 2 µg/ml (a dose able to completely block BM-MSCs proliferation without affecting their viability), we tested *in vitro*, by MTT assay, the anti-proliferation activity of their conditioned media (CM) on three human TCs lines: MOLT-4 (acute lymphoblastic leukemia), T98G (glioblastoma) and DU145 (prostate carcinoma). We evaluated also the direct inhibition of tumor growth by mixing primed BM-MSCs and the three TCs lines at different ratios (1:1, 1:10, 1:100) *in vitro* (through co-culture assays) and *in vivo* (in nod/scid mice, only with DU145 cell line). CM from primed BM-MSCs were further tested for their antiangiogenic properties by rat aorta ring assay: quantification of angiogenesis was performed by scoring the number of microvessels arising from aorta rings. In all the experiments, BM-MSCs not PTX-primed and their CM were used as negative control.

Our results demonstrate that BM-MSCs were able to rapidly incorporate PTX and then release it in the CM according to time-dependent kinetics. The internalization of drug into BM-MSCs and its release in CM were confirmed, respectively, by using fluorescent PTX and through a HPLC analysis. Primed BM-MSCsPTX gained a potent anti-tumor and anti-angiogenic activity *in vitro* that was dose-dependent as evidenced by testing their CM or by co-culture assay. *In vivo* experiments, performed by injecting, at the same time, cancer cells (DU145) and BM-MSCsPTX in immunodeficient mice, confirmed the *in vitro* observations and showed a significant delay in tumor takes together with a reduction of tumor growth.

Our data demonstrate that, without any genetic manipulation, BM-MSCs can be loaded with a chemotherapeutic molecule (paclitaxel) and, subsequently, released it. Because there are important evidences on the tropism of MSCs for tumour, these results open the way to develop new therapeutic strategies by localizing the drug effect in tumour microenvironment; this could increase the efficacy of cancer therapy minimizing, at the same time, the well known side effects related to chemotherapeutic agents.

## **P51. ROLE OF MESENCHYMAL STEM CELLS IN TUMOR CELLS GROWTH AND DRUG RESISTANCE IN CLASSICAL HODGKIN LYMPHOMA**

Cinzia Borghese<sup>1</sup> Marta Celegato<sup>1</sup>, Naike Casagrande<sup>1</sup>, Antonio Pinto<sup>2</sup>, Antonino Carbone<sup>3</sup>, Alfonso Colombatti<sup>1</sup>, Donatella Aldinucci<sup>1</sup>

<sup>1</sup>*Division of Experimental Oncology 2, and* <sup>3</sup>*Division of Pathology, Centro di Riferimento Oncologico, IRCCS-National Cancer Institute, Aviano, (PN);* <sup>2</sup>*Hematology-Oncology and Stem Cells Transplantation Unit, National Cancer Institute, Fondazione 'G. Pascale', Naples, Italy*

Classical Hodgkin Lymphoma (cHL) is a well studied model of tumour–microenvironment interactions, where the growth of malignant cells is regulated by interactions among tumour cells and reactive cells accumulating in HL-involved tissues. Fibrosis is considered a common morphologic feature of cHL lesions and stromal fibroblasts produce molecules capable to increase HL cells proliferation and drug resistance. Therefore, also Mesenchymal Stem cells (MSCs), that represent the pluripotent progenitor of stromal cells and tumor-associated fibroblasts, could be involved in the regulation of HL cells biologic behaviour and take active part in formation of tumor microenvironment. The aim of this preclinical study was to evaluate consequences of the cross-talk between MSCs and Hodgkin lymphoma cells. For this purpose we used a panel of Hodgkin lymphoma derived cell lines (L-1236, L-428, KM-H2, HDLM-2 and L-540) and Human Bone Marrow Derived-MSCs.

We demonstrated that conditioned medium from HL cells: increased the proliferation of human MSCs and inhibited senescence induced by serum starvation; increased osteoblastic differentiation; decreased adipocyte differentiation; increased MSCs movement and migration, this effect being mediated at least in part by the chemokine CCL5; increased CCL5 secretion by MSCs. These results suggest that MSCs might be: recruited into involved lymph nodes by CCL5 or other cytokines secreted by HL cells, induced to proliferate and to secrete CCL5, a cytokine involved in HL cells proliferation and microenvironment formation. Moreover we demonstrated that supernatants from MSCs (MSCs-CM) induced HL cells proliferation in a dose dependent manner and inhibited apoptosis induction by serum starvation. The combination chemotherapy ABVD (Adriamycin, Bleomycin, Vinblastine, and Dacarbazine) is now one of the most common chemotherapy regimens for the treatment of cHL. Co-cultivation with MSCs remarkable decreased growth inhibition and apoptosis induced by the treatment of HL cells with ABVD chemotherapy agents. IRF4 is a molecule involved in proliferation, survival and microenvironment formation of HL cells. Co-cultivation with MSCs remarkable decreased IRF4 down-regulation induced by both Adriamycin and Dacarbazine treatment in HL cells. MSCs-CM activated by HL cells increased the migration of L-540 cells, this effect being mediated at least in part by CCL5.

Taken together our data suggests that MSCs induced to proliferate or recruited from bone marrow by tumour cells, may represent a new cell type involved in HL cells proliferation, survival and drug resistance.

## **P52. ROLE OF AKT IN MESENCHYMAL STEM CELL MIGRATION**

Bulj Z<sup>1</sup>, Dozza B<sup>1</sup>, Cocco L<sup>2</sup>, Marmioli S<sup>3</sup>, Lucarelli E<sup>1</sup>, Donati D<sup>1</sup>

<sup>1</sup>*Osteoarticular Research Laboratory, Istituto Ortopedico Rizzoli, Bologna;*

<sup>2</sup>*Department of Anatomical Sciences, Cellular Signalling Laboratory, Bologna;*

<sup>3</sup>*Department of Laboratories, Pathological Anatomy and Forensic Medicine, University of Modena and Reggio Emilia, Modena, Italy*

**BACKGROUND:** Mesenchymal stem cells (MSCs) are adult multipotent cells isolated from various tissues with the ability to replicate and differentiate into multiple lineages. The huge potential and immunomodulatory capacities have made MSCs excellent candidates for regenerative medicine. MSC have been shown to migrate to damaged tissue and organs. Furthermore, MSCs have inherent tumor-trophic migratory properties, which allows them to serve as vehicles for delivering effective, targeted therapy to isolated tumors and metastatic disease.

**AIM:** Our aim is to investigate cell migration and the principal mechanisms that are underlying this complex phenomenon. Appropriated cell interaction with the surrounding microenvironment is influenced by coordination of different biological processes. Furthermore, abnormal cell motility contributes to pathological processes. Cell migration is context-dependent and cytokines found in microenvironment address cells to specific target and permit to complete reparative processes. Hitherto, the role of Akt protein kinase in migration is not clear and results from different laboratory are conflictual. Akt is activated by different growth factors and regulates pleiotropic cellular activities. We wanted to elucidate the contribution of Akt to MSCs' migration potential.

The goal of this work is to investigate mesenchymal stem cell migration and the involvement of PI3K/Akt pathway in this mechanism. We examined the consequences of down-regulating Akt activity using two different pharmacological inhibitors: LY-294002, a PI3K inhibitor, and the Akt inhibitor IV.

