



Istituto Zooprofilattico Sperimentale della  
Lombardia e dell'Emilia-Romagna  
"B. Ubertini"



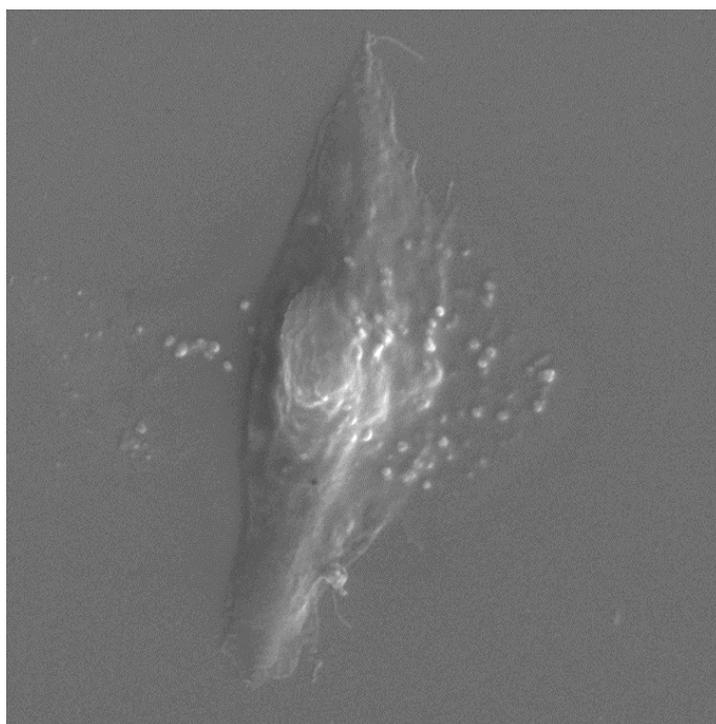
Centro Ricerca Eugenia Menni

## **26° Convegno Annuale della Associazione Italiana di Colture Cellulari (ONLUS-AICC)**

**PROGRESSI E PROSPETTIVE DELLE TERAPIE CELLULARI**

### **4th International Satellite Symposium AICC-GISM**

**MESENCHYMAL STROMAL CELLS ADVANCES**



**20-22 Novembre 2013**

**Brescia, Centro Pastorale Paolo VI  
Via Gezio Calini, 30  
25100 Brescia**

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di Colture Cellulari (ONLUS-AICC)**

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## Grafica e composizione abstract book

**Antonio Lavazza**, Brescia

**Francesca Sisto**, Milano

## Presentazione del Convegno

Il 26° Convegno Nazionale di AICC si svolge a Brescia con il supporto e la collaborazione dell'Istituto Zooprofilattico Sperimentale della Lombardia ed Emilia Romagna e della Fondazione Poliambulanza, Centro di Ricerca E. Menni di Brescia. Il Convegno presenta e discute gli avanzamenti nell'uso delle cellule come strumento terapeutico.

Numerosi sono i progressi conseguiti a partire dalle prime applicazioni, che risalgono ad alcuni decenni or sono, nella trapiantologia e nella cosiddetta "ingegneria tissutale". La maggiore conoscenza della biologia delle cellule staminali ha favorito la loro applicazione clinica sfruttandone le capacità differenziative, rigenerative e riparative. In questo processo evolutivo dalla sperimentazione di base a quella preclinica fino ai primi trials clinici anche gli aspetti regolatori hanno dovuto adeguarsi per garantire corrette procedure soprattutto relative alla sicurezza nell'uso di cellule in terapia. Un punto cruciale che ha generato dibattito è stato il forte orientamento nel considerare la cellula come farmaco che ha comportato l'applicazione di procedure di produzione, di controllo e di conservazione del tutto sovrapponibili a quelle adottate nell'industria per la produzione di farmaci di natura chimica. Il convegno apre con una valutazione critica di questi aspetti cercando di precisarne contenuti, problemi e sviluppi anche sul fronte della cellula considerata come laboratorio biologico per la produzione di sostanze terapeutiche. Prendendo spunto dai positivi risultati che ci consegna la storia dei trapianti di cellule emopoietiche la prima sessione è rivolta all' utilizzo di questo tipo di cellule staminali e a ciò che esse ci suggeriscono per altre applicazioni. Le successive sessioni sono dedicate ad un aggiornamento della ricerca in alcuni settori specifici di applicazione delle cellule staminali: quello neurologico, delle patologie renali e delle patologie oftalmiche.

Nell'ambito del Convegno, il Gruppo Italiano Staminali Mesenchimali (GISM) organizza un simposio satellite internazionale con la partecipazione di relatori europei dedicato a specifici progressi riguardanti le "Mesenchymal Stromal Cells (MSCs)" la cui facilità di isolamento ed espansione le ha rese di grande interesse in svariate applicazioni cliniche. Il simposio approfondisce il possibile utilizzo di MSCs nell'ambito di terapie neoplastiche e propone una sessione dedicata alle applicazioni muscolo-scheletriche ove il loro ruolo è da tempo consolidato. Una sessione è interamente dedicata ad aspetti critici essenziali riguardanti la loro espansione e produzione per l'uso clinico nel cui ambito saranno anche presentati i risultati del lavoro svolto da GISM con il personale coinvolto nella produzione ed il controllo delle MSC per uso clinico. La presenza di relatori altamente qualificati e di livello internazionale rende questo simposio una occasione proficua di aggiornamento per tutti i ricercatori anche quelli non direttamente impegnati in questi ambiti e di discussione tra esperti. Ci auguriamo che esso sia anche utile per generare nuove collaborazioni nell'ambito della ricerca e delle applicazioni cliniche di cellule staminali.

Augusto Pessina  
Maura Ferrari  
Enrico Lucarelli

# Programma Scientifico

## Mercoledì, 20 Novembre 2013

- 14.00 - 18.00 REGISTRAZIONE E COLLOCAZIONE POSTER
- 14.30 - 15.00 PRESENTAZIONE E APERTURA DEL CONVEGNO  
**Stefano Cinotti**, Direttore Generale, IZSLER, Brescia  
**Enrico Zampedri**, Direttore Generale Poliambulanza, Brescia  
**Carlo Leonetti**, Presidente AICC, Roma  
**Augusto Pessina**, Coordinatore GISM, Milano  
**Maura Ferrari**, Izsler, Brescia  
Invitato: **Mario Mantovani**, Assessore alla Salute ,Regione Lombardia
- 15.00 - 15.40 LETTURA MAGISTRALE: **Carlo Alberto Redi**, Pavia  
*La cellula: farmaco e laboratorio biologico?*
- 15.45 - 16.15 Coffee break
- 16.15 - 18.15 **I SESSIONE: Trapianto di cellule emopoietiche: cosa imparare per i trapianti di altre cellule staminali**  
Moderatori: **Michele Caraglia**, **Francesca Zazzeroni**
- 16.15 - 16.35 **Carmelo Carlo Stella**, Milano  
*Cellule Staminali Emopoietiche e Medicina Traslazionale*
- 16.40 - 17.00 **Franco Locatelli**, Roma  
*Trapianti di cellule emopoietiche in pediatria: dall'uso consolidato alle terapie di frontiera*
- 17.05 - 17.25 **Patrizia Comoli**, Pavia  
*Terapia cellulare in oncoematologia: il presente ed il futuro*
- 17.30 - 17.40 Discussione
- 17.40 - 18.10 **Comunicazioni selezionate:**
- 17.40 - 17.50 **Serena Delbue**, Milano  
*Amplificazione del genoma dei Polyomavirus JCV e SV40 in cellule staminali mesenchimali da cordone ombelicale*
- 17.50 - 18.00 **Maria Rosa Todeschi**, Genova  
*Cellule mesenchimali staminali derivate dalla gelatina di Wharton nella rigenerazione tissutale*
- 18.00 – 18.10 **Barbara Rossi**, Bologna  
*Studio dell'espressione di marcatori di pluripotenza in cellule staminali mesenchimali isolate da liquido amniotico, gelatina di wharton e sangue cordonale nella specie equina*

## **Giovedì, 21 Novembre 2013**

09.00 - 10.25 **II SESSIONE: Terapie cellulari in neurologia e neuro-oncologia**

Moderatori: **Sonia Emanuele, Carlo Leonetti**

09.00 - 09.20 **Erica Butti**, Milano

*Staminali neuronali dall'omeostasi alla riparazione*

09.25 - 09.45 **Antonio Uccelli**, Genova

*Cellule staminali mesenchimali per la sclerosi multipla: false speranze o realtà?*

09.50- 10.10 **Luigi Poliani**, Brescia

*"Stem cell signature" in neuro-oncologia: ruolo delle cellule staminali e staminali tumorali nella gliomagenesi e loro implicazioni terapeutiche*

10.15 - 10.25 Discussione

10.25 - 10.40 Coffee break

10.40 - 11.00 **Comunicazioni selezionate:**

10.40 - 10.50 **Martina Violatto**, Milano

*Metodi innovativi per la tracciabilità di cellule mesenchimali della parete del cordone ombelicale in un modello murino di sclerosi laterale amiotrofica*

10.50 - 11.00 **Elisabetta Donzelli**, Monza

*Cellule Staminali Mesenchimali e Progenitori Endoteliali umani promuovono la sopravvivenza di neuroni corticali di ratto in seguito a deprivazione di glucosio ed ossigeno*

11.00 - 12.40 **III SESSIONE: Prospettive di terapia cellulare in patologia renale**

Moderatori: **Guerino Lombardi, Stefania Meschini**

11.00 - 11.20 **Marina Morigi**, Bergamo

*Cellule stromali mesenchimali come terapia cellulare nelle malattie renali acute e croniche*

11.25 - 11.45 **Giovanni Camussi**, Torino

*Meccanismi paracrini nella terapia cellulare renale*

11.50 - 12.10 **Paola Romagnani**, Firenze

*Cellule staminali e rigenerazione renale*

12.15 - 12.20 Discussione

12.20 - 12.40 **Comunicazioni selezionate:**

12.20 - 12.30 **Claudia Lo Sicco**, Genova

*Caratterizzazione di microvescicole derivanti da cellule staminali mesenchimali umane e loro possibile ruolo nella rigenerazione muscolare*

- 12.30 – 12.40 **Marina Aralla**, Lodi  
*Cellule Mesenchimali Autologhe come possibile trattamento per 14 cani con sospetta mielopatia degenerativa: studio sulla sicurezza e applicabilità della metodica*
- 12.45 - 13.30 Assemblea dei Soci AICC e Cerimonia di consegna dei Premi AICC 2013, relazioni dei vincitori
- 13.30 - 15.00 Lunch e visione dei poster
- 15.00 - 16.25 **IV SESSIONE: Cellule staminali in patologie oftalmiche, presente e futuro**  
Moderatori: **Giulio Alessandri, Katia Scotlandi**
- 15.00 - 15.20 **Graziella Pellegrini**, Modena-Reggio Emilia  
*Terapie avanzate con colture di epitelio corneale: 15 anni di valutazioni biologiche*
- 15.25 - 15.45 **Paolo Rama**, Milano  
*Terapie avanzate con colture di epitelio corneale: 15 anni di valutazioni cliniche*
- 15.50 - 16.10 **Vania Broccoli**, Milano  
*Strategie sperimentali di terapia cellulare per le degenerazioni della retina*
- 16.15 - 16.30 Discussione
- 16.30 - 17.00 **Comunicazioni selezionate**
- 16.30 - 16.40 **Sabrina Renzi**, Brescia  
*Caratterizzazione di cellule limbal e Cellule Stromali Mesenchimali (CSM) isolate da tessuto adiposo*
- 16.40 - 16.50 **Chiara Bellotti**, Bologna  
*Lo "shuttling" di BAF e l'accumulo di precursori di Lamina A nella senescenza replicativa delle Cellule Stromali Mesenchimali durante la coltura in-vitro*
- 16.50 - 17.00 **Simona Artuso**, Roma  
*Cellule stromali mesenchimali come veicolo di paclitaxel per inibire la formazione di metastasi polmonari da melanoma B16*
- 17.30 - 18.00 LETTURA MAGISTRALE: **Ornella Parolini**, Brescia  
*Proprietà biologiche e potenzialità terapeutica di cellule derivate da placenta umana*

**Venerdì, 22 Novembre 2013**

**4th International Satellite Symposium AICC-GISM**  
*(Gruppo Italiano Staminali Mesenchimali)*

**Mesenchymal stromal cells advances**

- 09.00 - 09.25    **OPENING LECTURE: Peter J. Nelson**, Munich, Germany  
Mesenchymal stromal cells-based gene therapy in cancer research
- 09.30 - 11.00    **SESSION 1: MSCs and drug delivery**  
Chairpersons: **Augusto Pessina, Rosanna Supino**
- 09.30 - 09.50    **Arianna Bonomi**, Milan  
*Uptake and delivery of Paclitaxel by MSCs*
- 09.50 - 10.10    **Serena Duchi**, Bologna  
*MSC as vehicle of photodynamic therapy for osteosarcoma treatment*
- 10.10 - 10.30    **Luisa Pascucci**, Perugia  
*MSCs-tumor cell interaction: ultrastructural analysis*
- 10.30 - 10.50    **Gabriella Sozzi**, Milan  
*MSCs to repair lung injury after surgical cancer treatment*
- 10.50 - 11.00    General Discussion
- 11.00 - 11.30    Coffee Break
- 11.30 - 12.50    **SESSION 2: Clinical application of MSCs for musculoskeletal regeneration**  
Chairpersons: **Laura De Girolamo, Maura Ferrari**
- 11.30 - 11.55    **Elena Jones**, Leeds, UK  
*The use of native uncultured MSCs in bone repair applications: ex vivo and in vivo approaches*
- 12.00 - 12.20    **Laura de Girolamo**, Milan  
*ASCs for bone and cartilage regeneration: importance of donor features*
- 12.20 - 12.40    **Stefano Grolli**, Parma  
*MSCs in tendon repair*
- 12.40 - 12.50    General Discussion
- 12.50 - 14.00    Lunch

- 14.00 - 16.00 **SESSION 3: Critical issues in MSCs expansion and production**  
Chairpersons: *Enrico Lucarelli, Maria Luisa Torre*
- 14.00 - 14.25 **Karen Bieback**, Heidelberg, Germany  
*Xenogenic-free cell culture media for MSC expansion*
- 14.30 - 14.50 **Mariele Viganò**, Milan  
*Analytical procedures validation for good manufacturing practice (GMP) production of mesenchymal stem cells from different sources*
- 14.50 - 15.10 **Simona Guidi**, Siena  
*Cell factories and Cell culture according to the cGMP rules; tips and tricks to reach the compliance*
- 15.10 - 15.30 **Ivana Ferrero**, Torino  
*GISM work group report*
- 15.30 - 15.50 **Selected communications**
- 15.30 - 15.40 **Elisa Martella**, Bologna  
*Long-term culture without passaging of freshly-isolated human adipose-derived stromal cells (ASC) creates a niche environment which maintains their phenotypic and functional properties*
- 15.40 - 15.50 **Silvia Panseri**, Faenza  
*Magnetic remote control of mesenchymal stem cells as tool for nanomedicine applications*
- 15.50 - 16.00 General Discussion
- 16.00 - 16.20 POSTER AWARDS and Conclusion
- 16.20 - 16.30 Closing remarks

**Comunicazioni dei relatori invitati**  
**Convegno AICC**  
**International Symposium AICC-GISM**

## MSCs to repair lung injury after surgical cancer treatment

**F. Andriani**, F. Facchinetti, S. Furia, L. Roz, S. Bursomanno ,G. Bertolini, C. Carniti , G. Sozzi and U. Pastorino  
Fondazione IRCCS Istituto Nazionale Tumori, Milano, Italy

Surgical removal is the mainstay for early lung cancer treatment and persistent air leaks represent one of the most common clinical complications after lung surgery. Adipose tissue transplantation has been proposed as a new strategy for regenerative therapy after breast cancer surgery; however its efficacy and safety of lung tissue healing after lung resections are unknown. The purpose of our study was to test the biological activity of adipose tissue to facilitate lung tissue healing and evaluate its effect on cancer cells growth, thus providing insight for a possible clinical application. Different *in vitro* cellular models were used to prove the potential biologic effect of autologous fat tissue (AFT) in repairing injured lung tissue, and *in vivo* xenograft models were used to evaluate tumor promoting potential of AFT on putative residual cancer cells. Treatment of both embryonic (WI-38) and adult lung fibroblasts and of normal bronchial epithelial cells (HBEC-KT) with AFT samples, harvested from subcutaneous tissue layer of 20 patients undergoing pulmonary metastasectomy, improved wound healing and cell proliferation indicating a trophic effect on both mesenchymal and epithelial cell types. Conversely AFT-conditioned medium was unable to stimulate *in vitro* proliferation of a lung adenocarcinoma reporter cellular system (A549). Moreover, co-injection of AFT and A549 cells in nude mice did not promote engraftment and progression of A549 cells. These preclinical findings provide preliminary evidence on the potential efficacy of AFT to accelerate lung tissue repair without undesired tumor promoting effects on putative residual cancer cells.

## **Xenogenic-free cell culture media for MSC expansion**

**Karen Bieback**

Institute of Transfusion Medicine and Immunology, Medical Faculty Mannheim, Heidelberg University

Mesenchymal stem/stromal cells (MSC) are in the focus of intense research to develop novel cell-based therapies. To manufacture MSC applicable for use in patients Good Tissue/ Manufacturing/ Clinical Practice standards (GTP, GMP, GCP) need to be implemented. These standards ensure that a cell therapy product is safe, pure and potent.

With the need of ex vivo expansion the manufacturing process of MSC is complex. A variety of - also clinical - protocols rely on the use of fetal bovine serum (FBS), critically rated by the regulatory authorities. The high lot-to-lot variability and the risk of immunisation and infection call for xenogenic-free alternatives. While the development of GMP-compliant chemically defined media is on-going, “humanized” culture conditions appear highly encouraging. “Humanized” supplements include human serum, autologous or pooled allogeneic, cord blood serum as well as different platelet derivatives.

Any change of culture conditions can be of major impact on cellular qualities. The majority of studies confirm an accelerated proliferation of MSC in platelet lysate compared to FBS. Importantly this is not associated to chromosomal instabilities. These have only been monitored in FBS but not in humanized culture supplements. Although main characteristics of MSC, as defined by the ISCT, appear retained, continuative studies indicate that the choice of supplement for instance affects gene and protein expression. Adhesion as well as homing becomes modified by humanized culture systems compared to FBS.

To provide success of MSC-based therapies, the establishment of standardized manufacturing protocols and quality control parameters and assays is of utmost importance.

## Uptake and Delivery of Paclitaxel by MSCs

**Arianna Bonomi**

Università degli Studi di Milano – Dip. Scienze Biomediche, Chirurgiche e Odontoiatriche

Paclitaxel (PTX) is a first-line drug for the treatment of several tumors thanks to its capacity to promote cell death (by microtubule polymerization and by increasing the sensitivity to radiation) and to inhibit angiogenesis. Unfortunately, most of the side effects observed in cancer chemotherapy depend on the fact that the administered drugs are toxic not only for the tumor mass but also towards some other tissues and organs of the organism. In the attempt to solve this problem, in the last years several strategies have been proposed to deliver the drug in the tumor microenvironment and limit, by this way, the collateral toxicity: for instance, nanoparticles, toxic immunoconjugates and engineered stem cells. Because mesenchymal stromal cells (MSCs) are easy to *in vitro* manipulate and home to pathological tissues after being injected *in vivo*, these cells seem to represent one of the best devices to deliver anti-tumor compounds.

In a previous study, we demonstrated that exposure of mouse bone marrow derived stromal cells to Doxorubicin led them to gain anti-proliferative potential towards co-cultured haematopoietic stem cells (HSCs). We thus hypothesized whether MSCs isolated from different tissues (bone marrow, adipose tissue and dermis) after *in vitro* loading with the anti-cancer drug PTX and then localized near tumor cells (TCs), could inhibit the proliferation of these last ones.

Incorporation of PTX into MSCs was studied by using FITC-labelled-PTX and analyzed by FACS and confocal microscopy. Furthermore, the ultrastructural analysis performed by electron microscopy revealed that the loading with PTX did not induce morphological alterations in MSCsPTX. Release of PTX in culture medium by PTX primed MSCs (MSCsPTX) was investigated by HPLC. The anti-tumor activity of MSCsPTX was tested both *in vitro* (by evaluating the inhibition of the proliferation of different tumor cell lines) and *in vivo* (by co-transplanting MSCsPTX and cancer cells in mice or by treating tumor bearing mice with MSCsPTX). Also the anti-angiogenic activity was tested both *in vitro* (culture of endothelial cells and aorta ring assay) and *in vivo* (evaluation of angiogenic markers expression in tumors treated by MSCsPTX). The obtained results show that MSCs are able to rapidly incorporate PTX and slowly release it in the culture medium in a time dependent manner. PTX primed cells acquire, by this way, a potent anti-tumor and anti-angiogenic activity not only *in vitro* but also *in vivo*. Furthermore, the absence of any genetic manipulation makes these cells a safer tool in cancer therapy in comparison to engineered cells; indeed, although genetically modified cells provided encouraging results in animal models, they are not devoid of risks for clinical applications.

## **Homeostatic and reparative role of neural stem cells**

**Erica Butti**

Institute of Experimental Neurology (INSpe), Division of Neuroscience, San Raffaele Scientific Institute, Milan, Italy

Regenerative processes occurring under physiological (maintenance) and pathological (reparative) conditions are a fundamental part of life, and vary greatly among different species, individuals, and tissues. Physiological regeneration occurs naturally as a consequence of normal cell erosion, or as inevitable outcome of any biological process requiring the restoration of homeostasis. Reparative regeneration occurs as a consequence of tissue damage. Although the central nervous system (CNS) has been considered for years as a 'perennial' tissue, it is now becoming clear that both physiological and reparative regeneration occur within the CNS to sustain tissue homeostasis and repair. Proliferation and differentiation of neural stem and progenitor cells (NPCs) residing within the healthy CNS, or surviving to the injuries, are considered crucial in sustaining these processes. Thus, a large number of experimental stem cell-based transplantation systems for CNS repair have recently been established. The results suggest that transplanted NPCs might promote tissue repair not only via cell replacement but also via their local contribution to changes in the diseased tissue milieu.

## **The paracrine hypothesis of stem cell action in kidney repair**

**Giovanni Camussi, Vincenzo Cantaluppi**

Department of Medical Sciences, University of Torino

Multipotent mesenchymal stromal cells (MSCs) promote the recovery of acute and chronic kidney injury in several experimental models. The paracrine hypothesis of stem cell action is based on the observation that after infusion only few MSCs permanently engraft and differentiate into renal parenchymal cells. As the conditioned medium of MSCs may mimic the effect of the cells it has been suggested that the beneficial effect of MSC treatment be ascribed to the release of mediators. Indeed, MSCs produce several factors that may modulate inflammation and immune-response and stimulate regeneration. Apart from soluble factors MSCs release extracellular vesicles (EVs) that after receptor-ligand interaction can be incorporated by tissue injured cells and deliver biologically active components including, receptors, growth factors and nucleic acids. EVs by acting as signaling complexes may directly activate the recipient cells. In addition EVs may transfer mRNA and microRNA derived from stem cells able to modify the phenotype of recipient cells that acquire stem cell-like properties thus initiating tissue repair.

## Cell therapy in hematology-oncology: the present and the future

**Patrizia Comoli**

Oncoematologia Pediatrica, Fondazione IRCCS Policlinico San Matteo, Pavia

Among the novel biologic therapeutics that will increase our ability to cure human cancer and the complications associated to its cure in the years to come, adoptive T cell therapy (ATCT) is one of the most promising approaches. Although this is a complex and challenging field, there have been major advances in basic and translational research resulting in clinical trial activity that is now beginning to confirm this promise.

Early trials have focused on the treatment of viral infections and virus-related tumors developing in the immunocompromised host. Epstein-Barr virus (EBV)-associated malignancies offered a unique model to develop T cell-based immune therapies, targeting viral antigens expressed on tumor cells. In the last two decades, EBV-specific cytotoxic T-lymphocytes (CTL) have been successfully employed for the prophylaxis and treatment of EBV-related posttransplant lymphoproliferative disorders. More recently, this therapeutic approach has been applied to the setting of other hematologic and solid tumors. In these settings, with the possible exception of tumor-infiltrating lymphocytes therapy for melanoma, clinical trials of ATCT have so far provided only clear proofs-of-principle that objective clinical responses may be attainable. The results are encouraging, although further improvements to the clinical protocols are clearly necessary to increase anti-cancer activity.

Recent studies demonstrating that normal human lymphocytes can be genetically engineered to recognize cancer antigens and mediate cancer regression *in vivo* has opened opportunities for enhancing and extending the ACT approach to patients with a wide variety of cancer types. However, the high affinity for target antigens shown by some of these cell products, that is responsible for some dramatic, durable clinical responses recently reported, was also the cause of severe life-threatening adverse events.

ATCT offers a unique opportunity to restore antitumor and anti-pathogen immune surveillance, and it is therefore conceivable that application of this strategy will increase in the next few years. Despite the great potential, immunotherapy still has a marginal role in the management of patients with cancer. This is due to limitations inherent to the technologies and products employed, and to the financial and structural burden that are associated with cell therapy. Indeed, the extensive infrastructure needed for exploiting such approaches still restricts their use to academic centres with specific programs in the field. For progression to a widely used, effective and safe ATCT, cooperation in ATCT networks is crucial.

## **ASCs for bone and cartilage regeneration: importance of donor features**

**Laura De Girolamo**

I.R.C.C.S Istituto Ortopedico Galeazzi, via R. Galeazzi 4, Milano

Since 1994, when leptin was identified, adipose tissue was no longer just considered as a fat store, but as a true secretory tissue. About ten years later, the perception of adiposity changed again, thanks to the discovery of an abundant number of mesenchymal stem cells (ASCs, adipose derived-stromal cells), that possess similar properties to bone marrow mesenchymal stem cells. During the last decade, several preclinical studies have provided data on the safety and efficacy of ASCs, supporting the use of these cells in clinical applications. Adipose tissue is anatomically distributed throughout the human body, and the pattern of adipose tissue distribution is influenced by many factors, including sex, age, genotype, diet, physical activity level, hormones, and drugs; all these parameters may thus affect yield, proliferation and differentiation ability of human ASCs. In particular we studied the influence of age on hASCs immunophenotype, clonogenic ability and differentiation potential. The data showed that some MSC markers were differently expressed in cells derived from younger and elder women; moreover the yield of hASCs derived from younger women was significantly more abundant in comparison to elder ones, as well as characterized by a higher clonogenic potential. Osteogenic potential of hASCs derived from younger women appears more pronounced compared to cells derived from elder and obese ones. Little is known about ASCs derived from donors with chronic diseases, such as obesity. Indeed, adipose tissue of obese patients shows a reduced pressure of oxygen, involved in the up-regulation of pro-inflammatory genes that could affect the properties of these cells. In particular obesity-related inflammation may have an important role in the modification of the so called “stem cells niche” and thus may affect stem cell properties and differentiation potential of subcutaneous and visceral ASCs, thus possibly influencing the feasibility of autologous ASCs implantation in cell-based therapies.

Our study showed that obesity had a significant negative impact on the stem cell properties and the differentiation potential, in particular the osteogenic one, of both visceral and subcutaneous ASCs. However, despite this impairment, while subcutaneous obese-derived ASCs were able to differentiate toward osteogenic and adipogenic lineages, although to a small extent, visceral obese-derived ASCs lost most of the stem cell characteristics, including their multidifferentiation potential. Other causes should be further investigated to have a complete picture of the parameters influencing human ASCs behavior. A complete understanding of this phenomena could help to create a patient-specific biological solution, which would probably lead to a better final outcome.

## MSC as vehicle of photodynamic therapy for osteosarcoma treatment

Duchi S.<sup>a</sup>, Ballestri M.<sup>b</sup>, Dozza B.<sup>a,c</sup>, Sotgiu G.<sup>b</sup>, Dambruoso P.<sup>b</sup>, Varchi G.<sup>b</sup>, Lucarelli E.<sup>a</sup>

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Osteosarcoma(OS)is the most common primary tumor of the bone and the most frequent bone sarcoma in children and adolescents. Standard treatments include surgery and chemotherapy. Such treatments contribute to a current survival rate of 65%. The poor outcome in the efficacy of this therapy is due, in part, to an inability to deliver the drugs to the infiltrative tumor cells. To overthrow this limitation, one approach is to dispense therapeutic agents through Mesenchymal Stromal Cells (MSC) taking advantage of their unique ability to migrate, home and finally engraft in the tumor stroma. We therefore propose a combined strategy in which MSC are used as a delivery vehicle for photodynamic therapy (PDT), an approach that applies light and molecular oxygen in combination with a photosensitizing agent, to selectively eliminate cells.

For our purposes, we used biocompatible multi-functional core-shell (PMMA)-based nanoparticles (NPs) fluorescently labeled with fluorescein (FNPs) loaded with molecules well known to exhibit a high photo-activity and to generate ROS after excitation with specific emission wavelength source. ROS species represent the cytotoxic agents responsible of cell death.

In our previous study we successfully demonstrated that FNPs functionalized with meso-tetrakis(4-sulfonatophenyl) porphyrin(TPPS) on the external shell (TPPS@FNPs), can be efficiently and safely loaded into MSC *in vitro*. In addition, through laser confocal microscopy and time lapse imaging of TPPS@FNPs-MSC co-cultured with OS tumor cells *in vitro*, we demonstrated the ability of this system to induce cell death when stimulated with laser light.

The preclinical (*in vivo*) model is now essential for meaningful study of the efficacy of PDT for OS treatment. Hence in our present analysis, we tested the ability of FNPs functionalized with aluminium ftalocyanine tetrasulfonate cholorida (Ftl@FNPs) and loaded into MSC, to induce controlled and massive cells death of themselves and of OS cells in a short time frame right after light activation. The advantage of Ftl is the near infrared excitation wavelength (680 nm), which is a suitable light source for an appropriate penetration through the animal tissue into the tumor stroma. Cytofluorimetric analysis and confocal imaging clearly show the high efficiency MSC loading rate of Ftl@FNPs and the rapid cell death of MSC and OS tumor cells after light stimulus.

Our preliminary data demonstrate the excellent ability of MSC to function as a carrier of photo-killing agents *in vitro* with various photosensitizing agents and different light sources. Our system allows the controlled release of the drug since ROS are generated only after the occurrence of external light activation only at the desired body region.

Our results propose a novel therapeutic option for the treatment of bone sarcomas and tumors like breast cancer and gliomas, all of which currently require different therapeutic approaches to overcome recurring and drug treatment failures.

## GISM working group Report

**Ivana Ferrero**

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The success of advanced therapy-based approaches is highly dependent upon the development of standardized protocols according to Good Manufacturing Practice (GMP), including production and quality control processes.

The quality and safety of advanced therapy medicinal products (ATMPs), such as mesenchymal stem cells (MSCs), must be maintained throughout their production and quality control cycle, ensuring their final use in the patient. The particular characteristics and high plasticity of MSCs make them ideal candidates in cell therapy strategies to treat a number of degenerative and post-traumatic diseases caused by damage or cell loss.

The translation of research-based protocols into GMP-compliant procedures for large-scale production of clinical-grade MSCs requires careful analysis of all the risks and benefits to identify and control all critical aspects: source of MSCs and raw materials; culture media and supplements; quality control tests. The minimal criteria to identify MSCs were proposed by the International Society for Cellular Therapy (ISCT): adherence to plastic; expression of specific surface antigen (positive for CD105, CD73, CD90 and negative for CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II); and the multipotent capacity to differentiate into osteoblasts, adipocytes or chondroblasts under standard *in vitro* culture conditions (Dominici, 2006).

The GISM working group dedicated several meetings to discuss, with scientists from different Italian Cell Factories, the minimal quality requirements for *ex vivo* expanded MSCs in order to standardize methods and criteria for the clinical use of MSCs. The outcome of this work will be a document to submit to the Regulatory Authority. The quality attributes for MSCs proposed in this assessment are based on the quality attributes for cell substrates used for pharmaceutical manufacturing as described in the European Pharmacopoeia. The cell substrates used as starting/raw materials for biotech medicinal products are commonly used in pharmaceutical manufacturing and many licensed products in the European Market are manufactured using cell substrates (e.g. viral vaccines). Since the quality attributes of these cells are well defined in the European Pharmacopoeia, the GISM assessment for MSCs will be set up following the same approach, considering:

1. Isolation and expansion: cell source; quality control on tissue and materials; plating, seeding and expansion.
2. Quality control for the validation of MSC production: identity (morphology, immunophenotype, differentiative potential); growth characteristics; sterility (bacteriology, LAL test, mycoplasma); tumorigenesis; karyotype.
3. Quality control in process for MSCs: identity (morphology, immunophenotype); growth characteristics; sterility (bacteriology).
4. Quality control at batch release for MSCs: identity (morphology, immunophenotype); growth characteristics (number of cells, viability); sterility (bacteriology, LAL test, mycoplasma, virology).

The final document is aimed at providing a standardized process for the production of MSCs, according to GMP requirements, optimizing times and costs, without losing the safety of the cell therapy product.

## **Optimization of photoreceptor differentiation from human and rodent Muller Glia cell.**

**Serena G. Giannelli<sup>1,2</sup>, Gian Carlo Demontis<sup>3</sup>, Grazia Pertile<sup>5</sup>, Paolo Rama<sup>2,4</sup>, Vania Broccoli<sup>1,\*</sup>**

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There is growing evidence that Muller glia cells are a source of neural regeneration in both injured and diseased retinas. In fact, the process of reactive gliosis that occurs in these conditions has often and in different species been ascribed to a regenerative repair leading to replacement of retinal neurons and only in early vertebrates of photoreceptors. However, human MGC regenerative potential in retina is yet to be characterized.

We devised a method to obtain pure human and murine MGC culture that not only allows amplification of these cells for few passages but also recapitulates in-vitro reactive gliosis. Thanks to that, we were able to further manipulate culture MGCs toward differentiation: thus we confirmed mMGC neurogenic properties in-vitro and attributed for the first time these properties to hMGC. Furthermore photoreceptor commitment was detected in both differentiated murine and human MGCs. Therefore an extensive effort, including the optimization of a multi-step differentiating process, was carried out in order to significantly increase the efficiency of photoreceptor production over the entire differentiated progeny of hMGCs. These efforts led finally to a photoreceptor commitment efficiency as high as 54%. Intriguingly, human and mouse Müller cells need different procedures to be efficiently induced into the photoreceptor cell lineage, suggesting some intrinsic divergent sensibility to developmental molecular pathways. Human transplanted Muller glia derived photoreceptor progenitors integrate, survive and complete rod differentiation within immunodeficient mouse retinas. These data provide evidence that human adult Muller glia retains a remarkable plasticity and are therefore a promising source of novel therapeutic applications in retinal repair.