**RESULTS:** Down-regulation of Akt activity negatively influenced MSC motility in culture as demonstrated using scratch wound healing assay. Migration was observed by chemotaxis analysis using transwell chamber assay. Mesenchymal stem cells exhibited the most robust migration in basal conditions when Akt was activated. Furthermore, when cells were treated with specific PI3K/Akt inhibitors, migration was decelerated and spreading was inversely correlated with inhibitor concentration.

**CONCLUSIONS:** This study demonstrates that both inhibitors exhibited statistically significant differences in cell migration. These results indicate that reduction in total Akt levels suppresses cell migration. Our study establishes a pivotal role of Akt in MSCs migration, confirming pro-migratory effects.

**P53. PHENOTYPICAL AND FUNCTIONAL CHARACTERIZATION OF BONE MARROW-DERIVED MESENCHYMAL STROMAL CELLS IN PEDIATRIC PATIENTS AFFECTED BY ACUTE LYMPHOBLASTIC LEUKEMIA**

Conforti A, Cometa AM, Biagini S, Del Bufalo F, Incitti I, Starc N, Li Pira G, Bernardo ME and Locatelli F

*Ospedale Pediatrico Bambino Gesù, Roma*

**OBJECTIVE.** Acute lymphoblastic leukemia (ALL) is a malignant and clonal lymphoid disorder of the bone marrow in which early lymphoid precursors proliferate and replace normal hematopoietic cells. Alterations in the hematopoietic microenvironment of ALL patients have been reported, but little is known about the components of marrow stroma in these patients. In the present study, we phenotypically, functionally and genetically characterized ALL-Mesenchymal Stromal Cells (ALL-MSCs) isolated from pediatric patients. **METHODS.** MSCs were isolated and expanded *ex vivo*, from bone marrow (BM) of 10 ALL paediatric patients (mean age 6, range years 3-17). Morphology, proliferative capacity (cumulative cell count), immunophenotype (by flowcytometry), differentiation potential and immunomodulatory properties were analysed. Results were then compared to those obtained from BM-MSCs of 5 healthy donors (HD-MSCs; mean age 16, range 5-32 years). **RESULTS.** ALL-MSCs were isolated and expanded *ex vivo* at different time-points of the disease (*i.e.* at disease onset defined as day 0; day +15, +33 and +68 from the start of chemotherapy according to the Protocol of the Associazione Italiana Ematologia ed Oncologia Pediatrica, AIEOP, for pediatric ALL). Morphology and proliferative capacity did not differ between ALL- and HD-MSCs. ALL-MSCs displayed the characteristic spindle-shaped morphology and they were able to differentiate into both adipocytes and osteoblasts. Moreover, ALL-MSCs cultures were prolonged until P15 without observing any alteration in their morphology and surface phenotype, at any considered time-point. As for MSC typical surface immunological markers (CD90, CD73, CD13, CD105), ALL-MSCs and HD-MSCs immunophenotypes were comparable. The immunomodulatory properties of MSCs were evaluated in an allogeneic setting (ALL-MSCs/HD-PBMCs) by measuring PBMC proliferation induced by phytohemagglutinin (PHA, 4mg/ml). As for ALL-MSCs isolated at day 0 of the disease (8 patients), they were able to reduce PBMCs proliferation induced by PHA by up to 65% (with the ratio MSCs/PBMCs of 1:2) and 50% (with the ratio MSCs/PBMCs of 1:10); as for HD-MSCs (5 donors), they prevented PBMCs from proliferating by up to 65% (with the ratio MSCs/PBMCs of 1:2) and 60% (with the ratio MSCs/PBMCs of 1:10). Comparable results were obtained with MSCs isolated at subsequent time-points of the disease (day +15 in 6 patients; day +33 in 7 patients; day +68 in 2 patients). **CONCLUSIONS.** Our results demonstrate that MSCs expanded from BM of ALL paediatric patients at different time-points of their disease course and treatment maintain morphology, immunophenotype, proliferative capacity and immunomodulatory properties typical of HD-MSCs.

#### **P54. EFFECTS OF THE HYPOXIC MICROENVIRONMENT ON STEM CELLS ISOLATED FROM OMENTAL AND SUBCUTANEOUS ADIPOSE TISSUE FROM OBESE PATIENTS**

Giuseppe Coroniti<sup>1,2</sup>, Luisa Salvatori<sup>2,3</sup>, Francesca Caporuscio<sup>1</sup>, Laura De Girolamo<sup>4</sup>, Deborah Stanco<sup>5</sup>, Gianfranco Silecchia<sup>6</sup>, Carla Lubrano<sup>1</sup>, Stefania Mariani<sup>1</sup>, Lorenzo Principessa<sup>1,2</sup>, Linda Ravenna<sup>2,3</sup>, Anna Teresa Brini<sup>5</sup>, Matteo Antonio Russo<sup>1,7</sup>, and Elisa Petrangeli<sup>1,2,3</sup>

<sup>1</sup>Dpt Experimental Medicine, Sapienza University of Rome; <sup>2</sup>Dept Therapeutic Program Development, Regina Elena Cancer Institute, IRCCS IFO; <sup>3</sup>CNR, Inst Molecular Biology and Pathology; <sup>4</sup>IRCCS Galeazzi Orthopedic Inst, Milan; <sup>5</sup>Dpt of Medical Pharmacology, University of Milan; <sup>6</sup>Dpt of Surgery P. Stefanini, Sapienza University of Rome; <sup>7</sup>Dpt of Cellular and Molecular Pathology, IRCCS San Raffaele Pisana, Rome.

Human adipose stem cells (hASC) could be an important determinant in fat mass regulation and obesity-associated disorder. Patients with central obesity, characterized by omental fat accumulation, show a chronic inflammatory response and higher risk for obesity-related illnesses. In these patients, the release of pro-inflammatory cytokines is much greater in omental than in subcutaneous adipose tissue (AT). Moreover, AT of obese patients shows a reduced pressure of oxygen that could be involved in the expression of pro-inflammatory genes.

The aim of this study is to characterize hASC subpopulations isolated from subcutaneous and omental fat in normal and severe obese donors. The different features of the two kinds of donors could influence the behavior of hASC and their pro-inflammatory response to hypoxia. hASC isolated from the 4 ATs analyzed showed different biological and functional features. The clonogenic potential of cell populations, evaluated by Fibroblast-Colony Forming Unit assay, was higher in hASC isolated from normal donors than in obese ones and higher in subcutaneous than in omental tissues. These differences were confirmed by the analysis of proliferation rates.

We analyzed the effects of low oxygen concentration growing the hASC isolated from the 4 types of ATs in hypoxic conditions (2% oxygen) for 1 -24 hours. HIF1alpha activation, evaluated as nuclear translocation through Western Blot analysis, started earlier (2 hours) and appeared more intense in hASC from normal donors than from obese patients.

The hypoxic effects on the expression of inflammatory-related genes were analyzed by Real Time PCR. The expression of VEGFalpha, SOCS1 and EGFR was induced most strongly in hASC isolated from omental tissues of obese patients than from other ATs. The expression of damage receptors P2X7R and AGER appeared downregulated and induced in hASC from omental ATs of normal and obese donors, respectively. COX2 expression was induced by hypoxia in hASC from both subcutaneous ATs, while in omental ATs it was downregulated in hASC from normal donors and strongly upregulated in obese patients.