## Mesenchymal Stem Cells in tendon repair

**Stefano Grolli**

Dipartimento Scienze Medico-Veterinarie, Università degli Studi di Parma

In the last decade, regenerative medicine and mesenchymal stem cells (MSCs) have gained a strong interest in the therapy of musculoskeletal disorders in veterinary medicine. In particular, the application of adult MSCs to promote the healing of traumatic tendinopathies in the horse is considered a valuable model to study the therapeutic potential of these cells, in both animals and humans. Worldwide, probably several thousands of horse tendonitis have been treated by means of stem cell therapy. Nevertheless, the role of MSCs in tendon regeneration remains difficult to evaluate at the molecular and cellular level. For this reason, regenerative therapy of equine tendonitis can be considered a paradigm, illustrating how difficult it is to collect scientifically reliable data about the efficacy of cell therapy. As a matter of fact, several *in vivo* studies performed to date using equine MSCs to treat naturally occurring tendon injuries suggest a positive clinical outcome of cellular therapy, with a lower re-injury rate with respect to conventional, conservative therapies. Various therapeutic approaches have been proposed, based on the use of *in vitro* expanded MSCs derived from different tissue sources (bone marrow, adipose tissue, umbilical cord blood and peripheral blood), bone marrow concentrates and fat tissue-derived cell suspensions. Most of the stem cell therapies make use of autologous cells, although *in vitro* and *in-vivo* evidences are accumulating to suggest a possible use of allogeneic MSCs. Allogeneic MSCs could provide an “off-the-shelf” cell source, readily available without the time-demanding *in vitro* expansion of autologous cells, ensuring an immediate intralesional therapy after diagnosis of injury.

Whether MSCs primarily contribute to lesion healing by integrating into the injured tissue or indirectly by secreting immunomodulatory and bioactive trophic factors is still a matter of discussion. Experimentally induced tendonitis have demonstrated significant improvement in tissue strength, collagen fibers organization and quality of the reparative tissue. Furthermore, post mortem histopathologic examination of naturally occurring tendonitis treated with MSCs have not revealed any abnormal or neoplastic tissue within the implantation site. On the contrary, only partial data are available with regard to gene expression, survival rate and differentiation capability of the grafted cells. Furthermore, most of the studies suffer of a small sample size and lack of appropriate, randomized control groups.

In conclusion, MSCs have demonstrated a great potential for regenerative therapy of tendon injuries in the horse, although there is a strong need of rigorous experimental and clinical data to support the widespread application of the treatment.

## **Cell factories and Cell culture according to the cGMP rules; tips and tricks to reach the compliance**

**Simona Guidi**

CTP Tecnologie di Processo – Poggibonsi (SI)

Since the European Regulation 1394/2007 entered into force at the end of 2008, the world of cell therapies is in turmoil. After 5 years of trying to come into compliance, still many cell factories are not able to obtain authorization to produce according to the principles of Good Manufacturing Practices (GMP). The main obstacle however is not technical but cultural. Often those who have to design and manage a cell factory come from an academic and not from an industrial-pharmaceutical environment, thus they don't know the regulatory and technological possibilities to reach the result.

The synergy between researchers and technical and regulatory staff from pharmaceutical companies is the winning one. Working as a team the goal of growing the cells in accordance with the GMP can be achieved. The two crucial moments in the development of an ATMP are the initial phase, with the definition of the working environment (e.g. Clean Room, but not only), and the final stage, with the planning and execution of the final validations to prove that the process and structure are under control. The focus of this presentation is to give some suggestions to overcome some obstacles in the long and tiring journey to obtain the manufacturing authorization by the Competent Authority.

## **The use of native uncultured MSCs in bone repair applications: ex vivo and in vivo approaches**

**Elena Jones**

Leeds Institute of rheumatic and Musculoskeletal Medicine, UK

Cell therapy with MSCs is being increasingly used for the treatment of bone and cartilage disorders. Most commonly, MSCs are manufactured by culture-amplification of plastic adherent cells from bone marrow (BM) aspirates; this process is costly and time-consuming. In our laboratory, we have developed robust methods for the isolation and characterization of native BM MSCs based on the CD271 marker. Native MSCs have both similarities and differences from cultured MSCs; amongst most striking differences are alterations in cell morphology, surface phenotype and in their transcriptional profile, particularly in relation to Wnt-related and hematopoiesis-supporting genes. The advantage of using native MSCs as therapy for bone repair is a possibility to develop a rapid one-stage process whereby MSCs are extracted and re-implanted during the same surgical procedure. However, it is known that MSC are rare in BM aspirates necessitating the search for alternative MSC sources. We have previously shown that functionally competent MSCs could be also recovered, in large numbers, from digested bone fragments, including whole femoral heads removed following hip replacement surgery. More recently, we found large numbers of MSCs in human long bones, which could be recovered in reaming waste bags, another surgical by-product that is currently discarded. These surgical by-products contain massive numbers of uncultured MSCs (equivalent to one litre of marrow), which can be concentrated, purified and used as a single-surgery therapy for non-union fractures or large bone defects (latter in conjunction with osteoinductive scaffolds). Whereas most surgeons use simple 'concentrator' devices that are not specific for MSCs, we have optimized a GMP compatible process, based on anti-CD271 beads and a CliniMACS device, in order to isolate large numbers MSCs from the above-mentioned intra-osseous MSC sources.

In these 'ex vivo' approaches MSCs are removed and subsequently re-injected back into a patient. As an alternative, new in vivo/in situ approaches rely on targeting and re-directing patient's own MSCs towards the injury site. In this context our data show that a periosteum-like membrane naturally formed around a large bone defect can be used as a biological chamber to attract and 'house' MSCs as well as to enrich for growth factors and chemokines, potentially leading to faster repair. This is a good example of in situ tissue engineering in common orthopedic settings.

## **Cellule stromali mesenchimali come terapia cellulare nelle malattie renali acute e croniche**

**Marina Morigi**

Istituto di Ricerche Farmacologiche Mario Nergri, Bergamo

La prospettiva clinica di usare cellule stromali mesenchimali (CSM) da midollo osseo (MO) o da altre fonti ha creato enormi aspettative per la cura di malattie come l'infarto del miocardio, malattie neurologiche e l'insufficienza renale acuta (IRA). Il nostro gruppo è stato uno dei primi a documentare un effetto protettivo di MO-CSM nel modello sperimentale di IRA, una malattia che colpisce circa il 7% dei pazienti ospedalizzati con una mortalità piuttosto elevata. Un importante target renale della terapia cellulare nell'IRA è rappresentato dalle cellule epiteliali tubulari che in seguito ad un danno acuto ischemico o tossico, si disregolano e muoiono. Abbiamo osservato che l'iniezione di MO-CSM murine in topi con IRA indotta da cisplatino, un farmaco antitumorale nefrotossico, proteggeva la funzione renale e riduceva il danno necrotico tubulare stimolando la proliferazione delle cellule renali attraverso un meccanismo paracrino. Abbiamo confermato tali risultati anche con CSM umane, infuse in topi immunodeficienti con IRA. Tali cellule esercitavano un significativo effetto reno-protettivo e prolungavano la sopravvivenza degli animali con IRA, attraverso la produzione locale di citochine e fattori di crescita in grado di ridurre l'apoptosi e aumentare la proliferazione delle cellule renali residenti. Alla ricerca di sorgenti di CSM più efficaci ed accessibili, abbiamo studiato le CSM isolate da altri tessuti come il tessuto adiposo e il sangue del cordone ombelicale (CO). Le cellule staminali del tessuto adiposo ottenute da due diversi donatori, non hanno mostrato alcun effetto protettivo. Diversamente, le CO-CSM miglioravano significativamente la funzione e la struttura renale, aumentando la sopravvivenza degli animali in maniera molto più significativa rispetto alle cellule del MO-CSM. Le cellule del CO, localizzandosi prevalentemente a livello peritubulare, diminuivano in maniera più efficace lo stress ossidativo e l'apoptosi e aumentavano la proliferazione e il numero dei tubuli positivi ad una chinasi ad azione antiapoptotica e mitogenica, l'Akt. Tutti questi effetti, insieme alla loro attività antinfiammatoria e citoprotettiva per i capillari peritubulari suggeriscono quindi un più elevato potenziale rigenerativo delle CO-CSM rispetto alle altre cellule staminali studiate.

L'effetto renoprotettivo delle CSM è ben conosciuto nei modelli di danno renale acuto, tuttavia il loro effetto nelle malattie renali croniche non è così chiaro. Il nostro gruppo ha testato l'effetto delle MO-CSM in un modello di nefropatia indotto da adriamicina nel ratto. Abbiamo osservato che ripetute iniezioni di MO-CSM nel tempo limitavano la perdita dei podociti, normalizzavano la distribuzione glomerulare delle cellule parietali epiteliali e riducevano il danno endoteliale diminuendo la glomerulosclerosi, ma non erano in grado di migliorare la funzione renale e la proteinuria. Nuovi esperimenti sono in corso con CSM precondizionate o geneticamente modificate per aumentare il loro potenziale rigenerativo.

## **Engineered mesenchymal stem cells as therapeutic vehicles for the treatment of solid tumors**

**Peter J. Nelson** and Christiane Bruns

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Engineered mesenchymal stem cells (MSC) are being developed as a therapeutic modality for the treatment of solid tumors. Adoptively transferred MSC are recruited to tumor stroma where they promote tumor growth and metastasis. This biology has been harnessed to generate a class of therapeutic cellular vehicles that efficiently penetrate and target specific aspects of tumor biology. These cellular agents show potential utility if they can be properly engineered to selectively deliver therapeutic genes to tumor environments, limit damage to normal tissues, and be eliminated from the host in the context of therapy. To date, selective tissue targeting has been achieved through use of gene promoters linked to the activation and differentiation of stem cells that occurs within tumor microenvironments. The results show efficacy in the targeting of both primary tumors and metastatic disease. The selection of therapeutic genes used in this approach is a central issue. While a series of suicide genes have been developed, we are currently evaluating application of the sodium/iodide symporter (NIS) as a combined imaging/therapeutic gene. These and other issues will be discussed.

## **Proprietà biologiche e potenzialità terapeutica di cellule derivate da placenta umana**

**Ornella Parolini**

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Human placenta-derived tissues, and in particular the amniotic membrane, have been considered therapeutically valuable for more than 100 years. Indeed, the first successful use of human fetal membranes as a skin substitute was reported by the early 1900s, with this study opening the door to subsequent applications of the amniotic membrane as a surgical biomaterial for a variety of pathological conditions, including the treatment of ocular disorders, skin wound healing and reconstructive surgery. Over recent years, we have learned that different types of cells can be isolated from various regions of the human placenta. These cells, and particularly the amniotic epithelial cells (hAECs) and the mesenchymal stromal cells isolated from the amniotic and chorionic membranes (hAMSCs and hCMSCs, respectively), and from Wharton's Jelly and placental villi, have attracted increasing attention due to their interesting biological properties, including their pluripotency and immunological features. Consequently, the placenta is no longer considered as a mere waste material after birth, but is proving to be a valuable source of stem/progenitor cells with therapeutic potential, and hopefully, wide applicability in regenerative/reparative medicine. As for the characterization of stem cells isolated from other sources, researchers interested in the placenta have investigated the stem potential of human placenta-derived cells in order to assess whether they are endowed with the characteristics that are generally associated with "*stemness*", mainly focussing on their capacity to differentiate into several cell different types and to promote tissue regeneration by replacing defective host cells. Their multilineage differentiation capacity has been described mainly through *in vitro* studies, and is supported by scant evidence *in vivo*. Nevertheless, preclinical studies performed in animal models of various pathological conditions, including myocardial ischemia, liver fibrosis, lung fibrosis and rheumatoid arthritis, have demonstrated the therapeutic potential of placental cells. These studies have also suggested that the beneficial anti-inflammatory and anti-fibrotic effects and the improvement in function of damaged tissues which are observed after transplantation of placental cells are very likely associated with the immunomodulatory characteristics of these cells. These findings bring into discussion the question of whether the definition of "stem cells" also pertains to cells which act mostly by secreting paracrine-acting soluble factors to promote the improvement of degenerative conditions, such as inflammation and fibrosis, rather than through tissue-specific differentiation and replacement of the host's own damaged/defective cells. Although the mechanisms underlying the beneficial effects of placental cell transplantation still remain to be further investigated, the very promising results achieved so far are certainly a proof principle regarding the therapeutic potential of these cells, and are very encouraging as a basis for future studies.

## MSC -tumor cell interactions: an ultrastructural analysis

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Mesenchymal stromal cells (MSCs) have attracted great interest for their considerable therapeutic potential. When implanted in the site of injury or in a tumor, they exert their biological effects by a multitude of signals and complex interactions with resident healthy, damaged or transformed cells. Cell-cell interaction, in particular, may be achieved through some basic mechanisms like: 1) direct cell-cell interaction through the formation of mechanical adhesions, functional junctions or tunnelling nanotubes; 2) information exchange mediated by soluble trophic factors and signaling molecules; 3) transfer of molecules enclosed in membrane bounded vesicles (MVs).

In spite of the large number of papers dealing with the functional aspects and implications of MSC interactions, a few studies investigated their morphological counterpart.

In this work, the most relevant morphological features of MSC homotypic and heterotypic interactions evidenced by electron microscopy has been surveyed.

Our observations on cultured MSCs, demonstrated that they are often reciprocally interconnected by adhesion structures and by small points of contact located on cell body as well as along cytoplasmic processes. These cellular protrusions vary greatly in length and either get in touch with each other or insert into cytoplasmic invaginations of contiguous and distant cells. As cells proliferate and reach a higher density, these processes drastically reduce in length and appear as short intercellular bridges.

In order to explore the possible modulation of MSCs on tumor growth and progression, we recently performed a set of experiments aimed at investigating, morphologically, the relationships between MSCs and different type of tumor cells. In particular, the interaction between MSCs and the leukemia cell lines L1210 and Molt4, and between MSCs and the melanoma cell line B16 were analyzed by electron microscopy.

Co-incubation of L1210 or Molt-4 with MSCs resulted in the formation of mesenchymal cell aggregates surrounded by a "crown" of leukemic cells that seemed to be attracted by MSCs. Even if areas of contact between leukemia cells and MSCs were observed, no apparent specialization of the plasma membrane and of the cytoplasm beneath the sites of contiguity or contact was seen; therefore, the presence of junctional structures or complexes that could suggest a strong mechanical or functional interaction at the cell-cell interface was excluded.

When co-cultivated with B16, MSCs realize different kinds of interactions including adhesion and MV release. This latter aspect was revealed by the observation of a huge number of vesicles, shedding from the cell surface, and of exosomes, derived from multivesicular bodies located inside the cells.

The presence of nanometric cell surface projections has been occasionally detected and has been interpreted as a possible variety of communication referable to tunnelling nanotubes.

## **Biological parameters determining the clinical outcome of autologous cultures of limbal stem cells**

**Graziella Pellegrini**

Centre for Regenerative Medicine .University of Modena and Reggio Emilia

Limbal cultures restore the corneal epithelium in patients with ocular burns. We investigate biological parameters instrumental for their clinical success. We report a long-term multicenter prospective study on 152 patients, carrying severe burn-dependent corneal destruction, treated with autologous limbal cells cultured on fibrin and clinical-grade 3T3-J2 feeder cells. Clinical results were statistically evaluated both by parametric and non-parametric methods. Clinical outcomes were scored as full success, partial success and failure in 66.05%, 19.14%, and 14.81% of eyes, respectively. Total number of clonogenic cells, colony size, growth rate and presence of conjunctival cells could not predict clinical results. Instead, clinical data provided conclusive evidence that graft quality and likelihood of a successful outcome rely on an accurate evaluation of the number of stem cells detected before transplantation as holoclones expressing high levels of the p63 transcription factor. No adverse effects related to the feeder-layer has been observed and the regenerated epithelium was completely devoid of any 3T3 contamination. Cultures of limbal stem cells can be safely used to successfully treat massive destruction of the human cornea. We emphasize the importance of a discipline for defining the suitability and the quality of cultured epithelial grafts, which are relevant to the future clinical use of any cultured cell type.

## **"Stem cell signature" in neuro-oncologia: ruolo delle cellule staminali e staminali tumorali nella gliomagenesi e loro implicazioni terapeutiche**

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I gliomi di alto grado (HGG) rappresentano i tumori maligni più aggressivi dell'adulto e, nonostante le attuali terapie, incluso lo sviluppo di terapie personalizzate con farmaci capaci di aggredire selettivamente singole vie di segnale intracellulare (p.es. Cetuximab<sup>®</sup>, Avastin<sup>®</sup>), la prognosi resta infausta con un "overall survival" di soli 14.6 mesi. La Temozolomide (Temodal<sup>®</sup>) è l'unico farmaco che ha permesso un modesto aumento della sopravvivenza media in Pazienti con HGG associati a metilazione del gene O6-methylguanine-DNA-methyltransferase (MGMT) e trattati con chemoradioterapia concomitanti. Una delle ragioni di questo insuccesso è rappresentata dall'inadeguata stratificazione dei Pazienti in base ad un profilo biologico e molecolare altamente predittivo di risposta ad una specifica terapia. L'identificazione delle cellule staminali tumorali (CST) negli HGG, responsabili della trasformazione, progressione e mantenimento della neoplasia, fornisce un punto di vista nuovo sui meccanismi della gliomagenesi ed offre promettenti prospettive per lo sviluppo di nuove terapie. Le CST (o, meglio, "glioma initiating cells", GIC), sono resistenti alla chemioterapia ed altamente tumorigeniche. La comprensione dei "networks" di regolazione genica che controllano il mantenimento e la proliferazione delle GIC rappresenta un obiettivo fondamentale per lo sviluppo di nuovi approcci di terapia mirata e può essere ottenuto coordinando competenze diverse (biologia, genetica, neuropatologia, clinica) e utilizzando modelli sperimentali diversificati e complementari (modelli murini di HGG, GIC derivate da gliomi di Pazienti). Le moderne tecnologie di analisi molecolare "high-throughput" hanno già condotto all'identificazione di alcune interessanti "pathways" molecolari coinvolte nella gliomagenesi che hanno permesso di suddividere gli HGG in diversi sottotipi sulla base di differenti profili genetici, corrispondenti a diversa prognosi e risposta alla terapia. Questa osservazione sottolinea la necessità di poter accedere a modelli sperimentali che ricapitolino in modo più fedele la patologia umana. Tuttavia, target specifici e meccanismi molecolari correlati alla intrinseca aggressività delle GIC non sono ancora stati chiaramente individuati. Sebbene alcuni dati sperimentali suggeriscano che le GIC siano limitate ad ristretto pool di cellule CD133<sup>pos</sup>, recentemente è stato dimostrato che anche cellule tumorali CD133<sup>neg</sup> esprimono marcatori di staminalità (p.es. Sox2, BMI1, etc.), sottolineando, pertanto, l'estrema eterogeneità delle GIC nel contesto dello stesso tumore. Nei HGG umani, l'elevata espressione di EGFR e della sua variante mutata EGFRvIII contribuiscono all'acquisizione di un fenotipo più maligno e sono associate al profilo staminale. Abbiamo recentemente dimostrato che l'over-espressione di EGFR identifica una sottopopolazione di GIC altamente tumorigeniche (GIC-EGFR<sup>pos</sup>) ed è indispensabile per la gliomagenesi<sup>3</sup>. Queste GIC conservano i tratti specifici del tumore del Paziente, anche quando trapiantate in topi immunodeficienti, e rappresentano, quindi, un valido modello sperimentale pre-clinico per studiare il profilo delle CST. Recenti lavori hanno, inoltre, dimostrato che modulando i livelli di espressione di molecole "staminali" si limita il potenziale di "self-renewal" delle GIC, portando all'arresto della crescita ed alla differenziazione, favorendo l'apoptosi e limitando lo sviluppo del tumore in trapianti eterotopici sperimentali. Questi dati confermano il ruolo chiave delle GIC nella gliomagenesi e ne suggeriscono l'utilizzo come potenziali target molecolari per lo sviluppo di nuovi approcci terapeutici più efficaci.

<sup>3</sup>Mazzoleni et al. Cancer Research 2010 Oct 1;70(19):7500-13.

## **Terapie avanzate con colture di epitelio corneale: 15 anni di valutazioni cliniche**

**Paolo Rama**

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Dal 1998 al 2007 abbiamo operato di innesto di cellule staminali dell'epitelio corneale 112 pazienti. Tutti i pazienti presentavano una opacità corneale con vascolarizzazione secondaria ad un danno grave delle cellule staminali corneali. La causa più frequente era l'ustione da agenti chimici. La capacità visiva era fortemente compromessa in tutti i casi con un visus inferiore a 1/10. La gran parte dei pazienti inclusi nello studio aveva già fatto interventi precedentemente, compreso il trapianto tradizionale della cornea, senza alcun risultato. Il trapianto tradizionale di cornea non può avere successo in questi casi perché il lembo di cornea che viene innestato deve essere successivamente ricoperto dall'epitelio del ricevente. Se mancano le cellule staminali epiteliali, come nei pazienti considerati, anche il lembo trapiantato verrà nuovamente ricoperto da un tessuto vascolarizzato di origine congiuntivale con fallimento dell'intervento.

I pazienti sono stati seguiti negli anni e rivalutati tutti nel corso del 2008 in modo da avere un dato molto importante di sopravvivenza a lungo termine delle cellule staminali innestate.

Il risultato finale ha dimostrato una percentuale di successo nel 76.6% dei casi. Non sono state riportate complicanze maggiori né intraoperatorie né postoperatorie.

I successi e fallimenti sono stati inoltre correlati con la percentuale di cellule staminali presenti nella coltura: si è osservato che nelle colture che contenevano una percentuale di cellule staminali superiore al 3% era stato possibile ottenere un risultato positivo nell'80% dei casi, al contrario nelle colture povere di cellule staminali (>3%), la percentuale di successo scendeva sotto il 30%.

In conclusione possiamo dire che questo lavoro ha confermato che è possibile coltivare e trapiantare con un alta percentuale di successo cellule staminali dell'epitelio corneale, come è stato fatto per la ricostruzione dell'epidermide nelle ustioni cutanee. È stato inoltre possibile dimostrare che la percentuale di cellule staminali presenti nella coltura è fondamentale per il successo finale e quindi la procedura di espansione delle cellule coltivate in laboratorio è cruciale: per tale motivo si ritiene che la responsabilità della coltura delle cellule dovrebbe essere affidata solo a laboratori altamente qualificati e selezionati per garantire la sicurezza e il successo a lungo termine.

## La cellula: farmaco e laboratorio biologico?

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Vengono proposti alcuni dati fattuali per stimolare una proficua discussione al fine di giungere ad una valutazione critica degli aspetti che hanno portato nelle ultime decadi a privilegiare la visione della cellula quale farmaco; come ben noto quest'ultimo fatto ha determinato la necessaria adozione di procedure di produzione, controllo e conservazione delle cellule del tutto sovrapponibili a quelle adottate a livello industriale per la produzione di molecole ad azione farmacologica. Questa situazione è venuta stabilendosi sulla base delle conoscenze di biologia cellulare di cui disponevamo tempo addietro; oggi l'acquisizione della visione riduzionista e molecolare della cellula ai tempi delle post-omiche e della interattomica suggerisce un'ulteriore visione, non necessariamente in contrapposizione a quella precedente: la cellula quale piccolo e meraviglioso (per quanto miniaturizzato) laboratorio di biologia molecolare. E' questo un laboratorio che gli strumenti della biologia sintetica permettono di indirizzare verso la produzione, in vitro ed a volte già in vivo, di proteine ed acidi nucleici capaci di esplicare azioni farmacologiche; queste molecole (la cui natura a volte non è ancora ben definita) sono in grado di agire sia direttamente sia indirettamente sui sistemi biologici ai diversi livelli di strutturazione del vivente e conseguire azioni terapeutiche. Da questo insieme di dati la cellula può essere considerata sia farmaco sia produttore di farmaci, in dipendenza delle “condizioni al contorno” del suo impiego o meglio, delle condizioni fisiologiche prestabilite nelle quali porla ad operare. Vengono presentati esempi fattuali di “good medical practice” sia dell'una sia dell'altra opzione citologica, quali quelli derivanti dall'impiego innovativo di cellule staminali (da cordone ombelicale) per la neo-angiogenesi terapeutica dell'ischemia dell'arto e per la stampa in 3D di organi e quelli di cellule ingegnerizzate per essere sede di produzione di molecole ad azione farmacologica con rilascio in sede istologica prescelta grazie alla modificazione degli epitopi di superficie per selettivo riconoscimento mirato. Lo sforzo attuale della impresa scientifica si sta indirizzando a trovare tutte quelle condizioni al contorno compatibili con la fisiologia umana e capaci di stabilire la “occasionale”, “transitoria” natura della cellula sia quale farmaco (o veicolo di farmaci) sia quale produttore di farmaci; condizioni capaci di esaltarne la duttilità e plasticità citodifferenziativa. E' comunque evidente che tutte le possibili cautele per evitare impieghi dannosi al benessere del paziente devono essere messe in campo nell'una e nell'altra delle due visioni epistemologiche possibili: ciò significa che non è permesso derogare dagli imperativi della deontologia biologica e medica.

## **Targeting TRAIL receptors with genetically-engineered CD34+ hematopoietic stem cells**

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Preclinical studies demonstrating that tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) exerts a potent and cancer cell-specific cytotoxic activity prompted clinical development of recombinant soluble TRAIL. Despite a good toxicity profile shown in phase I/II clinical studies, limited evidences of antitumor activity have emerged for soluble TRAIL. Stem/progenitor cell-mediated gene delivery of anticancer therapeutics might represent an innovative approach to overcome the limitations inherent to TRAIL receptor targeting, i.e., pharmacokinetic of soluble TRAIL, pattern of receptor expression, tumor cell resistance. We have envisaged the use of CD34+ cells engineered by adenoviral transduction to express membrane-bound TRAIL (CD34-TRAIL+ cells). Transduced cells efficiently act as TRAIL-presenting vehicles and exert a potent tumor cell killing activity against a variety of hematopoietic (e.g., multiple myeloma, non-Hodgkin lymphoma) and nonhematopoietic (e.g., breast cancer) tumors, both in vitro and in vivo in xenograft models of human tumors. Following intravenous injection, CD34-TRAIL+ cells home in the tumor peaking at 48 hours after injection. Tumor homing of CD34-TRAIL+ cells is largely mediated by vascular cell adhesion molecule-1 (VCAM-1) and stromal cell-derived factor-1 (SDF-1). Computer-aided analysis of TUNEL-stained tumor sections demonstrates significantly greater effectiveness for CD34-TRAIL+ cells in increasing tumor cell apoptosis and necrosis over soluble TRAIL. Proteome array analysis indicates that CD34-TRAIL+ cells and soluble TRAIL activate similar apoptotic machinery. In vivo staining of tumor vasculature with sulfo-NHS-LC-biotin reveals that CD34-TRAIL+ cells but not soluble TRAIL target endothelial cells expressing TRAIL-R2, as shown by apoptosis of endothelial cells, appearance of hemorrhagic areas and marked reduction of vessel density. Overall, these results demonstrate that CD34-TRAIL+ cells induce potent antitumor effects by targeting tumor cells and tumor vasculature. Phase I/II clinical trials to test the safety and activity of CD34-TRAIL+ cells in patients with advanced solid tumors are planned to exploit the anticancer potential of cell-based TRAIL delivery.

## Mesenchymal Stem cells transplantation for multiple sclerosis

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Recent advances in our understanding of stem cell biology, such as the availability of innovative techniques, which allow stem cells to be obtained on a large scale, and the increasing pressure from patients for tissue repair strategies, have launched stem cell treatments as one of the most exciting and difficult challenges in the MS field. Results obtained from small clinical studies of transplantation of autologous hematopoietic stem cells (AHSC) have demonstrated that this procedure is feasible and possibly effective in severe forms of MS, but tackles only inflammation without affecting tissue regeneration. The adult stem/progenitor cells from bone marrow and other tissues referred to as mesenchymal stem cells or multipotent mesenchymal stromal cells (MSC) display a significant therapeutic plasticity as reflected by their ability to enhance tissue repair and influence the immune response both *in vitro* and *in vivo*. Pivotal experiments have been carried out in experimental autoimmune encephalomyelitis (EAE), a model for human multiple sclerosis (MS) demonstrating that intravenous MSC administration induces tolerance to myelin antigens and promote as well neuroprotection and tissue repair. Recent studies have emphasized that MSC secretome may suffice to recapitulate many of the effects carried out by these cells on immune and tissue resident cells, thus suggesting that even engraftment in the target organ could be dispensable. This new paradigm predicts MSC clinical translation based on their ability to work mainly as drug-stores. However, MSC priming by local cues, upon interaction with the host environment, represents a prerequisite to maximally enhance their therapeutic plasticity and may still support stem cells transplantation over the administration of their secreted factors.

Overall, current experimental evidence suggests that the sound clinical exploitation of MSC for MS may lead to novel strategies aimed at blocking uncontrolled inflammation, resetting the immune system, protecting neurons and promoting remyelination, but not at restoring the chronically deranged neural network responsible for irreversible disability typical of the late phase of MS. Based on this rationale, an international phase II clinical trial is currently on going to address the safety and efficacy of MSC in MS individuals with active disease not responsive to the currently approved therapies.

## **Analytical procedures validation for good manufacturing practice (GMP) production of mesenchymal stem cells from different sources**

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The production of Mesenchymal stem cells (MSC) for cellular therapy should fulfil the requirements of GMP rules. In this context, the production process and the analytical methods for quality control should be validated to demonstrate standardization and compliance with quality requisites. In our hospital-based GMP facility we produce MSC from different sources and we developed and validated a set of quality control assays for final products: sterility and endotoxins assay (<0,25 EU/mL), absence of mycoplasma and adventitious viruses, cell dose, purity (as percentage of cells CD90+/CD105+/CD45- > 80%) and viability (as percentage of cells PI- > 80%). Sterility was validated following Eu. Ph. 2.6.27 with both the standard microbial strains and those isolated during environmental monitoring. The endotoxin method (Eu. Ph. 2.6.14) was validated at different dilutions of the final product to exclude inhibition. The method to detect mycoplasma was compliant to the Pharmacopoeia requirements (Ph 2.6.7). We also performed a viral validation to exclude the accidentally introduction of respiratory viruses, Cytomegalovirus and Epstein-Barr Virus during production. For cell counting, an automated method (NucleoCounter, Chemometec) was found to be more accurate ( $R^2 > 0.99$ ) and precise ( $CV < 10\%$ ) than standard manual method (Burker chamber). Moreover, we assessed the repeatability and reproducibility ( $CV < 5\%$ ) of the immunophenotyping analysis of MSC by flow cytometry to determine the purity of our products as the percentage of cells that co-express CD90 and CD105 and is negative to CD45. Finally, cell viability by Annexin-PI analysis was evaluated on MSC after expansion and cryopreservation. All these methods have been included in the Investigational Medicinal Product Dossier of all the MSC-based products currently provided by our Institution in several phase I/II clinical trials already ongoing.

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## **Cellule Mesenchimali Autologhe come possibile trattamento per 14 cani con sospetta mielopatia degenerativa: studio sulla sicurezza e applicabilità della metodica**

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Canine degenerative myelopathy (DM) is a late onset, progressive spinal cord axonopathy that occurs in many dog breeds. Dogs with DM show a progressive ataxia and UMN (upper motor neuron) spastic paraparesis of the pelvic limbs leading to loss of the ability to ambulate within 6 to 12 months of the onset of signs. The disease progresses to paraplegia until reaching lower motor neuron (LMN) signs. If euthanasia doesn't occur early (usually 3 up to 6 months after diagnosis) clinical signs ascend to involve the thoracic limbs and urinary and fecal functions. Genetic, metabolic, nutritional, vascular, and immune-mediated etiologies have been proposed, but an evidence for a specific pathogenic mechanism is lacking. A definitive diagnosis of DM can only be confirmed by postmortem histopathologic examination of the spinal cord: neuropathologic lesions occur in the spinal cord myelin and axons in all funiculi with a prevalence in the mid thoracic region. Similarities between DM in dog and neurodegenerative diseases such amyotrophic lateral sclerosis (ALS) were discovered. The use of mesenchymal stem cells (MSCs) in patients with ALS or DM is justified by their high degree of plasticity, the ability to release growth factors and modulate the immune system. This study aims to evaluate the safety and feasibility of the intrathecal and intravenous administration of autologous MSCs in dogs with suspected DM. Fourteen dogs with chronic and progressive UMN clinical signs of the pelvic limbs were included in the study. In all the patients radiographs, magnetic resonance imaging (MRI) and cerebrospinal fluid (CSF) analysis were negative. DNA exam for superoxide dismutase 1 (SOD-1) mutation was performed. Autologous bone marrow MSCs were isolated from each dog, cultured and expanded. In each dog  $2 \times 10^6$  MSCs was injected intrathecally in cisterna magna,  $2 \times 10^6$  between L5-L6 space and  $0.5 \times 10^6$  MSCs/kg intravenously. Follow up of 6 and 12 months included clinical evaluation, complete blood and biochemistry work up, neurological evaluation and MRI. MSCs consistently (>98%) expressed their classical surface markers and were negative for lymphocytes and hematopoietic cells (FACS analysis). Blood workup and MRI did not reveal changes compared to previous exams nor onset of new neurological pathologies. Neurological evaluation evidenced static clinical signs in 9 cases, 2 dogs died by natural causes and 3 dogs were euthanized after 6 and 9 months due to owner's request. 4 of them underwent autopsy and DM was confirmed by histology. Implantation of MSCs in dogs with DM is a feasible and a relatively safe procedure. Clinical relevance of the immunological effects is yet unclear. The results of this study encourage the selection of a greater, statistically more relevant, group of dogs for similar evaluation, even to apply the results obtained from the canine model in human medicine.

## Cellule stromali mesenchimali come veicolo di paclitaxel per inibire la formazione di metastasi polmonari da melanoma B16

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It has been suggested that, due to their tumor-tropic and migratory properties, mesenchymal stromal cells (MSCs) may home to tumor microenvironment. Additionally MSCs can be loaded in vitro with Paclitaxel (PTX) and when co-transplanted with tumor cells in mice release the anticancer drug and inhibit both tumor takes and growth. Based on this background, we here investigated whether systemic (i.v.) injection of MSCs loaded with PTX were able to reduce the formation of lung metastasis induced by B16 melanoma in syngeneic mice and to inhibit the growth of tumor mass.

Green Fluorescent Protein-transduced SR4987 murine stromal cells were used to load PTX (GFP+SR4098-PTX). According to a previous published procedure, PTX was loaded in GFP-SR4987 by incubating the cells for 24h with the drug. The amount of PTX release by the cell in the conditioned medium (CM) was checked by HPLC and its anti-tumor activity was tested in vitro on B16 cells. Anti-proliferative activity of GFP+SR4098-PTX on B16 melanoma cells was also evaluated by a transwell co-culture system. In vivo experiments were performed by injecting  $2.5 \times 10^5$  i.v. or i.m. B16 melanoma cells in syngeneic C57Bl6 mice. On day 5, 10 and 15 after tumor injection, mice divided into four groups, were injected i.v. with  $5 \times 10^4$  GFP+SR4987-PTX, control GFP+SR4987, pure PTX (10 mg/kg) and saline respectively. The effect of treatments was evaluated by counting lung metastasis and by measuring diameters of tumor mass in mice i.m. B16 injected.