Our data show that hASC isolated from omental tissues of obese patients displayed major differences with respect of those of normal donors. This finding suggest that specific *in vivo* microenvironmental signalling determine a lasting conditioning of hASC biological behavior, of their responsiveness to specific triggers and of gene expression: the strong enhancement of hypoxia-induced pro-inflammatory gene expression could depend on a differential interaction of HIF signaling with other activated pathways, such as that of NF-kB. This effect could result from an adaptation of hASC to the microenvironment of omental AT in obese donors, characterized by chronic hypoxic conditions and presence of inflammatory cytokines.

Further studies will investigate the role of NF-kB and the effects of specific inhibitors to identify novel therapeutic targets in order to heal obesity.

## **P55. Survival of Mesenchymal Stem Cells in Collagenase Induced Tendonitis in an Ovine Model**

Crovace A<sup>1</sup>, Lacitignola L<sup>1</sup>; Rossi G<sup>2</sup>; Francioso E<sup>1</sup>

<sup>1</sup>*Department of Emergency and Organs Transplantation (D.E.O.T), Faculty of Veterinary Medicine, University of Bari, Valenzano (BA);* <sup>2</sup>*Department of Veterinary Sciences, Faculty of Veterinary Medicine, University of Camerino, Matelica (MC)*

Mesenchymal stem cells (MSCs), also referred to as connective tissue progenitor cells or multipotent mesenchymal stromal cells, have demonstrated significant potentials for clinical use. This clinical utility is due to their convenient isolation, their lack of significant immunogenicity permitting allogenic transplantation without immunosuppressive drugs, their lack of ethical controversy, and their potential to differentiate into tissue-specific cell types with trophic activity, to promote vascularization and potent immunosuppressive effects. The purpose of this study was to evaluate the efficacy and the survival of local injection of allogenic MSC marked with Red Fluorescent Protein (RFP) (Lentigen-Italy) in collagenase induced tendonitis in the ovine Achilles' tendon. The study was performed after the approval by the National Animal Care and Use Committee

Four sheep (2 years old, female, 45 bwt) have been enrolled in the study. After some days for the acclimatation, the sheep have been investigated to exclude any previous Achilles' tendon lesion. Three weeks before starting of the study one sheep was randomly selected for Bone Marrow harvesting for MSCs cultivation. The MSCs obtained has been transfected with a lentivirus for integration of a gene for expression of Red Fluorescent Protein (RFP). After a week the other 3 sheep was injected in both Achilles' tendon with 400 U.I. of Collagenase IA of *Cl. histolyticum* (Sigma-Aldrich-Italy). After two weeks the left Achilles' tendon of each sheep was injected with a solution of  $6 \times 10^6$  RFP-MSCs ( $MSC_{RFP}$ ) in 1 ml of fibrine glue (TISSUCOL, Baxter). The remaining tendons were used as negative control and received the same volume of saline solution as placebo. At 3-4-6 weeks from the treatment the tendons were harvested and evaluated for morphology, collagen I and III expression, presence of CD34+ cells and visualized at fluorescence microscope to assess RFP expression of the grafted  $MSC_{RFP}$ .

The results of these investigations evidenced the presence of  $MSC_{RFP}$  in the treated tendons respect to the control ones at 3, 4 and 6 weeks after the treatment. Moreover, the RFP positive tissue showed high expression of collagen I and low collagen III with good morphology in comparison to the lesions treated with placebo. The presence of high expression of collagen I and low collagen III with good morphology, in term of restored tendon architecture, can be related to the  $MSC_{RFP}$  injected into tendon lesions, as a large number of cells can survive in the site of injection. In  $MSC_{RFP}$  treated tendons marked expression of CD34+ has been detected too at each interval of time. These results showed that intralocal administration of MSCs into the tendon lesion can lead to a good effect on injured tendon. The local infusion delivery entails injecting MSCs directly into the tissue of interest and guarantees a higher number of engrafted cells and optimal therapeutic effect. Besides the survival of high numbers of positive RFP-MSCs in treated samples have been demonstrated at 3, 4 (Fig.1) and 6 weeks from the treatment. We have evaluated also that quality of tendon healing in  $MSC_{RFP}$  treated tendons has been based on a better architecture of collagen fibers and high expression of collagen I respect to collagen III (Fig.2), related to the control tendons. Moreover, in control tendons, no RFP cells have never been detected. This phenomenon has shown that even when there is a lesion in another tendon, cells injected intralocally survive in the treated area and persist in that zone until 6 weeks from treatment (excluding the systemic recruitment). Interestingly we evaluated that in  $MSC_{RFP}$  treated tendons there was a high expression of CD34+ cells. This findings can be explained to a chemotactic effect of MSCs on CD34+ cells, but their role is still unknown. The data obtained in this study confirm that MSCs allograft have a positive effect on tendon healing, its lack of significant immunogenicity permitting allogenic transplantation without immunosuppressive drugs and that the local injection in the tendon allows the survival of MSCs with good engraftment efficiency.

## **P56. LARGE ANIMAL MODEL (SWINE) FOR CELL-BASED CARDIOVASCULAR REPAIR IN ACUTE MYOCARDIAL INFARCTION: ONE YEAR FOLLOW UP**

A. Crovace\*, G. Rossi°, G. Alessandri°, F. Staffieri\*, L. Lacitignola\*, E. Francioso\*, G. Ferlan\*

\**Department of Emergencies and Organs Transplantation, University of Bari Aldo Moro*; °*Department of Veterinary Science, University of Camerino (MC)*; °°*Neurological Institute Carlo Besta, Milano*

The Aim of this study was to evaluate the results obtained after one year follow up, with the use of Bone Marrow derived Stromal Cells (BMSCs) and of Human Omentum – derived Fat Stromal Cells (HOFSCs) in the repairing of myocardial infarct in pigs.

**MATERIAL AND METHODS.** The BMSCs were harvested from bone marrow collected from ileal crest of pigs while the HOFSCs were obtained from human patients undergoing abdominal surgery. After the isolation the cells were cultured and characterized, were also detected their production of growth factors and cytokines and their angiogenic potential *in vitro*. After the approval of the Italian Ministry of Health, 30 pigs were enrolled in the study. The myocardial infarct was obtained by a permanent ligation of the inter ventricular artery (IVA). The pigs were divided in three groups: group 1 – treated with BMSCs, group 2 – treated with HOFSCs and group 3 - control group treated with placebo (Saline). In the group 1 and in group 2 after two hours from the ligation of IVA, the cells were injected into the proximal ischemic border zone. After surgery the animals were monitoring periodically by echocardiography, myocardial scintigraphy and cardiac MRI. The animals were euthanized at 3, 6 and 12 months and the hearts were harvested for histological and immunoistochemistry evaluations. In the infarcted area the microvessel density was evaluated using sections labelled for the endothelial markers.

**RESULTS.** *In vitro* conditions the cells showed the capacity to differentiate into osteogenic, adipogenic and cardiomyogenic cell lineages and were homogeneous for many markers and produce growth factors, cytokines and an high level of angiogenic factors. The instrumental evaluations of the heart functionality (echocardiography, myocardial scintigraphy and cardiac MRI) showed an improvement of myocardial function at 3 months post infarct and a significant decrease of distress symptoms in all pigs treated respect to the control group, but at 6 and at 12 months post infarct they do not give indications of amelioration of the hearts condition and these aspects seemed to be the same to the ones of the control group. The histological examinations at 3 months evidenced, in the treated groups, a reduction of a fibrotic and necrotic tissue and an increment of myogenic, cardiomyogenic and vascular markers that had suggested a better vascularitation and cardiomyogenesis respect to the control group. At 6 and 12 months after the surgery whereas it was possible to observe a major presence of necrotic tissue and an high reduction of cardiomyogenesis with a tickness of the infarcted area and with heart lesions similar to those observed in the control group.