GFP+SR4987-PTX cells released about 0.5 pg/cell of PTX so that  $5 \times 10^4$  cells/ml allow to reach in vitro a concentration of PTX that is able to induce 80% of B16 growth inhibition ( $IC_{50} = 7.07 \pm 1.3$  ng/ml). Injection of GFP+SR4987 cells i.v. in mice showed a significant homing of the cells into lung at 24h and their presence persisted at 48 and 72h after injection.

The systemic treatment of mice bearing B16 melanoma with GFP+SR4987-PTX inhibited the primary tumor mass at higher level (56%) than that observed with the PTX alone (44%) ( $p=0.027$ ) or untreated mice ( $p<0.001$ ). GFP+SR4987-PTX administration showed a marked efficacy in reducing B16 lung metastasis. The treatment decreased of about 90% the number of artificial metastasis induced by i.v. injection of B16 melanoma cells, compared to the 70% reduction observed after the treatment with PTX alone ( $p<0.001$ ). No significant reduction was produced by GFP+SR4987 compared to untreated control. Our data show for the first time that MSCs loaded with PTX may home directly into metastatic lung and strongly reduce metastasis growth. Studies are in progress to investigate the mechanism of lung recruitment of MSCs and whether SDF-1-CXCR4/CXCR7 axis may have a role.

## Lo “shuttling” di BAF e l'accumulo di precursori di Lamina A nella senescenza replicativa delle Cellule Stromali Mesenchimali durante la coltura *in-vitro*

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Human mesenchymal stromal cells (MSC) aging represents a growing issue, both for advanced therapy implications of *in-vitro* senescence and for MSC role in maintenance and repair of tissues in aging people.

Lamin A is a component of nuclear envelope. Mutations or modifications of its molecular processing are responsible of a group of diseases named laminopathies, which includes Hutchinson-Gilford progeria syndrome that is characterized by premature aging.

*In-vitro* over-expression of wild type or mutant lamina A in immortalized human MSC and skin fibroblasts has been associated with molecular and morphological signs of accelerated aging. Other investigators reported nuclear accumulation of lamin A precursor in Vascular Smooth Muscle Cells (VMSC) after their prolonged *in-vitro* culture or in tissue specimens from aged donors.

Recently it has been reported that the chromatin associated protein Barrier-to-autointegration factor (BAF) localization is affected by the presence of prelamin A.

In our study we used primary cultures of MSC isolated from the bone marrow of healthy donors (BM-MSC) to analyse by immunostaining prelamin A and BAF localization in correlation with other markers of cellular senescence such as decreasing proliferation rate,  $\beta$ -galactosidase activity and cytoskeleton disruption.

To overcome the known donor variability of MSC behaviour we organized our observations in three stages identified as “early”, “middle”, “late” according to the level of completion (50%, 50-80%, > 80%) of the total lifespan of each cell culture respectively.

As already shown in others cell types by different authors, in our BM-MSC lines we observed different BAF localization patterns: 1) equally distributed between cytoplasm and nucleus, 2) strongly localized in the nucleus and weakly in cytoplasm, 3) exclusively localized in the nucleus.

As the number of  $\beta$ -Gal positive senescent cells increased with time in culture, we observed an increase of prelamin A positive cells.

Moreover, we detected a direct correlation between prelamin A positive cells and BAF nuclear localization but conversely prelamin A positive MSC resulted negative for cell cycle progression marker Ki67.

Overall, our preliminary data suggest that prelamin A and BAF co-localize in the nucleus only in cells that have acquired a senescent phenotype and do not proliferate. Apparently this is in contrast with previous evidence showing that BAF shuttling between nucleus and cytoplasm is related with cell cycle progression. However, the observed nuclear localization of BAF in senescent cells can be explained by the concurrent presence of prelamin A that impair BAF dynamic association with its numerous partners.

Our findings help to elucidate the possible mechanism that underlies proliferation arrest in *in-vitro* cultivated MSC.

Further investigations will characterize the molecular interaction between prelamin A and BAF and place it in the broader landscape of cellular senescence that also embraces details as reduced antioxidant activity, dysfunctional mitochondria and altered cytoskeleton.

## **Amplificazione del genoma dei Polyomavirus JCV e SV40 in cellule staminali mesenchimali da cordone ombelicale**

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Multipotent stromal cells are present in the Wharton's jelly matrix (WJSC) of the umbilical cord and can be used as an allogeneic source of cells to treat immunological disorders. Recently it was demonstrated that adult Bone Marrow (BM)-derived Mesenchymal Stromal Cells (MSC) are susceptible to infection with viruses showing potential oncogenic properties, such as the Polyomavirus JC (JCV).

The aim of this study was to investigate the presence of human Polyomaviruses (JCV, BK Virus-BKV, SV40 and Merkel cell polyomavirus-MCPyV) in WJSC, and explore the risk of infection.

MSC samples from 35 umbilical cords were investigated by quantitative Real Time PCRs for the presence of DNA sequences of JCV, BKV, SV40, and MCPyV.

JCV DNA was detected in 1/35 (2.8%) of MSC samples, while SV40 DNA was found in 3/35 (8.6%) of the examined samples. None of the samples showed sequences of BKV and MCPyV.

The present study is the first to demonstrate the in vivo ability of polyomaviruses to infect WJSC. Since the therapeutic approach with the WJSC has high potentiality and a more intensive use can be easily hypothesized, the need to get consensus and guidelines to detect rare viral infections in MSC is pressing.

## **Cellule Staminali Mesenchimali e Progenitori Endoteliali umani promuovono la sopravvivenza di neuroni corticali di ratto in seguito a deprivazione di glucosio ed ossigeno**

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Oxygen and glucose deprivation (OGD) due to ischemic events or trauma in the brain result in neuronal loss. The therapeutic approaches available for the treatment of these conditions are not and often the outcome is unfavorable for the patient or at least unpredictable.

Stem cells could be useful for the treatment of OGD injured-neurons. Mesenchymal Stem Cells (MSCs), isolated from bone marrow as well as from various tissues, have poor immunogenicity and neuroprotective properties being able to alleviate ischemic brain injuries in animal models. The Endothelial Progenitor Cells (EPCs) are present at low frequencies both in the bone marrow and in the peripheral blood and can be mobilized by the administration of drugs such as statins. They are thought to play a role in the recovery of cerebrovasculature integrity after stroke.

In the present study we evaluated the potential neuroprotective effect of human MSC and human EPCs on rat embryonic cortical neurons injured by OGD.

OGD was induced by incubating the cortical neurons in a hypoxia chamber in a 95% N<sub>2</sub> + 5% CO<sub>2</sub> atmosphere at 37°C without glucose for periods ranging from 30 minutes and 6 hours. When the neurons were returned in normoxic atmosphere they were 1) co-cultured with either MSCs or EPCs seeded on a cell culture avoiding neurons and MSCs or EPCs direct contact while sharing the same medium, or 2) cultured in a medium previously conditioned by either MSCs or EPCs. Neuronal survival was evaluated by MTT assay and viable cellular counting. Also neuronal morphology was taken into account to evaluate the potential MSCs and EPCs neuroprotective effect.

Both MSCs and EPCs increased neuronal survival after ODG. This effect was observed in absence of a direct contact between MSCs or EPCs and the injured neurons, suggesting that the release of soluble factors may be the main mechanism of action.

In conclusion both MSCs and EPCs could represent a potential therapeutic approach for the treatment of brain ischemic injury. Further studies are needed to identify the specific molecules involved in the neuroprotective effect of MSCs and EPCs.

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## **Caratterizzazione di microvescicole derivanti da cellule staminali mesenchimali umane e loro possibile ruolo nella rigenerazione muscolare**

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The role of mesenchymal stem cells (MSCs) in recovery of a variety of tissue injuries has been widely investigated. MSCs are considered effective therapeutic agents inducing the functional improvement in the repair of injured tissues, not only through direct engraftment and differentiation, but mainly through their paracrine activity. However, the nature of the factors responsible for the beneficial paracrine effects of MSCs is not fully elucidated. Increasing evidences show that besides the secretion of trophic factors, the release of microvesicles (MVs) constitutes an alternative paracrine mechanism adopted by MSCs. The recognition of MVs as carriers of genetic information has opened a new era in the field of cell biology. The roles of MVs has been evoked in regenerative medicine as possible mediators of anti-apoptotic and pro-regenerative effects associated to MSCs administration. In this preliminary study, we aim to carry out a detailed characterization of MVs released by human bone marrow-MSCs, to study their possible involvement in a mouse model of muscle regeneration.

The isolation method is based on repeated ultracentrifugation steps of the MSC-conditioned medium, followed by characterization of the resulting pellet enriched in MVs through electron microscopy, flow cytometry and western blot for the expression of the proteins typically enriched in MVs. Size- and Truocount- beads were used in the flow cytometric analysis to define the proper size and number of isolated MVs, respectively. We observed that the MSC-derived MVs showed highly different distribution of size and shape: 1) exosomes with smaller diameter (< 110 nm), cup-shaped morphology and bilayer membrane, and 2) ectosomes with larger size (110-210 nm). In addition, culturing the muscle cell line C2C12 with the MSC-derived MVs previously stained with the fluorescent lipophilic dye PKH67 resulted in the uptake of MVs by C2C12. These data may provide a starting point for further work to explore the biological significance of human MSC-derived MVs in cell-cell signaling and skeletal muscle regeneration. The use of MSC-MVs instead of whole living cells could therefore offer a series of advantages, both in terms of safety and ease of handling.

## Long-term culture without passaging of freshly-isolated human adipose-derived stromal cells (ASC) creates a niche environment which maintains their phenotypic and functional properties

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The stromal vascular fraction (SVF) of adipose tissue contains progenitor cells, referred to as adipose stromal/stem cells (ASC) able to differentiate into different mesenchymal lineages. *In vivo*, ASC are identified as CD34+/CD105<sup>-</sup> cells, but their phenotype changes during culture and expanded ASC are described as CD34<sup>-</sup>/CD105<sup>+</sup> cells. In order to preserve ASC progenitor properties, we aimed at mimicking the native ASC environment by favoring cell-extracellular matrix and cell-cell interactions.

In our model SVF is plated on dishes to leave cells to adhere, to grow and to reach the confluence for 28 days (Unpass cells). Unpass cells are compared to “sister” cells obtained according to guidelines for *ex vivo* expansion (Pass cells).

After 28 days of culture, Unpass-cells showed a significant increase of clonogenic capacity ( $33.33 \pm 10.12$ ) compared to pass-cells ( $16.93 \pm 5.16$ ). Cumulative population doublings (PDN) showed that Unpass cells have a minor PDN ( $9.47 \pm 1.29$ ) compared to pass cells ( $25.79 \pm 3.25$ ), and this can explain the significant difference between clonogenic data, but cells (p0) with a comparable population doubling numbers ( $6.94 \pm 1.63$ ) have also a reduced clonogenic ability.

Unpass-ASC expressed mesenchymal markers, such as CD90 and CD73, but remained negative for the expression of CD105. Furthermore, a percentage of Unpass-ASC ( $22.3 \pm 18\%$ ) maintained the expression of CD34 while it was lost by pass-ASC after the first passage. Compared to pass-ASC, Unpass-ASC expressed higher levels of Nanog, Sox2, KLF4 and OCT4 and differentiated better under adipogenic induction *in vitro* and under osteogenic conditions both *in vitro* and *in vivo*. FACS analysis and functional adhesion assays showed that pass-ASC expressed all alpha integrins, which recognize molecules as collagen, fibronectin and laminin, while Unpass-ASC only expressed alpha5 integrin (fibronectin receptor) and at lower levels. We observed a similar pattern of integrin expression in SVF cells, suggesting different adhesive and migratory properties.

Interestingly, the data got from colony forming unit of SVF after the double selection for CD73 (marker of mesenchymal stromal cells) and CD49e (integrin  $\alpha 5$ ) confirmed that ASC positive for CD49e are clonogenic.

Moreover, human fat tissue immunofluorescence highlighted that CD49e positive cells are located close to the vessel confirming results described in literature where they described ASC as perivascular cells.

These results are important from a biological point of view because they suggest a fundamental role of the *in vivo* microenvironment for these cells. Moreover, this work hypothesizes that there is an *in vitro* method to reproduce *in vivo* niche, which can be used to further investigate the molecular pathways involved in the process of maintenance of MSC properties.

From a clinical point of view the data have possible clinical applications because they prompt a more standardized protocol to expand *in vitro* ASC with the advantage that they are phenotypically more similar to the *in vivo* ones.

## Magnetic remote control of mesenchymal stem cells as tool for nanomedicine applications

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Nanobiotechnology, the integration of nanotechnology with molecular biology and medicine, is an emerging research area that has experienced rapid growth over the past decade because it offers exciting opportunities for discovering new science, producing novel materials, and developing efficient processes.

We focused here on the development of a completely novel way for *in vitro* 3D cell culture, which is pivotal for tissue engineering and nanomedicine. Despite its importance in cell behaviour understanding, it is not easy to achieve a proper 3D cell culture system, given the necessity to provide cell support and allow the exchanges between cells and medium, of oxygen, nutrients, growth factors. In order to reproduce *in vivo*-like physiological conditions, a nanostructured collagen scaffold was used in synergy with fully novel magnetic nanoparticles (MNPs). Among the variety of promising nanomaterials, collagen is widely used due to its biomimesis and biocompatibility and furthermore MNPs represent very fascinating tools due to their ability to respond to a magnetic field providing a “action at a distance” or “remote control”.

The project idea is to obtain a 3D cell culture where cells guiding and growth factors/drugs delivery are precisely remote controlled by a low external magnetic field. In this study we employed novel bioactive and completely bioresorbable nanoparticles, recently developed in our group by doping hydroxyapatite with Fe ions (FeHA MNPs). They do not have poorly tolerated magnetic secondary phase (i.e. magnetite and maghemite) and consequently they do not need any coating. We studied the magnetization of mesenchymal stem cells with different concentrations of FeHA MNPs (from 10 µg/ml up to 2000 µg/ml). Neither of each affected cell behaviour. In fact, cell viability, proliferation and morphology remained comparable to the control throughout the experimental time. Moreover these particles strongly enhanced MSCs osteogenic differentiation as demonstrated by gene expression analysis. In addition applying a low external magnetic field, we were able to remote control cell migration demonstrating that magnetic cells were obtained. Subsequently, we seeded these cells into a 3D porous collagen scaffold and applying an external magnetic field we were able to move them in a well localized area of the biomaterial. Even in this 3D cell culture system cells showed behaviour comparable to the control. Further improvement will rely on the use of a co-culture system (e.g. mesenchymal stem cells, macrophages, endothelial cells) and, always using an external magnetic field, deliver FeHA MNPs functionalized with specific growth factors and/or drugs in controlled timing and area of the scaffold.

This study showed the great potential that novel MNPs may offer in biology and medicine applications.

## Caratterizzazione di cellule limbal e Cellule Stromali Mesenchimali (CSM) isolate da tessuto adiposo

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Le prime evidenze pratiche dell'efficacia delle cellule isolate dal limbo nella rigenerazione corneale sono state ottenute a seguito del trapianto di frammenti dello stesso in una cornea danneggiata. Successivamente il tessuto limbale è stato coltivato *in vitro* e le cellule così ottenute sono state utilizzate per la cura di difetti visivi. Parallelamente, è stato accertato che anche le CSM limbal possono essere espanse *in vitro*, ed utilizzate per il trapianto nella sede corneale danneggiata, al fine di ripristinare le funzionalità visive compromesse. Nel presente studio le indagini sono state effettuate utilizzando il limbo di origine cunicola, quale modello per l'applicazione terapeutica negli animali. Il tessuto limbale è stato isolato e trattato come descritto da Ghafar 2013. La sospensione di cheratociti stromali è stata centrifugata ed il *pellet* lavato con PBS. Infine, i cheratociti isolati sono stati inoculati in una piastra per colture cellulari a 6 pozzetti ( $5 \times 10^3$  cells/cm<sup>2</sup>) previamente stratificate con cellule murine irradiate (3T3), diluiti in terreno colturale specifico ed incubati a 37°C, 5% CO<sub>2</sub>. Il tessuto adiposo retrobulbare ed addominale è stato disgregato enzimaticamente ed il *pellet* cellulare è stato inoculato ed incubato a 37°C, 5% CO<sub>2</sub>. I cheratociti limbal sono stati valutati morfologicamente ed hanno evidenziato una forma dendritica/stellata a 7 giorni dall'inoculo. Le CSM derivate dal grasso retrobulbare ed addominale presentavano una forma di tipo fibroblastico. L'espressione genica è stata valutata mediante metodica EVA Green Real-Time PCR, al fine di accertare la presenza di marcatori specifici per i cheratociti corneali. L'analisi ha evidenziato la presenza di RNA di lumicano, aldehyde dehydrogenase (ALDH) e collagene tipo I (Coll I). Infine, è stata eseguita la prova di differenziamento cellulare per valutare la capacità delle CSM di tessuto adiposo delle due differenti sedi di assumere le caratteristiche tipiche delle cellule corneali. Tali cellule sono state inoculate con terreno raccolto dalle colture di cellule limbal (terreno condizionato) ed incubate per 7 giorni. La coltura è stata monitorata giornalmente ed è stata evidenziata una variazione nella morfologia da fibroblastica a dendritica/stellata dopo 4 giorni di incubazione.