**CONCLUSIONS.** The results obtained demonstrated that after a seeming amelioration at the initial stage (3 months) of the anatomical and clinical aspects, the cell therapy at the other interval of time (6 and 12 months) did not induce the expected improvement.

## **P57. REAL TIME IMAGING OF HUMAN MESENCHYMAL STEM CELLS IN LIVING RATS: FEASIBILITY AND PROSPECTIVES**

Valentina Diana<sup>1</sup>, Giovanna Levandis<sup>2</sup>, Patrizia Bossolasco<sup>3</sup>, Silvia Cerri<sup>2</sup>, Vincenzo Silani<sup>1,4</sup>, Giorgio Lambertenghi Delilieri<sup>3</sup>, Elio Polli<sup>3</sup>, Fabio Blandini<sup>2</sup>, Marie-Therese Armentero<sup>2</sup> and Lidia Cova<sup>1</sup>

<sup>1</sup>IRCCS Istituto Auxologico Italiano, Department of Neurology and Laboratory of Neuroscience, Milan; <sup>2</sup>IRCCS National Neurological Institute "C. Mondino", Laboratory of Functional Neurochemistry, Interdepartmental Research Center for Parkinson's Disease, Pavia; <sup>3</sup>Fondazione Matarrelli, Università degli Studi di Milano, Dipartimento di Farmacologia, Chemioterapia e Tossicologia Medica, Milan; <sup>4</sup>Centro "Dino Ferrari", Università degli Studi di Milano - IRCCS Istituto Auxologico Italiano, Department of Neurology and Laboratory of Neuroscience, Milan

Parkinson's disease (PD) is a degenerative disease of the central nervous system (CNS), commonly treated with pharmacological therapies yet unable to slow down the pathological progression. A perspective approach could reside in the regeneration/preservation of the damaged CNS in PD; several studies have indeed demonstrated that stem cell (SC) transplantation may represent a feasible tool to replace/support cells affected by the pathology. We have previously shown that human mesenchymal stem cells (hMSC) grafted in a rodent PD lesional model can reduce neurodegeneration. However, tracking transplanted SC to monitor their location and interaction within host tissues still appears an unsolved challenge.

In this study we used the Odyssey platform to develop an *in vivo* imaging paradigm that allows a rapid and long-term real time detection of grafted hMSC in living PD rats.

hMSC were previously labeled with a membrane intercalating dye that emits in the near infrared spectrum (NIR, CellVueNIR815) as well as with the nuclear dye Hoechst 33258, and thereafter *in vitro/in vivo/ex vivo* experiments were performed.

Our *in vitro* studies indicated that Hoechst/CellVue labeling was stable for a long time, and did not interfere with the essential biological functions of the cells, such as proliferation, metabolism and survival.

Double-labeled hMSC were then transplanted in the striatum of PD rats and traced *in vivo* at different time-points with the Odyssey platform fitted with the MousePod adaptor. Fourteen days later, rats were sacrificed and brains were isolated and processed by a cryostat to obtain serial coronal slices observable by Odyssey platform. Hoechst/CellVue+ transplanted cells resulted properly localize inside the lesion site, as demonstrated by our *ex vivo* experiments.

This study demonstrates that CellVueNIR815 cell labelling tracked by Odyssey platform constitutes an effective, safe and non-invasive method to monitor *in vivo* grafted SC. This procedure enables real time evaluation of hMSC distribution after transplantation and will support the development of efficient therapeutic strategies promptly applicable to patients.

**P58. THE DEVELOPMENT OF A NOVEL METHOD FOR THE ISOLATION AND CRYOPRESERVATION OF HUMAN UMBILICAL CORD WHARTON'S JELLY MONONUCLEAR CELLS THAT CONTAIN A SUB-POPULATION OF MESENCHYMAL PROGENITOR CELLS AND UMBILICAL CORD VASCULAR CELLS THAT CONTAIN A SUB-POPULATION OF ENDOTHELIAL PROGENITOR CELLS FOR THE PRODUCTION OF 2 CELLULAR THERAPY PRODUCTS**

Zacharias G Kallis<sup>1</sup>, Efthymia Yiacoumi<sup>2</sup>, Petros Petrou<sup>2</sup>, Maria Menikou<sup>3</sup>, Kathrin Pütsch<sup>4</sup>, Craig Donaldson<sup>5</sup>

<sup>1</sup>*C.B.B. Lifeline Biotech Ltd (Lifeline Umbilical Cord Blood and Tissue Bank), Nicosia Cyprus;* <sup>2</sup>*Lifeline Cellular Therapy Products Laboratory, Nicosia, Cyprus;* <sup>3</sup>*University College London Division of Surgery & Interventional Science, UCL Royal Free Hospital, London, UK;* <sup>4</sup>*Research & Development, Miltenyi Biotec GmbH, Bergisch Gladbach Germany;* <sup>5</sup>*Associate Head of Department for Human, Biomedical & Sports Sciences, Department of Applied Sciences, University of West England, Bristol, UK*

**OBJECTIVES:** The creation of an inexpensive processing and cryopreservation protocol for the in-parallel banking of Mesenchymal Progenitor Cells and Vascular endothelial cells with umbilical cord blood.

**MATERIALS AND METHODS:** Human Umbilical cord sections were trimmed and the Wharton's Jelly was separated from the vein and two arteries under GMP conditions. Each type of tissue was then mechanically dissociated in Miltenyi C Tubes using Miltenyi GentleMACS™ Dissociator to create single cell suspensions in sterile Phosphate-buffered saline. The suspensions were sieved using the BD Falcon™ 100µm cell strainers and the volume is increased to 20ml with the addition of autologous plasma obtained as waste product from umbilical cord blood processing. The Wharton's jelly cells in plasma suspension were then administered into OriGen CS25N cryobags and 55% DMSO/ 5% Dextran-40 solution was added as cryoprotectant (OriGen Biomedical cryopreservative solution Ph. Eur. Grade). The unit was cryopreserved as per umbilical cord blood buffy coat Standard Operating Procedure and banked in the same inventory and Liquid nitrogen cryostorage vessels. The vascular tissue suspension with added cryoprotectant was cryopreserved in Greiner Bio-One cryotubes in Liquid nitrogen.

**RESULTS:** The viability, efficacy and proliferative activity of the progenitor cells has been shown to be retained both post processing and at thawing.

**CONCLUSIONS:** Scientific literature suggests that there is an increased potential of mesenchymal stem cells applications in the fields of regenerative medicine, tissue engineering and gene therapy. Furthermore, it has been suggested that mesenchymal stem cells have a beneficial role when co-administered with cord blood in transplantations due to their immunoregulatory properties and their effect on the severity of GVHD. In addition, such cells could constitute the stroma layer in cord blood *ex vivo* expansion protocols. The protocol offers the cryopreservation at cellular level of unmanipulated progenitor cells, ready for future more efficient expansion, culture, manipulation and application techniques. These products can be cryopreserved using existing cryo-inventory and logistics as per cord blood banking which makes the introduction of such a production line alongside with cord blood processing and banking very attractive with minimal investment. We suggest that the in parallel banking of these cellular therapy products complement umbilical cord blood banking. The described approach offers a feasible option for both public and family banks as this protocol offers an inexpensive and efficient methodology for the introduction of the banking of such additional cellular therapy products that would compliment cord blood banking.

## **P59. RELIABLE PROTOCOL TO TRACK MESENCHYMAL STEM CELLS USING A LENTIVIRAL VECTOR EXPRESSING LUCIFERASE PROTEIN**

I.V. Libani<sup>1,2</sup>, C.Bossio<sup>3</sup>, R.Mastrangelo<sup>3</sup>, F.Bianco<sup>3</sup>, G. Lucignani<sup>1,2</sup>, L. Ottobri<sup>1,2</sup>

<sup>1</sup>Department of Biomedical Sciences and Technologies, Section of Radiological Sciences, Università degli Studi di Milano, Italy; <sup>2</sup>Centre of Molecular and Cellular Imaging - IMAGO, Università degli Studi di Milano, Segrate, Italy; <sup>3</sup>Neuro-Zone srl, Milano, Italy

Adult mesenchymal stem cells (MSC) isolated from adipose tissue represent a useful tool to regenerate damaged tissues in regenerative medicine protocols due to their high proliferative and differentiation potential and easily accessible, ethically-approved source. Nevertheless, interactions between transplanted cells and host tissues, is not completely understood.

Imaging is a new *in vivo* approach that allows to investigate overtime some important parameters (such as cell distribution, survival, localization and fate of injected cells) thereby reducing inter-individual variability and the number of experimental animals needed.

In this study the feasibility of luciferase lentiviral infection has been evaluated in order to achieve the direct visualization of mouse adipose tissue-derived adult mesenchymal stem cells by optical imaging, as a proof of principle for their long-term tracking in pre-clinical models.

Adipose-derived MSCs from CD1 mice were infected with different concentrations of a lentiviral vector carrying the luciferase gene under the control of Phospho Glycerate Kinase promoter (PLW vector).

Labeled MSCs were analyzed for viability, morphology, and osteogenic differentiation capability along with maintenance of luminescence labeling. Moreover, intracellular calcium dynamics following 1mM ATP stimulation was analysed by means of single cell calcium imaging recordings.

Our analysis showed that MSCs can be efficiently transduced with PLW vector maintaining the proportion with the amount of virus used.

The infected cells showed that biological features of luciferase-positive MSC were not altered. Moreover, cells maintained their physiological differentiation potential, quantitatively assayed by analyzing calcium deposits via Alizarin Red staining. Moreover, just like non-infected cells, they showed responsiveness to stimulation by extracellular ATP.

Our protocol efficiently labeled Adipose-derived MSCs without altering their biological properties and could allow direct cell detection *ex vivo* and *in vivo* by optical imaging. Insertion of luciferase probe appears as a reliable technique to follow the fate Adipose-derived MSCs upon transplantation and for studying their behaviour *in vivo* and *ex vivo* in order to establish efficient therapeutic strategies.

## **P60. EX VIVO VISUALIZATION OF TRANSFECTED HUMAN MESENCHYMAL STEM CELLS AFTER TRANSPLANTATION: A RELIABLE CELL-LABELING PROTOCOL FOR OPTICAL IMAGING**

I.V. Libani<sup>1</sup>, L. Cova<sup>2</sup>, V. Diana<sup>2</sup>, M-T. Armentero<sup>3</sup>, F. Blandini<sup>3</sup>, G. Lucignani<sup>1,4</sup>, L. Ottobriani<sup>1,4</sup> and V. Silani<sup>2,5</sup>

<sup>1</sup>Department of Biomedical Sciences and Technologies, Section of Radiological Sciences, Università degli Studi di Milano, Italy; <sup>2</sup>Department of Neurology and Laboratory of Neuroscience-IRCCS Istituto Auxologico Italiano, Cusano Milanino, Italy; <sup>3</sup>Laboratory of Functional Neurochemistry, Interdepartmental Research Center for Parkinson's Disease, IRCCS Neurological Institute "C. Mondino", Pavia, Italy; <sup>4</sup>Centre of Molecular and Cellular Imaging - IMAGO, Università degli Studi di Milano, Segrate, Italy; <sup>5</sup>Department of Neurology and Laboratory of Neuroscience, Centro "Dino Ferrari" - Università degli Studi di Milano - IRCCS Istituto Auxologico Italiano, Milan, Italy

Stem cells, due to their high proliferative and differentiation potential, have been transplanted into different animal models of neurodegenerative diseases with uneven results since an exhaustive comprehension of the interactions between transplanted cells and host tissues is still missing. Imaging is a new approach that allows to investigate overtime some important parameters (such as cell distribution, survival, localization and fate of injected cells) *in vivo*, thereby reducing interindividual variability and the number of experimental animals needed. Here we evaluate the feasibility of m-cherry lentiviral infection (transduction) as specific labelling protocol for the real time visualization of bone marrow-derived human Mesenchymal Stem Cells (hMSC) *in vivo* by Optical Imaging as a proof of principle for their long-term tracking in pre-clinical models. Commercial hMSC were infected with different concentrations of a lentiviral vector carrying the m-cherry gene under the control of Phospho Glycerate Kinase promoter (PGK). Labeled hMSC were analyzed for viability, morphology, and differentiation capability along with maintenance of fluorescent labeling after extensive culture *in vitro*. Thereafter, we transplanted them in a rodent model of Parkinson's disease based on the unilateral intrastriatal injection of 6-hydroxydopamine, a procedure that causes a progressive and retrograde degeneration of the nigrostriatal pathway. Our FACS analysis showed that a high percentage of hMSCs expressed the reporter gene (87,2% and 92.4% with a MOI equal respectively to 2,5 and 5; non infected control cells: less than 1%) indicating that the cells can be efficiently transduced with the lentiviral vector bearing the m-cherry. Infected cells showed a high level of vector copy number inserted in their DNA able to stably express the corresponding mRNA, as estimated by real-time PCR. Biological features of m-cherry-positive hMSC were not altered, even in long term cultures, since their doubling time and metabolic rates were comparable to control cells and no morphological alterations were detected by confocal analysis. Upon striatal transplantation, it was possible to visualize hMSC *ex vivo* in the whole brain by a sensitive CCD camera for fluorescent imaging. Finally, the presence of the m-cherry-positive cells at the injection site was also confirmed using human specific antibodies on frozen microscope slides.

Our protocol efficiently labeled hMSC without altering their biological properties and allowed direct cell detection *ex vivo* by optical imaging. Insertion of m-cherry fluorescent probe appears as a reliable technique to follow the fate hMSC upon transplantation and for studying their behaviour *in vivo* and *ex vivo* in order to establish efficient therapeutic strategies promptly applicable to patients.