I risultati ottenuti dovranno essere confermati mediante analisi di citofluorimetria, utilizzando marcatori specifici, quali: p63, ALDH, CK3, collagene I in quanto noti essere specifici per i cheratociti corneali e confermare la capacità differenziativa delle CSM.

Lo studio *in vitro* delle CSM del tessuto adiposo retrobulbare ed adiposo e del loro differenziamento in cheratociti rappresenta un importante progresso nell'ambito della terapia cellulare rigenerativa, con potenzialità da approfondire e da valutare.

## Studio dell'espressione di marcatori di pluripotenza in cellule staminali mesenchimali isolate da liquido amniotico, gelatina di wharton e sangue cordonale nella specie equina

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Foetal adnexa contain a heterogeneous mixture of cells including a stem cell population that express a panel of pluripotency markers, such as Oct4, Nanog, Sox2 and SSEA4 (Pappa and Anagnou, 2009; Dyvia *et al.*, 2012). These extra-embryonic tissues and fluids represent viable, accessible and safe sources. The aim of this work was to evaluate pluripotency markers expression in mesenchymal stem cells isolated from equine amniotic fluid (eAF-MSCs), Wharton's jelly (eWJ-MSCs) and umbilical cord blood (eUCB-MSCs) as continuation of the characterization of these cells previously performed by our research group (Iacono *et al.*, 2012). Equine AF, WJ and UCB samples were recovered at parturition from 3 mares and (46.7 ± 14.4) ml of AF, (2.4 ± 2.2) g of WJ and (23.3 ± 14.4) ml of UCB were collected at foaling. Isolated cells were isolated and cultured as previously described (Iacono *et al.* 2012). Briefly, cells were cultured in DMEM-TCM 199 (1:1) plus 10% FBS and incubated in a 5% CO<sub>2</sub> humidified atmosphere at 38.5 °C. At passage 3 characterization was performed by immunocytochemistry (ICC) and RT-PCR. ICC was carried out for SSEA4 protein fixing cells with paraformaldehyde (4%), doing an overnight incubation at 4°C with anti-SSEA4 primary antibody and then incubating cells with FITC-labeled secondary antibody for 1 hr at room temperature in the dark. Nuclei were stained with Hoechst and the percentages of positive cells were obtained doing ratio between FITC+ cells and nuclei. Equine skin fibroblasts were used as negative control. RT-PCR was performed to analyze mRNA expression of Nanog and Sox2. Equine blastocysts and equine skin fibroblasts were used respectively as positive and negative controls and GAPDH was employed as reference gene. ICC analysis showed expression of the embryonic stem cells antigen SSEA4 in eAF-MSCs (14.8 ± 9.5 %), eWJ-MSCs (8.9 ± 2.6 %) and eUCB-MSCs (7.1 ± 4.8 %). On the other hand, expression of Nanog and Sox2 was not found in all samples, unlike human AF-MSCs and WJ-MSCs. ICC and RT-PCR analyses for the embryonic marker Oct4 (data not reported) gave unclear results so further studies are necessary. Results obtained in the present study reveal that equine MSCs derived from foetal adnexa are an heterogeneous population and express low level of the embryonic marker SSEA4, but do not express embryonic pluripotent markers like Sox2 and Nanog as described in the literature for other species. So these cells could represent an intermediate stage between pluripotent embryonic stem cells and lineage-restricted adult stem cells, as previously demonstrated for human MSCs (De Coppi *et al.* 2007; Pappa and Anagnou, 2009). Further work is ongoing to find specific primers and antibodies to assess the expression of OCT4.

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## Cellule mesenchimali staminali derivate dalla gelatina di Wharton nella rigenerazione tissutale

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The umbilical cord represents a widely available source of mesenchymal stem cells (UC-MSC) which could be promising candidates for tissue regeneration therapies. Current literature on the osteogenic capacity of UC-MSC is still controversial. Hence, the goal of the present work was to clarify the role of UC-MSC in bone regeneration. We used clinical-grade UC-MSC isolated from Wharton's Jelly and cultured with human Platelet Lysate (hPL). A cytokine array assay of UC-MSC conditioned medium revealed the presence of numerous cytokines such as IL-8, IL-6, MCP-1, PAI-1, CXCL1, G-CSF and MIF indicating a pro-inflammatory role. It also revealed high concentrations of VEGF as detected by ELISA. This pro-angiogenic feature was confirmed using a metatarsal angiogenesis assay. Since it has now been established that a pro-inflammatory initial burst is important for neoangiogenesis and consequently bone regeneration, we hypothesized that this characteristic of UC-MSC secretome could influence the behavior of the cells in vivo. UC-MSC were seeded on ceramic scaffolds and implanted subcutaneously in immunocompromised mice. Histological analysis showed that UC-MSC were *not* able to form bone tissue but numerous blood vessels were noted. Interestingly, live-cell tracking in vivo showed that UC-MSC remained viable and proliferative for only 21 days after implantation. However, preliminary flow cytometric analysis of retrieved cells have not demonstrated an involvement of UC-MSC in the recruitment of host putative osteoprogenitor cells. We speculate that an orthotopic rather than ectopic site of implantation could create the proper environment to support UC-MSC mediated angiogenesis and recruitment of host osteoprogenitors in the lesion.

## Metodi innovativi per la tracciabilità di cellule mesenchimali della parete del cordone ombelicale in un modello murino di sclerosi laterale amiotrofica

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La Sclerosi Laterale Amiotrofica (SLA) è una patologia neurodegenerativa del motoneurone, caratterizzata da paralisi muscolare e compromissione di molte funzioni vitali. La prognosi infausta e la terapia inefficace hanno spinto la comunità scientifica a cercare metodi innovativi compresa la terapia cellulare.

Trattamenti con cellule staminali neurali e mesenchimali sono già stati effettuati nei pazienti SLA con risultati controversi e insoddisfacenti. Principale limite degli studi clinici è la mancanza di informazioni riguardo l'interazione cellula/ospite. L'utilizzo di modelli preclinici di SLA è perciò fondamentale per comprendere le potenzialità/rischi del trattamento.

E' stata recentemente comprovata l'efficacia di cellule staminali mesenchimali (MSCs) e cellule del sangue del cordone ombelicale in modelli preclinici di SLA dopo somministrazione intracerebroventricolare (ICV) o sistemica (IV). Il nostro gruppo ha focalizzato l'attenzione sul tracking longitudinale di cellule mesenchimali, amniotiche ed ematopoietiche in topi sani e malati. Il presente studio genera da un progetto più ampio atto a valutare biodistribuzione, tossicità ed effetto terapeutico di cellule mesenchimali della parete del cordone ombelicale (UC-MSCs) in un modello murino di SLA (il topo SOD1G93A) e in topi sani.

Al fine di ottimizzare il sistema di tracking longitudinale abbiamo co-incubato le UC-MSCs con l'Hoechst 33258, per visualizzare il nucleo, e con nanoparticelle polimeriche rodamate (NPs), per marcare il citoplasma. Gli studi *in vitro* hanno confermato che le NPs internalizzano efficientemente nelle UC-MSCs e non ne alterano i principali parametri funzionali.

La somministrazione ICV (250.000 UC-MSCs) mostra una selettiva biodistribuzione delle cellule all'interno del sistema ventricolare. In entrambi i gruppi sperimentali si osservano aggregati sferoidali di cellule che si riducono in dimensioni seguendo un gradiente rostro caudale (maggiore nel corno anteriore dei ventricoli laterali e minori nel 4° ventricolo) e diminuiscono nel tempo pur permanendo fino al termine dell'osservazione (21 giorni). La clearance cellulare sembra essere maggiore nei topi controllo rispetto ai SOD1G93A.

La somministrazione IV (500.000 UC-MSCs) induce una rapida distribuzione delle cellule nel polmone, milza e in minor quantità nel fegato. Diversamente da quanto osservato dopo ICV, tali cellule non formano aggregati. E' interessante notare la presenza di segnale nel parenchima cerebrale che permane nel tempo. Negli organi periferici la clearance è invece molto rapida in entrambi i gruppi sperimentali.

L'analisi longitudinale effettuata in questo progetto ha rivelato che: 1) l'utilizzo delle NPs fluorescenti e dell'Hoechst 33258 come marcatori cellulari, rappresenta un sicuro e affidabile sistema di tracciabilità cellulare; 2) le UC-MSCs presentano una risposta simile, in termini di localizzazione e permanenza nei tessuti a cellule precedentemente testate in modelli preclinici di SLA; 3) entrambe le vie di somministrazione risultano essere potenzialmente sfruttabili per trattamenti terapeutici.

Gli studi di efficacia clinica saranno perciò fondamentali per comprendere le potenzialità terapeutiche di questi approcci e designarne la possibile traslabilità clinica.

**Poster**

**Convegno AICC**

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## **A comparative study of demineralized bone matrix powders for tissue engineering: influence of different particle size on mesenchymal stromal cell attachment and metabolism**

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Demineralized bone matrix (DBM), which is a form of allograft, possesses the properties of osteoinductivity and osteoconductivity. A high number of data obtained through preclinical studies have clearly supported the utility of DBM in different human clinical settings.

In this study, DBM powder was produced from sheep cortical bone and was evaluated for its ability to support sheep mesenchymal stromal stem cell (MSC) adhesion, metabolism, and proliferation in order to assess its potential use in clinical tissue engineering strategies.

DBM powder was composed of granules of three different particle size ranges: (1) DBM 1L (large): 1mm-2mm, (2) DBM 2M (medium): 0.5 mm-1 mm, and (3) DBM 3S (small): < 0.5mm, all of which were evaluated.

Chemical physics and structural characteristics of these three samples were carried out in order to evaluate the differences among samples.

Four different cell numbers were used in the granule seeding process, and the impact of the particle size on adhesion, metabolism, and proliferation of MSC was investigated upon seeding at day 1 (d1) and after the initial stage of cell proliferation at day 3 (d3). Our data revealed that all three DBM particle sizes allow for very efficient seeding to occur in all cell densities tested (> 85%) with a slightly better result given by DBM 2M. Cell adhesion on the granules was also demonstrated for all three particle size ranges using SEM analysis. Moreover, DBM 2M showed a higher level of cell metabolism than both DBM 1L and DBM 3S both at d1 and d3 and at all cell densities, as revealed by Alamar Blue reagent, an indicator used to visualize metabolic cell activity. Cell proliferation was qualitatively evaluated by SEM and confocal laser microscopy after a *Live & Dead* staining. For all of the DBM samples tested, the highest proliferation rate was found at the lowest density.

These results suggested that the DBM particle size played a significant role in influencing *in vitro* metabolism of MSC, while it did not significantly affect cell adhesion. We concluded that selecting a DBM particle size between 0.5 mm and 1 mm was the best choice to support the highest MSC metabolism. In addition, it was concluded that the particle size of a granulated biomaterial might be an important factor for the development of optimal constructs for bone tissue engineering.

## **The dedifferentiation program of melanoma cancer initiating cells predisposes to a major degree of invasion.**

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There is enormous interest to target cancer initiating cells (CIC) for clinical treatment because these cells are highly tumorigenic and resistant to chemotherapy. Oct4 is expressed by CIC in different type of cancer, as a positive regulator of cell dedifferentiation. The aggressive clinical behavior and their metastatic propensity of malignant melanoma (MM) could be related to the developmental origins of melanocytes from the neural crest. In fact, many studies show that the MM initiating population is a subset of tumor cells defined by the expression of the neural crest marker CD271. Therefore, dedifferentiation and reacquisition of stemness are pivotal event in MM development.

In our study, we employed *in vivo* Reflectance Confocal Microscopy (RCM) for the morphologic characterization of MM cell populations in three different types. RCM represents a new imaging tool in Dermatology applied for more accurate clinical diagnosis of skin malignancies. We sought to evaluate the expression of CD271 in different morphological subtypes of MMs, and to correlate them with MM cytological aspect.

The presence of MM type organized in nests with cerebriform fashion or dense & sparse cell aggregation leads to a high probability of CD271 expression (sensitivity 66.6%, specificity 100%). Interestingly, these peculiar morphologies of cell aggregates also represent a characteristic pattern for nodular MM, known for its rapid growth and invasion, and major biological aggressiveness.

Moreover we showed, by using immunofluorescence or western blot analysis, that different human primary melanoma cancer cells cultured *in vitro* express Oct4 and other markers of stemness (CD271, CD133, CD20, ABCB5), in different ways that correspond to the morphologically heterogeneous population of cells present in MM types. These different features might indicate a peculiarly CIC susceptibility toward specific treatments.

These results suggest that CIC dedifferentiation would be an efficient target for innovative MM therapy pending a full dissection of the molecular events connecting dedifferentiation, stemness and tumorigenicity.

## Effect of tributyltin (TBT) on porcine vascular progenitor cells

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Tributyltin (TBT) is a chemical compound extensively used in the past as a biocide especially as anti-fouling paints for ships and fishing nets (Sarkar et al. *Ecotoxicology*. 2006; 15(4):333-40). Because of its long half-life TBT is strongly persistent in the sea environment and it has been shown that it can contaminate mammals, including man, through the food chain (Okoro et al. *Rev Environ Contam Toxicol*. 2011; 213:27-54). Furthermore, TBT has been classified as an endocrine disruptor displaying obesogenic properties (Pagliarani et al. *Toxicology in vitro* 2013 978-990; Grun and Blumberg *Mol Cell Endocrinol* 2013 304 19-20).

To date, few studies have been investigated the effect of TBT on stem/precursor cells; the purpose of the present research was to observe the effect of TBT on vascular progenitor cells (pVPCs) isolated from pig aorta (Zaniboni et al., 2013, *Proceeding of IV SCR Italy Society Meeting*). In order to evaluate the cytotoxicity of TBT, pVPCs (third passage) were seeded in a 96 wells plate and exposed to increasing doses of TBT (1, 10, 50, 100, 500, 750, 1000 nM) for 48h. Cell viability was assessed by LDH determination and annexin V/PI binding assay was used to evaluate the percentage of apoptotic and necrotic cell through a flow-cytometric approach. The ability of TBT to influence the adipogenic differentiation was investigated, too: pVPCs were seeded in a 24 wells plate and cultured using a specific medium (Stem Pro Medium Adipogenesis, Life Technologies) in order to induce adipogenesis in presence (100nM TBT group) or not (CTR group) of TBT for 14 days. Adipogenic differentiation was then evaluated through the Oil Red O staining.

Results showed that cell viability decreased in a dose dependent way, in particular 1000 nM TBT reduced cell viability to  $57,53 \pm 8,42$  %. After 3 hours of TBT treatment (1000 nM) flow cytometric analysis showed that  $10,81 \pm 1,21$  % of cells undergone apoptotic death.

After 14 days of 100 nM TBT treatment a significant difference was observed in lipid droplet production; in particular  $6,4 \pm 2,8$ % of cells in CTR group vs  $13,6 \pm 2,2$ % of cells in TBT group. Further gene expression analysis are needed to investigate the kinetic of expression of transcripts involved in adipogenesis, in order to confirm the obesogenic proprieties of TBT.

In conclusion, our results suggest that TBT could interfere in the adipogenic differentiation pathway of porcine vascular progenitor cells.

## Application of a new-generation cell selector for the standardization of a Regenerative Medicine product

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**Key terms:** mesenchymal cells; cell sorting; homogeneous cell preparation;

**Introduction:** Mesenchymal stem cells (MSCs) are immature multipotent cells that have the ability to self-renew and differentiate into several cell types; the therapeutic property that makes them one of the major candidates for Regenerative Medicine is their attitude to stimulate host tissue regeneration through paracrine secretion of a series of biomolecules that both stimulate the proliferation of tissue-specific progenitors and induce powerful localized immunomodulation and antiinflammatory effect.

Although bone marrow has been considered for years the classical reservoir of MSCs (BM-MSCs), the adipose tissue (AT) has been proven to be an increasingly attractive source of MSCs for tissues regeneration since fat is easily obtainable in large quantities, using non-invasive techniques.

**Background:** Classical MSCs isolation techniques from AT (Adipose derived MSCs - ASCs), are based on enzymatic digestion of the latter. Obtained suspension is a complex heterogeneous cell product that requires further selection for specific clinical application; indeed homogeneous preparations are strongly recommended.

**Aim:** The purpose of this research is the characterization of an ASCs product obtained by purification technique using a non-equilibrium, Earth gravity-assisted, dynamic flow fractionation (NEEGA-DF) technology, which is able to select cells based on differences in their dimensional/morphological properties. This simple, fast and non-invasive cell sorting methodology is able to purify homogeneous ASCs subpopulations, maintaining their properties and functions unmodified.

**Methods:** A few-microliter volume of the cell suspension was injected into the NEEGA-DF device by a continuous flow. In a relatively short time (about 30 min), sorted ASCs populations were collected. Fractionated cells were collected and biologically characterized in order to define proliferation rate, viability, surface markers and multi-differentiation abilities. Unfractionated cells were used as reference control.

**Results:** Separation between ASCs and “blood cells” has been demonstrated, as well as a noticeable target cell purification from other important cell contaminants. Different isolated cell fraction characterizations displayed a dissimilar proliferation yield and disparate differentiation potential after induction treatments. Evaluation of results demonstrates a highly reproducible profile of the NEEGA-DF sorting technology.

**Conclusions:** Results demonstrate that the NEEGA-DF cell sorting technology can be used to purify ASCs populations. The biocompatible highly reproducible fractionation process of the innovative selector represent a scalable opportunity to obtain a bed-side tool able to highly purify homogenous and viable ASCs fractions for Regenerative Medicine applications.