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## **P61. USE OF HUMAN ADIPOSE-DERIVED STEM CELLS IN A MOUSE MODEL OF NEUROPATHIC PAIN**

S. Niada<sup>1,2,3</sup>, A. Rossi<sup>1</sup>, E. Arrigoni<sup>1,2</sup>, S. Franchi<sup>1</sup>, M. Colleoni<sup>1</sup>, A.E. Panerai<sup>1</sup>, P. Sacerdote<sup>1</sup>, A. T. Brini<sup>1,3</sup>

<sup>1</sup>Department of Medical Pharmacology, Università degli Studi di Milano; <sup>2</sup>PhD Student in Pharmacological Science, Università degli Studi di Milano; <sup>3</sup>IRCCS Galeazzi Orthopaedic Institute, Milano

Neuropathic pain is a complex disease and, so far, there are no pharmacological treatments acting in a definitive way. Recent studies suggest that this disease is associated with neuronal-tissue damage. Adipose-derived Stem Cells (ASCs) have shown the capacity of limiting neuronal damage through their anti-apoptotic effect, together with the reported capacity of releasing neurotrophic molecules. These cells are adult, undifferentiated, multipotent cells capable of self-renewal, with low immunogenicity and immunosuppressive properties; moreover, they are easily available in large amount. Our study aimed to detect the effect of human ASCs in a mouse sciatic nerve chronic constriction injury (CCI) model. hASC were isolated from subcutaneous adipose tissue of 5 healthy women (mean age 37±12); all the populations were characterized phenotypically and their clonogenic and differentiative potential were evaluated in order to verify their stemness. Particularly these cells shown a clonogenic capacity of about 3% at early passages and, when cultured in osteoinductive medium for 14 days, they presented an increase in phosphatase activity and in calcified extracellular matrix deposition of 252% and 193%, respectively. 10<sup>6</sup> hASC were injected into the rodents caudal vein 7 days after CCI, and, at 1,3,7,14,21 and 28 days post injection, we assessed their effect on mechanical allodynia (Dynamic Plantar Aesthesiometer) and thermal hyperalgesia (Plantar test) and correlated it with the alterations in pro- and anti-inflammatory cytokines (ELISA assay). hASCs were able to completely reverse hyperalgesia and reduce allodynia starting 24 hours after injection. The effect began to fade 21 days after administration, but it could be restored by a new cell injection (1x10<sup>6</sup>). In parallel we observed a recovery of cytokines balance both for pro- and anti-inflammatory ones. In particular the levels of IL-1 $\beta$  and IL-6, that are significantly enhanced in CCI mice, are restored when mice are treated with cells. As far as anti-inflammatory cytokines are concerned, hASCs treatment induces a significant increase in IL-10 level respect to both sham-operated and CCI animals. In this study, we demonstrated that hASCs treatment is able to reduce neuropathic pain symptoms and to re-establish cytokine balance in a CCI mouse model; this phenomenon might be due to the recruitment of the cells in the lesion area and to their interaction with the resident ones inducing a modulation of pain and inflammation.

## **P62. FORSKOLIN PROMOTES NEURONAL DIFFERENTIATION OF HUMAN WHARTON'S JELLY MESENCHYMAL STEM CELLS**

<sup>1</sup>Emanuela Paldino, <sup>2</sup>Carlo Cenciarelli, <sup>3</sup>Giulio Maira and <sup>1</sup>Patrizia Casalbore  
<sup>1</sup>*Istituto di Biologia Cellulare e Neurobiologia-CNR, Roma*; <sup>2</sup>*Istituto di Farmacologia Traslazionale-CNR, Roma*; <sup>3</sup>*Istituto di Neurochirurgia, Università Cattolica del Sacro Cuore, Roma*

Wharton's jelly mesenchymal stem cells (hWJMSCs) represent an interesting source of stem cells for clinical applications because they can be easily accessible, have a reduced immunogenicity and retain a high proliferative growth and multilineage differentiation properties for long term *in vitro*. The purpose of this study is to investigate the hWJMSCs differentiation ability toward neuronal fate. hWJMSCs have been isolated by different methods from fresh full-term birth human umbilical cords with parents consent and subsequently have been characterized at early and late passages by FACS analysis and resulted positive for mesenchymal stem markers CD105, CD73, CD90 and negative for CD31, CD45 and CD117, endothelial and hemopoietic markers respectively. Karyotype analysis demonstrated even at late passages a chromosomal stability, furthermore it was observed that hWJMSCs didn't induce tumor formation when injected into immunodeficient SCID mice. hWJMSCs's ability to differentiate in osteogenic, chondrogenic and adipogenic lineages was assessed in standard differentiation media. The trans-differentiation of hWJMSCs into neural lineage was investigated in presence of forskolin which is known to increase the intracellular levels of cAMP. In this conditions for 7-10 days *in vitro* (DIV) we observed remarkable changes in cell morphology: hWJMSC had lost their typical flat morphology and displayed more elongated cellular branching. Western blot analysis revealed a down modulation of Sox2, Oct 4 and nestin expression levels, suggesting a specific induction towards a neural lineage. Besides, immunocytochemistry analysis showed a complete positivity to early neuronal marker III- $\beta$ Tub and at a lesser extent to markers for neurons MAP2 and NF-M respect to control cells. Furthermore, hWJMSC differentiated for 7 DIV showed a significant release of BDNF respect to control. A molecular profiling of hWJMSCs was performed by microarray analysis which revealed 1532 statistically up-regulated genes compared to vehicle-treated cells. These genes are mainly involved in neuronal signaling pathway (MAPK, SHH, CREB) and a part of them are found expressed in dopaminergic lineage as LMO3, WNT5A, SLIT3, NPTX1, FOXO1, GABRB1, NR4A2. Taken together, these findings indicate that hWJMSCs may be playing a significant role in therapies for neurological diseases.

### **P63. PPAR-ALPHA NUCLEAR RECEPTOR/APOE AXIS: A NEW TARGET OF BREAST CANCER STEM CELLS**

Alessio Papi<sup>1</sup>, Gianluca Storci<sup>2,4</sup>, Tiziana Guarnieri<sup>1,2,7</sup>, Donatella Santini<sup>5</sup>, Mario Taffurelli<sup>6</sup>, Claudio Ceccarelli<sup>5</sup>, Marina Orlandi<sup>1</sup>, Massimiliano Bonafé<sup>2,3</sup>.

<sup>1</sup>Dept. of Experimental Biology, University of Bologna, Bologna, Italy; <sup>2</sup>Center for Applied Biomedical Research (CRBA), St. Orsola-Malpighi University Hospital, Bologna, Italy; <sup>3</sup>Departments of Experimental Pathology, <sup>4</sup>Department of Hematology, Oncology and Laboratory medicine "L&A Seragnoli Institute", <sup>5</sup>Department of Radiology and Hystocytopathology, <sup>6</sup>Department of Surgical and Anesthesiological Sciences, University of Bologna, Bologna, Italy; <sup>7</sup>Istituto Nazionale Biostrutture e Biosistemi (INBB), Rome, Italy.

The study of the subpopulation present in human breast tumor tissues, called "Breast cancer stem cells (CSCs)", is the new goal of cancer therapy. In fact, the recurrence of tumor after chemotherapy is probably due to the presence of this subpopulation that escapes from pharmacologic treatment.

Breast CSCs can be studied *in vitro* by expanding mammospheres (MS), multicellular spheroids obtained from breast cancer specimens.

We used MS expanded from human normal specimens (Normal mammospheres, N-MS), from human tumoral breast tissues (Tumor mammospheres, T-MS) and MCF7-MS obtained from the cancer cell line MCF7.

We measured nuclear receptors expression and we observed that Peroxisome Proliferator-Activated Receptor-alpha (PPAR-alpha) was over expressed in MCF7-MS. We demonstrated that the PPAR-alpha specific agonist (Wy14643) sustains MS formation, induces the expression of stem cell genes (Slug, Notch-3 and Jagged-1) and down-regulates the expression of the differentiation markers Estrogen Receptor-alpha and cytokeratin-18 in MCF7-MS. These effects are associated with the hampering of an inflammatory Nuclear Factor- $\kappa$ B/Interleukin-6 (NF- $\kappa$ B/IL-6) axis.

We also found that PPAR-alpha promotes the stem cell phenotype and MS formation by activating the Hypoxia-Inducible Factor-2alpha pathway through Hypoxia Response Element (HRE) and Carbonic Anhydrase-IX induction in hypoxic (1% pO<sub>2</sub>) conditions.

Moreover, PPARalpha regulates the expression of the cholesterol transporter ApoE, that is over expressed in T-MS compared to N-MS. Due to the role of ApoE in a variety of inflammatory age-related diseases, including cancer, we thus speculate that this PPAR-alpha/ApoE crosstalk may take part to the inflammatory stem cell phenotype.

Finally, we observed that a ligand of retinoid X receptor (RXR), the 6-OH-11-O-hydroxyphenantrene (IIF), have opposite effects on MS with respect to WY14643 action. In fact, IIF reduced the number of colonies of T-MS and MCF7-MS through the inhibition of the NF- $\kappa$ B/IL-6 pathway.

These data highlight a crucial role of PPAR-alpha in sustaining the inflammation-dependent survival of breast CSCs and suggest the possible chemotherapeutic use of IIF against breast cancer recurrence.

**P64. DECIPHERING THE TRANSCRIPTIONAL COMPLEX CRITICAL FOR THE EPIGENETIC REGULATION OF EPITHELIAL TO MESENCHYMAL TRANSITION AND METASTATIC PROCESS DRIVEN BY ENDOTHELIN A RECEPTOR/ $\beta$ -ARRESTIN-1 IN OVARIAN CANCER**

<sup>1</sup>Rosanò L, <sup>1</sup>Cianfrocca R, <sup>1</sup>Tocci P, <sup>1</sup>Di Castro V, <sup>1</sup>Spinella F, <sup>2</sup>Salvati E, <sup>2</sup>Biroccio A, <sup>1</sup>Natali PG and <sup>1</sup>Bagnato A

*Laboratories of <sup>1</sup>Molecular Pathology, and <sup>2</sup>Experimental Chemotherapy, Regina Elena National Cancer Institute, Rome, Italy*

In epithelial ovarian cancer (EOC), endothelin (ET)-1/ET A receptor (ET<sub>A</sub>R) axis controls epithelial to mesenchymal transition (EMT), invasion, and metastasis. We recently showed the ability of scaffold protein  $\beta$ -arrestin-1 to create signalling platforms driving EOC progression, but its role in EMT and invasion is still undefined. To decipher the transcriptional core complex that is governed by ET-1 axis in EMT, here we dissected the yet uncovered function of  $\beta$ -arrestin-1 as nucleus chaperone in mediating ET<sub>A</sub>R-dependent nuclear responses. Because ET<sub>A</sub>R controls  $\beta$ -catenin signaling in EMT, we investigated the relationship of  $\beta$ -arrestin-1 with  $\beta$ -catenin in the nucleus. As shown by confocal and biochemical analyses, ET-1/ET<sub>A</sub>R activation induces nuclear translocation of both  $\beta$ -arrestin-1 and  $\beta$ -catenin and their colocalization, which were impaired by introduction of nuclear export signal by a single point (Q394L) mutation in  $\beta$ -arrestin-1 and by nuclear export inhibitor Leptomycin B. Furthermore, ET-1-dependent  $\beta$ -arrestin-1 nuclear translocation correlated with an increase in  $\beta$ -catenin transcriptional activity and in the enhanced expression of  $\beta$ -catenin target genes involved in tumor progression, such as ET-1, matrix-metalloproteinase (MMP)-2 and Cyclin D1.  $\beta$ -arrestin-1 silencing or expression of cytoplasmic mutant  $\beta$ -arrestin-1Q394L results in reduced  $\beta$ -catenin-dependent transcription, cell migration and invasion, and in the regulation of EMT determinant expression, indicating a new function of  $\beta$ -arrestin-1 in ET<sub>A</sub>R-driven transcriptional responses in EMT. A thorough characterization of the molecular events by which  $\beta$ -arrestin-1 contributes to regulation of these genes revealed that  $\beta$ -arrestin-1 and  $\beta$ -catenin participate in the formation of a nuclear complex on the promoter regions of these  $\beta$ -catenin target genes, as shown by ChIP and IP assays. This nuclear complex includes the p300 histone acetyltransferase to increase H3 and H4 hyperacetylation and the reorganization of chromatin, thereby increasing gene expression. In an i.p. model of human EOC metastasis in mice, transfection of EOC cells with sh- $\beta$ -arrestin-1 or  $\beta$ -arrestin-1-Q394L significantly reduced the intravasation of tumor cells and the numbers of metastatic nodules. Furthermore, ChIP assays on the metastatic nodules showed reduced association of  $\beta$ -catenin and  $\beta$ -arrestin-1 on above promoters in nodules from cells lacking  $\beta$ -arrestin-1 compared to control, in an extent similar to that induced by treatment with the ET<sub>A</sub>R antagonist, zibotentan. Moreover, the occupancy of  $\beta$ -arrestin-1 and  $\beta$ -catenin on ET-1 and MMP-2 promoters is enhanced in EOC tissues compared to normal tissues, indicating that  $\beta$ -arrestin-1 transcriptional complex is critical for EMT and metastasis. Overall, our study provides for the first time a new mode by which ET<sub>A</sub>R may regulate gene transcription achieved via a nuclear interaction connecting ET<sub>A</sub>R and  $\beta$ -arrestin-1 to  $\beta$ -catenin transcriptional activity in EOC cells. Supported by AIRC, AstraZeneca

**P65. DNA PROFILING FOR THE MONITORING OF CROSS-CONTAMINATION IN MESENCHYMAL STEM CELLS FOR CLINICAL APPLICATION**

Marta Serra<sup>1</sup>, Livia Roseti<sup>1</sup>, Francesca Canella<sup>1</sup>, Carmen Munno<sup>1</sup>, Alice Tosi<sup>1</sup>, Susi Pelotti<sup>2</sup>, Carla Bini<sup>2</sup>, Pier Maria Fornasari<sup>3</sup>, Alessandra Bassi<sup>1</sup>

<sup>1</sup>Cell Factory, Prometeo, Rizzoli RIT (Research Innovation Technology), Istituto Ortopedico Rizzoli (IOR), Bologna, Italy; <sup>2</sup>Department of Medicine and Public Health, Section of Legal Medicine, University of Bologna, Italy; <sup>3</sup>Musculoskeletal Cell and Tissue Bank, Prometeo, Rizzoli RIT, IOR, Bologna, Italy

Adult mesenchymal stem cell (MSCs) display the ability to both self-renew and differentiate along multiple lineage pathways, which makes them an attractive source for cell therapy. In the musculoskeletal system there is a wide variety of cell-based applications: the repair or regeneration of injured tissues (bones, cartilage, tendons, ligaments, muscles, etc..) or the treatment of chronic conditions such as Rheumatoid Arthritis. In Europe, *in vitro* expanded MSCs for clinical use are considered advanced therapy medicinal products (ATMPs), as defined by European Regulation (European Commission [EC], No 1394/2007). Consequently, being considered as drugs with a therapeutical effect, it is mandatory that they have to be manufactured in accordance with “specific medicinal rules” named Good Manufacturing Practice (GMP) in dedicated environments built as real “pharmaceutical factories”.