## **Determinazione e localizzazione di cellule derivate dalla membrane amniotica dopo trapianto in utero in ratto**

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Le cellule della membrana amniotica umana (hAMCs) vengono attualmente considerate di grande interesse per la loro possibile applicazione nella medicina rigenerativa. Inoltre, queste cellule, se trapiantate in utero, potrebbero rappresentare un'importante soluzione al trattamento di malattie genetiche a livello fetale. Lo scopo della ricerca è stato quello di valutare la migrazione, la localizzazione ed il chimerismo delle hAMCs dopo xenotrapianto in utero in un modello murino, attraverso tecniche di immunistochemica e di biologia molecolare. Lo xenotrapianto è stato effettuato inoculando un pool di cellule amniotiche ottenute da placenta umana nell'utero di 5 femmine di ratto Sprague Dawley a 18 giorni di gravidanza. I feti ed i ratti sono stati sacrificati, con un eccesso di anestetico, rispettivamente dopo 24h dall'inoculo, alla nascita, a 7 e a 14 giorni dopo la nascita. I feti e gli organi dei ratti sono stati fissati in formalina per processazione istologica o congelati a -80°C per indagini biomolecolari. Il chimerismo cellulare è stato valutato mediante l'utilizzo dell'anticorpo monoclonale Human Cytoplasmic Marker (STEM 121) in grado di identificare antigeni citoplasmatici di cellule umane e tramite metodiche di PCR. Il 100% dei feti (8/8), a 24 h post-inoculo, mostrava immunopositività citoplasmatica all'anticorpo STEM 121, confermando il diffuso chimerismo ottenuto in PCR. Alla nascita si osservava positività citoplasmatica nel 43% (3/7) dei ratti neonati, prevalentemente a carico della pleura e del polmone e il DNA chimerico era presente nella maggior parte degli organi dei ratti analizzati (7/7). A 7 giorni dalla nascita si osservava una ridotta positività sia in immunistochemica che in PCR. A 15 giorni dalla nascita il segnale in PCR era ridotto e pressochè assente quello rivelabile in immunistochemica. L'anticorpo STEM 121 utilizzato per rilevare le cellule amniotiche umane è risultato altamente specifico e ha consentito, unitamente ai risultati di biologia molecolare, di identificare la presenza delle hAMCs in organi e tessuti di ratto dopo xenotrapianto. Questo studio apre nuovi scenari relativi alle capacità di migrazione, di attecchimento, ed eventualmente, di differenziazione di queste tipologie cellulari.

## **In vitro production of differentiated Endothelial Cells from Mesenchymal Stem Cells, collected from adult rat Adipose Tissue (AD-MSCs): preliminary results**

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Mesenchymal Stem Cells (MSCs) are multipotent cells that reside within various tissues, including adipose tissue and bone marrow. Research concerning the potential therapeutic role of MSCs has advanced significantly over the last decade. Many studies have demonstrated that adipose tissue is an excellent source of MSCs (AD-MSCs), easily accessible in experimental animals. AD-MSCs can be expanded *in vitro* over the short term and they can differentiate toward a variety of cell types of mesodermal lineage. Therefore, they are thought an attractive tool for cell therapy. Selection of appropriate animal models for preclinical evaluations is crucial for optimization and validation of therapeutic protocols in regenerative medicine. Therefore, *in vitro* studies are necessary to future *in vivo* applications of MSCs. Rat is an animal model useful for a large number of *in vitro* studies. Aim of this work is the *in vitro* production of endothelial cells differentiated from rat ADMSCs. For this purpose, subcutaneous adipose tissue samples were collected from 5 Wistar rats, in accordance to the European Directives (86/609/CEE and 63/2010/EU) concerning the protection of experimental animals. These samples were processed for *in vitro* AD-MSCs isolation. After 2 days, nonadherent cells were removed and medium ( $\alpha$ -MEM) was replaced. The adherent AD-MSC population was expanded for 5 passages. To assess their characteristics, the expression of stemness markers, such as OCT4, SOX2 and NANOG, was evaluated by Real Time Sybr Green PCR. The analysis of gene expression was performed from 0 to 5 passage. For this purpose, mRNA was extracted from each passage using a commercial kit. Successively, mRNA was retrotranscribed in cDNA and stemness genes were detected using specific primers pairs, designed by the use of Primer Express software. Gene expression analysis was normalised by the GADPH gene housekeeping expression. To induce endothelial differentiation, stem cells at passage 0 and 1 were cultivated in D-MEM medium for 18-24 h. Successively, D-MEM medium was replaced with an enriched medium with VEGF-C. Cells were cultivated for 15 days. To verify the begin of differentiation, gene expression analysis for endothelial markers CD31, eNOS and vWF was performed by Real Time Sybr Green PCR. Specific primers pairs were designed by the use of Primers Express software. Gene expression results show an increase of expression levels for stemness markers, up to the *in vitro* passage 3. The begin of the endothelial differentiation is proved by the initial increase of expression levels for CD31, eNOS and vWF, associated to a decrease in the expression levels for stemness markers. These are preliminary results of a project aimed to the *in vitro* production of endothelial differentiated cells from AD-MSC. Further studies are required to confirm the acquisition of the endothelial phenotype by AD-MSC cells, induced to differentiate.

## **Adoptive immunotherapy in colon cancer: generation of tumor-specific cytotoxic T lymphocytes (CTLs) from peripheral blood of colorectal cancer patients**

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**Background:** Colorectal cancer (CRC) is the third most common cancer worldwide and the fourth most common cause of death in the developed Western countries. Although a clear association between autologous tumor-infiltrating T lymphocytes (TIL) and clinical outcome has been documented in this neoplasia, active and adoptive immunotherapy do not play an important role in the treatment of metastatic CRC (mCRC).

Adoptive T-cell transfer (ACT) using autologous TIL, grown ex-vivo and then infused into the cancer patient, has emerged as an effective treatment for patients with metastatic melanoma and other solid tumors. However, in vitro studies performed on bulk TIL cultures purified from mCRC patients demonstrated contrasting preliminary results on the lytic activity against autologous cancer cells.

**Methods:** 76 patients affected by CRC were enrolled. Tumor biopsies were obtained at surgery, together with 100 ml of heparinized peripheral blood. Tumors were dissociated to a single-cell suspension and cultured in order to obtain tumor cell line from each patient. Dendritic cells (DCs) were generated from previously separated Peripheral Blood Mononuclear Cells, using a magnetic positive selection of CD14<sup>+</sup> monocytes, cultured in presence of Interleukin-4 and recombinant human Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF). Anti-tumor CTLs were elicited using DCs as antigen-presenting cells, autologous apoptotic tumor cells as source of antigens and T CD-8 lymphocytes enriched effectors, with weekly stimulation. To evaluate the cytotoxic activity of CTLs, Interferon- $\gamma$  (IFN- $\gamma$ ) secretion was assessed by ELISPOT.

**Results:** Tumor cell lines and DCs were obtained from 20 out of 76 patients. To date ELISPOT was performed for 6 patients: strong IFN- $\gamma$  secretion was detected at the third, fourth and fifth stimulations for one patient and at the second for another patient, whereas for three patients a weak secretion was detected during the second and third stimulations. T-cells from one patient did not react to the stimulations.

**Conclusions:** Generation of CTLs suitable for ACT immunotherapy is feasible from peripheral blood in patients with CRC.

## Effects of bioreactors and biomaterials on bone-marrow mesenchymal stem cells proliferation and differentiations for tissue engineering applications

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Bone marrow and Adipose tissue are now recognized as an important source of postnatal mesenchymal stem cells (MSCs) for regenerative medicine applications. Here we described several applications of tissue engineering in enhancing proliferation and differentiation of MSC towards osteoblasts to reduce the gap between the process of bone formation *in vitro* and the subsequent graft of bone substitutes *in vivo*. These techniques include: three-dimensional organic or inorganic scaffolds and the bioreactors, that improve the static culturing cell methods by regulate the homogenous tissue growth. In particular we demonstrated that the combination of 3D-nanostructured biomaterials and bioreactors, such as mechanical vibration and electromagnetic stimulation, improves the amount of cell adhesion, osteoblastic differentiation and bone matrix deposition to engineer bone for clinical use. We used high frequency vibration (HFV) to accelerate MSC differentiation and pulsed electromagnetic field (PEMF) to induce differentiation of bone marrow MSCs (BM-MSCs) and adipose-tissue MSCs (ASCs). Among the biomaterials considered to produce the 3D scaffolds, pectin hydrogels resulted particularly suitable as injectable cell carriers. Macrogels with different rheological properties and microspheres were produced with different degree of crosslinking. The injectability of the gels cross-linked with different salts (CaCl<sub>2</sub>, CaCO<sub>3</sub>, ZnCl<sub>2</sub> and FeCl<sub>3</sub>) through different needle size was tested by texture analysis and rheological characterization. The rheological parameters confirmed that pectin gels behave as soft-gels with mechanical properties similar to soft tissues. Such properties may provide minimally invasive implantation and the ability to fill a desired shape. The tight control over the gelation kinetics allows loading cells or drugs in the hydrogels before injection. The obtained results support the idea that pectin gels can recreate adequate microenvironments for cell delivery. All these models represent a good starting point to engineer bone constructs for potential clinical use in regenerative medicine.

## Alginate microcarriers and films for adipose-derived stromal cell delivery

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Stem cells-based therapies applied to tissue engineering represent a promising approach to the treatment of several degenerative diseases and tumours in humans. In particular, the ability of Adipose Mesenchymal Stem Cells (ASCs) to interact with different environments, along with the immune tolerance, and their migratory abilities to pathologic tissue, make ASCs an attractive platform for both cellular therapy and drug delivery in humans. In order to exploit their functions, ASCs need to be localized at the target site, especially because there is the risk that ASCs are washed out by uptake of the cells at the level of major organs (lung and liver). Microcarriers and films can be used as Stem Delivery Systems (SDSs) since they offer a large surface area for the cell anchorage and promote cell attachment depending on their properties (e.g. microcarrier diameter, typically 100-400 µm; density, typically from 1.02 to 1.10 g/ml) and composition. In this study alginate microcarriers and films were proposed as SDSs for vehiculation, targeting and controlled release of ASCs, with the aim of optimizing the therapeutic effect of the cells.

Different formulations of alginate microcarriers were obtained by spray technique and SDSs were characterized in terms of particle size distribution (Beckman Coulter LS230 granulometer). Microcarriers were then freeze-dried, films were obtained and analyzed under a scanning electron microscopy (Mira 3, Tescan). Films were sterilized and used for cell cultures: ASCs at P3 passage were seeded onto films (800000 cells/cm<sup>2</sup> of film) in DMEM/F12 1:1, 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin e 0.25 µg/ml amphotericin, and cultured for 41 days at 37°C and 5% CO<sub>2</sub>. Cell viability were assessed directly on the construct through the use of a microscope coupled to excitation-emission filters for fluorescent dyes: propidium iodide (2 micrograms/ml, Invitrogen) and calcein-AM (5 micrograms/ml, Invitrogen) were used for staining of died and viable cells, respectively.

Microcarriers presented a mean diameter ranging from 154.3 µm to 474.2 µm, depending on formulation: this particle size was suitable for cell attachment and culture. After freeze-drying, films had a rough surface and maintained structural integrity also after sterilization process. ASCs adhered on the film surface, formed cell aggregates and were viable until the end of culture.

In conclusion, technological resources are available to obtain alginate microcarriers and films for adipose-derived stem cells delivery, for the controlled release of cells and active factors at the damage site, as injectable or implantable products.

## WEB-BI, Business Intelligence and Regenerative Medicine

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The WEB-BI project, co-funded by the Piedmont Region in the context of POR FESR 2007/2013, has the objective to deliver an innovative framework to extract affordable information from web sites. The ICT techniques employed in this framework have been applied in the specific context of Regenerative Medicine, exploring information on the therapeutic employment of stem cells and medical products for advanced therapies.

The project objectives emerge from the observation that the web contains a huge amount of data, and that converting these data into useful information is a difficult and time consuming task. This is especially true for young research sectors such as Regenerative Medicine. Researchers and professionals working in this field spend a lot of time exploring the web to find new useful information, and relating it to their own knowledge base.

Automatic exploration and summarization of web data comes with many problems and obstacles, which include the necessity to deal with multi-lingual, unstructured and potentially unreliable sources of information.

The WEB-BI project applies advanced ICT techniques to solve these problems, and applies Business Intelligence techniques to the data extracted from the web, in order to extract statistical information in real time, and to make the relevant sources available from where this information has been extracted. In particular, we applied this concept to the search of clinical trials; instead of exploring the web to gather information on trials performed on a specific disease, with WEB-BI one can immediately see how many trials have been performed, where, when, and then read more detailed information of the referred-to sources.

The WEB-BI project has delivered a prototype of the basic tools and techniques that will be later employed to create a publicly available web site, that will provide the following features:

- real-time statistical information about trials, published papers, therapies and medical products linked to a specific disease/therapeutic agent
- automatic digests of clusters of related sources, such as papers published on a specific field of research
- direct access to sources of information used to create statistics and digests
- exploration of the web to find new relevant sources of information about Regenerative Medicine
- automatic evaluation of the affordability of the new sources
- natural language processing techniques to extract information from both structured and unstructured documents

The WEB-BI research project has been successfully completed in September 2013, and the involved partners are currently approaching the necessary activities to convert the research result into a product that can be made available to the public.

# Murine fetal membrane mesenchymal stromal cell and murine embryonic fibroblast grafts in the central nervous system of immune competent mice: a comparative analysis

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**Background:** Immune-modulating properties of human mesenchymal stem cells isolated from fetal membranes are now subject of increasing interest and major investigations. Human fetal membrane mesenchymal stromal cells grafted in preclinical animal models showed promising results, but determination of their exact physiological properties and immune modulating effects in vivo remains limited due to the xenogeneic nature of this type of grafts.

**Objectives:** this study is focused on characterization of immune-modulating properties of murine fetal membrane mesenchymal stromal cells (FMSCs) compared with embryonic fibroblasts (MEFs), after syngenic grafting in the central nervous system (CNS) of immune competent mice.

**Methods:** eGFP- and eGFP+ embryos were obtained by inter-crossing of male C57BL/6-eGFP transgenic mice with female wild type C57BL/6 and FMSCs or MEFs were respectively isolated from fetal membranes and embryonic tissue. Growth curves for eGFP+ FMSCs and MEFs have been established and analyzed; flow cytometric immune phenotype characterization on eGFP-FMSCs and MEFs has been performed. In vitro immune modulating properties has been investigated by co-cultures of FMSCs or MEFs with activated murine microglial cells (BV2 cell line), evaluating TNF- $\alpha$  production levels by stimulated microglia. Finally eGFP+ FMSCs and MEFs were transplanted in immune competent mice CNS in order to perform syngenic cell grafting experiments and to investigate innate immune response.

**Results:** phenotypical analysis of FMSCs and MEFs did not show significant differences (both populations are Sca1+, CD44+, CD90+, MHCI-, MHCII+, CD106-, CD31- and CD45), but FMSCs did not display proliferative potential as compared to MEFs. In vitro immune response analysis evidenced that both FMSCs and MEFs are able to reduce TNF- $\alpha$  production by activated microglial cells. Cell grafting experiments, in the CNS of immune competent mice, showed reduced survival levels for FMSCs compared to MEFs grafts, but quantitative histological analysis found out no significant differences in endogenous innate immune responses observed after FMSC or MEF grafting.

**Conclusion:** isolation and further characterization of murine FMSCs will allow a more exhaustive analysis on their intrinsic immune-modulating properties and possibly on their regeneration-inducing properties after autologous (or allogeneic) grafting in preclinical animal models.

## Encapsulation of tendon stem/progenitor cells (TSPC) and adipose-derived stem cells (ASC) for the treatment of tendinopathies

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Tendons are poorly vascularized and are characterized by a low mitotic activity; for this reason the spontaneous healing process of the tissue in case of tendinopathy occurs slowly and in an ineffective way. Current therapeutic strategies do not always allow to obtain long term satisfactory results; as a consequence alternative approaches, including the one involving mesenchymal stem cells (MSC), have been recently proposed. Recently, a new cell population, named *Tendon stem/Progenitor cells* (TSPC), with stem cells characteristics such as self-renewal capacity, clonogenicity and multipotency, has been identified in tendons. These features make these cells a potential new tool to be employed in the treatment of tendinopathies. Three-dimensional cell culture in alginate beads seems to be a very promising technique to allow not only optimal cell connections and organization, but also to achieve an efficient local cell delivery; thus, the aim of this study was to evaluate the influence of a method of encapsulation and culture of stem cells in alginate beads, on proliferative, and tenogenic potential of TSPC.

TSPC were used in mono-culture and also in co-culture with adipose derived stem cells (ASC), in order to evaluate a possible synergistic effect of their interaction. ASC were isolated from liposuction of abdominal subcutaneous adipose tissue by enzymatic digestion (n=3), whereas TSPC were isolated by spontaneous migration from discarded fragments of semitendinosus and gracilis tendons obtained from anterior cruciate ligament (ACL) reconstruction with autologous hamstrings (n=3). Mono-culture, as well as co-culture of TSPC and ASC (three different ratio, 50:50, 70:30, 30:70) were prepared and maintained in culture both in control and in tenogenic medium. Both mono and co-culture were cultured in three-dimension in alginate beads; monolayer cultures were prepared as control.

Viability and proliferation of encapsulated cells were evaluated by MTT, Trypan Blue and CyQuant Assay during the culture time (time 0, 1 day, 5 days, 7 days, and 14 days). The results showed that all the cultures present high levels of viability and that the encapsulation process does not provoke cytotoxic effects. Moreover, expression of specific tendon cell markers (scleraxis and collagen type I) measured at two different time points, 7 days and 14 days, was not significantly up-regulated in tenogenic-treated monolayer cell culture, whereas encapsulation and co-culture promoted the expression of scleraxis but not of collagen I.