One main requirement that should be guaranteed for MSCs clinical use is the absence of cross-contamination. This is a serious and often unrecognized problem of both clinical and research fields. In fact, many studies concern contamination coming from tumor cell cultures, while the possibility of mix-up between normal cells (from other types of cultures or from the same type but different patients) usually has little or no consideration.

The aim of this study was to verify if human MSCs cultures, validated in compliance to GMPs for clinical application in bone regeneration, were free of cross-contamination by means of DNA profiling.

Expanded MSCs were analyzed for Nuclear DNA Short Tandem Repeats and miniShort Tandem Repeats and for Mitochondrial DNA hyper variable regions. Patient related control blood samples were used as controls.

The results showed the absence of cross-contamination between MSCs cultures and thus their identity maintenance until the end of manipulation.

Our findings demonstrated that DNA profiling can be a suitable test for quality control in cell therapy applications. In fact, this technique gives the possibility to obtain the results before implantation, allows to detect small amounts of contaminating DNA and can be performed with a few cells.

## **P66. DNA PROFILING: GMP VALIDATION OF AN IDENTITY TEST FOR HUMAN CELL LINES**

Simone Sponza, Pamela Ferro, Giovanni Perono, Chiara Ferrandi e Vittoria Ardissona  
*Procelltech srl, Colletterto Giacosa (TO), Italy*

Recent insights from Regulatory Authorities request application of Good Manufacturing Practice (GMP) guidelines for advanced therapy medicinal product (ATMP) production and quality control (QC). ATMPs can be produced only in Cell Factories that, to be compliant with GMP, need to validate their production process and to identify appropriate QC points all along their path (in process controls) and before releasing batches for injection into patients. To this aim, sample aliquots at the concentration requested for therapeutic use in patients are assayed for sterility, identity and functionality.

Procelltech validated the molecular method “DNA profiling” based on DNA fingerprinting to analyze the genetic profile of human cell lines.

This method is based on multiplex PCR of human genome regions (loci) consisting of DNA repeated in tandem (Short Tandem Repeats, STR). By identifying STR of a specific sequence at specific locations in the genome, it is possible to create the individual genetic profile.

The DNA profiling method used the “AmpFISTR® Identifier™ PCR Amplification Kit” from Applied Biosystems. This kit is a short tandem repeat (STR) multiplex assay that amplifies 15 tetranucleotide repeated loci and the Amelogenin, a gender determining marker, in a single PCR amplification. The kit uses a five-dye fluorescent system for automated DNA fragment analysis through Genetic Analyzer ABI 310 - Applied Biosystems.

In compliance to the international guide line ICHQ2, the validation strategy for DNA profiling was aimed to verify the specificity of the assay: *interspecies specificity* and *intraspecies specificity*.

*Interspecies specificity* was performed to demonstrate that the method could detect only human alleles. To this aim, DNA samples from different animal cell lines and from one bacteria strain have been compared to in-house Reference cellular preparation StemPro® (Human Adipose-Derived Stem Cell, Invitrogen). Only with the StemPro® cellular preparation a full STR profile was obtained.

*Intraspecies specificity* was performed to demonstrate that the method was able to discriminate between two different human cell lines. In-house Reference cellular preparation StemPro® has been compared to the human cell line MRC-5/ATCC CCL171. Two complete, independent STR profiles have been obtained for both human cell lines: StemPro® and MRC-5.

For Repetability, all the experiments have been repeated three times by the same operator.

The approach followed in this study to validate the DNA profiling showed that this method is an highly specific and rapid tool to determine and control genetic identity of human cell lines during production processes.

## PREMIO ONLUS-AICC 2011 “Junior”

**Le colture cellulari nella ricerca biomedica**

*L'Associazione Italiana di Colture Cellulari bandisce un premio di*

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- curriculum vitae (laurea con indicazione del voto, dottorato, specializzazioni, altri titoli e riconoscimenti scientifici, eventuale associazione all'AICC);
- elenco delle pubblicazioni edite dal 2006 al 2011 su riviste internazionali, con indicazione dell'*impact factor*;
- elenco delle pubblicazioni edite dal 2006 al 2011 su riviste nazionali;
- relazione sulla ricerca svolta redatta secondo il seguente schema: Titolo, Basi scientifiche (non più di 140 parole), Scopo della ricerca (140), Metodologie impiegate (70), Risultati (400), Contributo originale delle colture cellulari (100), Conclusioni e prospettive future (100). Le domande contenenti relazioni che non rispettano tale schema verranno escluse dal concorso.

La domanda deve essere spedita entro il **30 settembre 2011** (farà fede il timbro a data dell'Ufficio Postale accettante) al Presidente dell'AICC: Dr. Carlo Leonetti, Istituto Regina Elena CRS, Via delle Messi d'Oro 156, 00158 Roma (Tel.: 06 52662534, Fax: 06 52662592, e-mail: [leonetti@ifo.it](mailto:leonetti@ifo.it)).

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La commissione giudicatrice sarà composta dai Membri del Consiglio Direttivo della ONLUS-AICC e da eventuali esperti designati dal Consiglio stesso. Il giudizio della commissione è inappellabile.

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Roma, 10 giugno 2011

Il Presidente della ONLUS-AICC  
Carlo Leonetti

Si ringrazia per il generoso contributo economico offerto per la realizzazione del Premio

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- curriculum vitae (laurea, partecipazione a corsi, altri titoli e riconoscimenti scientifici, eventuale associazione all'AICC);
- elenco delle pubblicazioni su riviste internazionali, con indicazione dell'*impact factor*;
- elenco delle pubblicazioni su riviste nazionali;
- elenco delle comunicazioni a congressi;
- relazione sulla ricerca svolta redatta secondo il seguente schema: Titolo, Basi scientifiche (non più di 140 parole), Scopo della ricerca (140), Metodologie impiegate (70), Risultati (400), Contributo originale delle colture cellulari (100), Conclusioni e prospettive future (100). Le domande contenenti relazioni che non rispettano tale schema verranno escluse dal concorso.

La domanda deve essere spedita entro il **30 settembre 2011** (farà fede il timbro a data dell'Ufficio Postale accettante) al Presidente dell'AICC: Dr. Carlo Leonetti, Istituto Regina Elena CRS, Via delle Messi d'Oro 156, 00158 Roma (Tel.: 06 52662534, Fax: 06 52662592, e-mail: [leonetti@ifco.it](mailto:leonetti@ifco.it)).

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Roma, 6 giugno 2011

Il Presidente della ONLUS-AICC  
Carlo Leonetti

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## **PREMIO ONLUS-AICC 2011 “Senior”**

**Le colture cellulari nella ricerca biomedica**

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- curriculum vitae (laurea, dottorato, specializzazioni, altri titoli e riconoscimenti scientifici, eventuale associazione all'AICC);
- elenco delle pubblicazioni edite dal 2006 al 2011 su riviste internazionali, con indicazione dell'*impact factor*;
- elenco delle pubblicazioni edite dal 2006 al 2011 su riviste nazionali.

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Roma, 10 giugno 2011

Il Presidente della ONLUS-AICC  
Carlo Leonetti

Si ringrazia per il generoso contributo economico offerto per la realizzazione del Premio



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*Si ringraziano tutti i soci sostenitori AICC per il loro contributo:*

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