The high levels of expression of scleraxis of encapsulated co-cultures confirm the hypothesis that the surrounding microenvironment, the cell-cell interactions and the exchanges of macromolecules, allowed by the characteristic shape of the scaffold and by his high porosity, are important and decisive factors in promoting tenogenic differentiation.

# Conditioned medium from amniotic mesenchymal stromal cells reduces lung fibrosis in bleomycin-injured mice: methodological approaches for lung fibrosis evaluation.

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**Background and aims:** Our previous data indicated that placenta-derived cells are able to reduce bleomycin-induced lung fibrosis in mice through the paracrine action of soluble factors released by these cells. Indeed, we found that conditioned medium (CM) generated from the culture of human amniotic mesenchymal stromal cells (AMSCs), exerts a specific anti-fibrotic effect, which was not observed when administering CM generated from other cell types, preserves pulmonary gas exchanges and reduces lung pro-inflammatory cytokine levels and macrophage recruitment to the lung.

In these experiments, lung fibrosis was evaluated both by the histological analysis of mice lung sections applying Ashcroft's score system and by the determination of lung collagen content with the Sircol assay.

Considering that Ashcroft's scale was originally set up to evaluate pulmonary fibrosis in human biopsies and that in our studies we obtained very variable results depending on the observer's experience, we intend to analyze lung tissues from bleomycin-challenged mice, treated or not with AMSC-CM, with an additional score system, by Hübner, in order to compare the results obtained and to have a reliable system to evaluate fibrosis in mice lungs. Hübner and colleagues modified Ashcroft's scale by improving distinction between different levels of fibrosis in murine lung tissues and by adopting a 20-fold instead of 10-fold objective to avoid the wide heterogeneity of the field.

In parallel, we determined lung collagen with both Sircol assay and a self-made reagents method modified from Kliment et al (Int J Clin Exp Pathol, 2011).

**Methods:** Mice were intratracheally instilled with bleomycin (4 U/kg). After 15 min, 8 mice (AMSC-CM Group) were intrathoracically injected with AMSC-CM, 10 mice (Bleo Group) remained untreated and 4 mice (Saline Group) received saline, instead of bleomycin. After 14 days, lungs were removed, one hemi-lung was formalin-fixed, embedded in paraffin block, sectioned and stained with Haematoxylin-Eosin and with Masson-Goldner trichrome. The remaining hemi-lungs were stored at -80°C and used for collagen content determination.

**Results:** AMSC-CM treated mice showed higher body weight and lower lung weights with respect to mice of the Bleo Group. The histological analysis indicated that mice of the AMSC-CM Group developed lower lung fibrosis than mice of Bleo Group and in parallel, lungs of these mice showed lower collagen content. The two applied score systems gave similar results but it is noteworthy that, inside each experimental group, the scores obtained with Hübner's scale display less variability with respect to those obtained with Ashcroft's one. Similar data were obtained from Sircol assay and self-made reagents.

**Conclusions:** These results confirm the anti-fibrotic action of AMC-CM. Although the results obtained from Ashcroft and Hübner's scales are comparable, the Hübner's one show the advantage to give less variable values. Sircol assay and self-made reagents method appear equivalent.

## Cancer Stem Cells: a new target for tumor treatment

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Cancer Stem Cells (CSCs) comprise a cellular subpopulation involved in tumor development, recurrence and metastasis, representing an ineludible pharmacological target to eradicate tumors. CSCs, are able to self-renew and are highly resistant to both conventional chemo- and radio-therapy causing cancer relapse. Therefore, development of specific therapies targeted to CSCs might improve treatment efficacy in cancer patients. Increasing evidence suggest that breast cancer, as other tumor types, may be driven by CSCs. A recent study showed that patients treated with the oral antidiabetic drug metformin had a lower cancer incidence and mortality compared to diabetic patients treated with other drugs, suggesting a potential antiproliferative activity of this drug. In this study we evaluate CSCs, obtained from spontaneous mammary tumors of cats and dogs, as cell target for metformin treatment.

Spontaneous feline mammary carcinomas (FMCs) and canine mammary tumors (CMTs) were included in this study. The great majority of tumor samples gave rise to primary cultures, that were maintained in stem permissive medium to enrich cultures in FMC- and CMT-CSCs. CSCs grew as non-adherent mammospheres but, in the presence of serum, they adhered to the plastic support and acquired a differentiated phenotype. In both cellular types, this process caused down regulation of the expression of the stem cell marker CD44. CSCs, but not differentiated FMC and CMT cells, were tumorigenic when injected in NOD/SCID mice, an essential feature of CSCs. Therefore, we tested the cytotoxicity of different concentrations of metformin (0.1-100 mM) on CSCs and their differentiated counterpart, by MTT assay. The results show that metformin induces a significant reduction on CSCs cell viability after 48h of treatment.

Our results suggest that feline and canine mammary carcinomas contain CSCs that could represent a suitable experimental model to evaluate novel pharmacological approaches in human and animal studies.

## ***In vitro* study on equine amnion-derived mesenchymal stem cells for potential application in endometrial regeneration**

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Equine amniotic membrane-derived cells (AMCs) exhibit progenitor/stem cell-like properties, expressing specific markers and differentiating *in vitro*. In addition, AMCs and their conditioned medium (CM) show immunomodulatory properties *in vitro* and tendons regeneration *in vivo* when allogeneically transplanted. In the present study, we further characterise AMCs in view of their potential application in endometrial regenerative medicine. AMCs and cells isolated from endometrium (EDCs) at diestrus were studied for their proliferative capacity. Moreover, proliferative potential of EDCs co-cultured with AMCs in a transwell system or in the presence of CM secreted by AMCs was also investigated. Since endometrial stromal cells proliferate and specialize during pregnancy under the control of progesterone, the expression of endometrial genes involved in early pregnancy (AbdB-like *Hoxa* genes), in conceptus pre-implantation development (*ERα*, *ERβ*, *PR*, *PGRMC1* and *mPR*), and their regulators (*Wnt7a*, *Wnt4a*) were evaluated on AMCs cultured with and without progesterone.

Our results demonstrated that AMCs and EDCs showed very similar proliferative capacity in the first two passages with an average doubling time (DT) value of 1.11 days for AMCs and 1.56 days for EDCs. In the next passages, DT significantly increased ( $P < 0.05$ ) until P5 for EDCs, while for AMCs the proliferative ability was very active between P3 and P5. However, EDCs, when in co-culture with AMCs by transwell system or by CM, showed a 1.23 and 1.34 fold increase in proliferation, respectively, compared to baseline EDCs in standard medium ( $P < 0.05$ ).

All studied genes were expressed abundantly in endometrial tissue (ED) and amniotic membrane (AM), whereas *ERβ* was not detected. Similarly, AMCs at P0 expressed the same genes identified in tissues, excepted for *ERα* and *ERβ*. *Hoxa-9* was the only gene observed in AMCs at passage 1 (P1), but after culture of AMCs with progesterone, *Hoxa-9*, *PGRMC1* and *mPR* expressions were conserved in this cell line until P5 that was the last passage studied.

These data suggest that AMCS or their CM can promote EDCs proliferation and may be used in regenerative medicine when poor endometrial proliferation is associated with infertility or poor pregnancy outcome. In addition, AM and AMCs are similar to ED tissue in the expression of the studied genes until after cells isolation. Unfortunately, the expression of many of the genes evaluated is lost at P1 supporting the hypothesis that culture conditions play an important role in limiting AMCs behaviour, probably inducing epigenetic effects. The culture with progesterone preserves the expression of membrane-bound intracellular progesterone receptors and *Hoxa-9*, one of the primary steroid hormone-responsive genes in the uterine stroma in humans and mouse. Since the endometrial receptivity depends on the number of progesterone receptors in epithelial and stromal cells, these data provide an intriguing indication of the possible implication of AMCs in endometrial regeneration.

## Characterization of Spleen-derived Mesenchymal Stem Cells in Ph-neg MPN patients

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Extramedullary hematopoiesis, as consequence of bone marrow microenvironment dysregulation, is a key feature of advanced stage disease in Philadelphia negative myeloproliferative neoplasms (Ph-neg MPN), and in particular of myelofibrosis. In patients with myelofibrosis, extramedullary hematopoiesis occurs mainly in the spleen, where a microenvironment that provides a residence for circulating hematopoietic progenitor/stem cells (HSCs/HPCs) may be derived from endogenous splenic cells and/or from the mobilization of bone marrow mesenchymal stromal cells (MSCs). In order to investigate Ph-neg MPN splenic microenvironment, we evaluated the possibility to *in vitro* isolate MSCs from the spleen of 23 patients with myelofibrosis undergoing therapeutic splenectomy for progressive splenomegaly and of 7 healthy donors (HDs) undergoing splenectomy for trauma surgery. Written informed consent was obtained from both patients and HD. Following the standard procedure for BM-MSC expansion, we were able to isolate and *in vitro* expand MSCs from the spleen of 9 patients with myelofibrosis (39%) and of 3 HDs (43%). MSCs were characterized for morphology, clonogenic efficiency (CFU-F), proliferative capacity (population doubling, PD), immunophenotype (flow-cytometry), osteogenic and adipogenic differentiation potential (histological staining), and ability to reach senescence. Moreover, the capability to support hematopoiesis of HD-derived CD34+ cells by co-cultures on feeder layers of irradiated spleen MSCs from both patients and HDs will be evaluated. Preliminary data suggest that: *i.* spleen MSCs from both patients and HDs show a morphology typical of aged cells, and they enter senescence phase at earlier passages (p) (median value: p4, range: p2-p10 and p4, range: p4-p10, respectively), in comparison with BM-MSC; *ii.* CFU-F number is higher in patients than in HDs (median: 0.07, range: 0.03-0.1 and median: 0.03, range: 0.03-0.04/10<sup>6</sup> seeded cells, respectively) showing that higher number of MSC precursors are present in myelofibrosis splenic tissues; *iii.* patients MSC proliferative capacity is lower than that of HDs. Experiments aimed to assess the differentiation potential and the support to *in vitro* hematopoiesis are in progress. How these characteristics of spleen-derived MSCs from patients may affect local microenvironment and support extramedullary hematopoiesis need further investigations.

## Study *in vitro* on porcine Stem Cells from Buccal Fat Pad and Subcutaneous Adipose Tissue for Periodontal and Oral Bone Regeneration

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Oral bone lost is an important issue in maxillo-facial and dental surgery. In our previous study we have characterized human mesenchymal stem cells from Bichat's fat pad, a convenient tissue for dentists, and proposed them for future therapies of periodontal defects and oral bone regeneration. Considering the need for preclinical study and the swine as an accepted animal model in tissue engineering applications, here we compared the swine Adipose-derived Stem Cells (ASCs) from Buccal fat pad and from subcutaneous adipose tissue both alone and in combination with synthetic scaffolds.

$5.5 \times 10^4 \pm 3.3 \times 10^4$  and  $3.0 \times 10^4 \pm 9.3 \times 10^3$  cells/ml were isolated from interscapular subcutaneous adipose tissue (Scl) and buccal fat pad (BFP) of 6 swine. All the ASCs, with MSC fibroblast-like morphology, proliferated constantly during culture with an average doubling time (DT) of  $82.9 \pm 11.5$  hours and  $72.5 \pm 8.2$  hours for Scl- and BFP-ASCs, respectively, and showed a high clonogenic ability ( $+10.1 \pm 1.4\%$  for Scl- and  $+8.9 \pm 1.5\%$  for BFP-ASCs). By FACS analysis both cell populations appeared similar in size and granularity, and expressed CD90, whereas the CD14, CD45 and CD271 were not detectable. Furthermore, after osteogenic induction, all the ASCs showed an increase of either collagen and calcified extracellular matrix (ECM) production of about 87% and 118% for Scl-ASCs, and of about 254% and 116% for BFP-ASCs, compared to undifferentiated cells. Alkaline phosphatase activity ( $+126\%$  and  $+201\%$  for Scl- and BFP-ASCs, respectively) and osteonectin expression ( $+336\%$  for Scl- and  $+306\%$  for BFP-ASCs) were also upregulated, confirming their capacity to osteo-differentiate. When induced, both cell types were also able to differentiate towards the adipogenic and chondrogenic lineages, as revealed by lipid vacuoles production and GAGs deposition. Additionally, the osteoinduction of cells in the presence of titanium disks and silicon carbide-plasma-enhanced chemical vapor deposition (SIC-PECVD) fragments, significantly increased the amount of calcified ECM compared to control cells; moreover, titanium is osteoinductive per se on ASCs ( $+91$  and  $+284\%$  for Scl- and BFP-ASCs).

Finally, porcine autologous or heterologous sera, did not improve cell growth compared to standard condition.

No significant difference between cells from the different harvesting sites was observed, concerning all the features described.

In conclusions, swine buccal fat pad contains mesenchymal stem cells, and we suggest them for preclinical studies of periodontal and bone defects regeneration.

## Multipotent mesenchymal stromal stem cells derived from waste human ovarian follicular fluid: bioengineering applications *in vitro*

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Modern tissue engineering strategies combine living cells and scaffold materials to develop biological substitutes that can restore tissue functions. Biomaterials and bioreactor systems provide both the technological means to reveal fundamental mechanisms of cell function in a 3D environment, and the potential to improve the quality of engineered tissues. Mesenchymal stem cells (MSC) have a large capacity for self-renewal while maintaining their multipotency and represent a promising tool for regenerative medicine. Our studies showed the possibility to derive MSC from the human ovarian follicular fluid (FF cells) that picked-up during human assisted reproduction techniques and usually wasted during IVF procedures. FF cells were collected from healthy women undergone an IVF treatment during oocytes retrieval and cultured with DMEM + 10% FBS in *in vitro* minimal medium conditions, without any growth factor, including leukemia-inhibiting factor (LIF), previously considered essential. FF cells showed typical mesenchymal stemness markers (including CD-90, CD-44, CD-105, CD-73) and multipotent differentiation capacity in osteogenic, chondrogenic and adipogenic lineages. Now we also tested capability of FF cells to grow on gelatin cryogel scaffolds, promising new biomaterials owing to their biocompatibility, compared to MSC derived from human bone marrow. Data collected demonstrated that FF cells could grow on biomaterial not only on the top but also in the layers below till 60  $\mu\text{m}$  of deepness. Results suggested that cells observed were mesenchymal ones due to their positive immunostained for vimentin and CD-44, typical markers for MSC. Finally we evaluated if ultrasound treatment on FF cells cultures have positive effects on proliferation activity. To study effects of mechanical conditioning, we tested two different times of ultrasound stimulus (LIPUS), 2 or 5 minutes, corresponding respectively to 17.88 and 44.70 joule per day on cell culture and compared data to control without any kind of mechanical stimulation. The percentage number of cells showing intensive proliferative activity was  $11.26 \pm 5.19\%$  in non-stimulated control cultures,  $15.60 \pm 1.70\%$  in LIPUS stimulated cultures for 2 min, and  $46.43 \pm 15.04\%$  in LIPUS stimulated cultures for 5 min. Comparisons with Bonferroni test showed statistically significant differences between control and LIPUS stimulation for 5 min ( $p=0.003$ ) and between the two LIPUS stimulations ( $p=0.021$ ); on the other hand, the proliferation did not increase with the shorter ultrasound treatment. Our results suggest that cells provided by mesenchymal plasticity can be easily isolated by waste follicular fluid, avoiding scraping of human ovaries. Moreover, successfully growth of putative MSC derived from follicular fluid on three-dimensional cryogel scaffold and positive effect on proliferation activity induced by ultrasound stimulus, open potential developments in biotechnological or medical applications. Therefore, FF cells may be introduced as a valuable model system with which to study the mesenchymal lineages for basic research and tissue engineering.

## Semi-quantitative monitoring of adhesion of mesenchymal stromal cells on calcium-phosphate granules through a computer vision system.

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The interaction between cells and biomaterials is the basis of Regeneration Medicine and Tissue Engineering. In particular, the adhesion of cells onto scaffolds and, in general, material surfaces, is crucial for their survival, growth and proliferation.

Mesenchymal Stromal Cells (MSC) are the cells mainly used in clinical bone reconstruction applications for the property to be self-renewing and able to differentiate towards specific phenotypes, among them the osteoblastic. Iliac crest autologous bone is considered the gold standard to obtain large volumes of bone marrow, which is a natural reservoir of MSC. However, MSC progenitors represent only a small fraction of the total population of cells in adult bone marrow. This determines the necessity of an intermediate step of *in vitro* cell expansion in order to obtain a relevant number of MSC to guarantee clinical success, mainly dependent on the presence of MSC and mostly on the properties of the scaffolds or the biomaterials employed.

The quantitative estimation of the biomaterial surface covered by cells is still an open issue due to difficulties in determining the area of the cells with a good statistics. Our research aims at studying adhesion and proliferation of MSC on OSPROLIFE<sup>®</sup> hydroxyapatite-tetra calcium phosphate (HA-TTCP, Eurocoating SPA, Cirè-Pergine, Italy), a commercial bone substitute biomaterial in form of granules. In particular, in this work we propose a non-invasive approach based on Computer Vision techniques to monitor cells adhesion and provide automatically a semi-quantitative estimation of the percentage of the visible part of the single granules covered by cells.

In order to acquire a sufficient statistics, we analyse wide-area images (namely, “mosaics”) built by collecting several single microscopic images using *MicroMos v2.0*, an open source software tool we developed for stitching images acquired with a standard, even manual, widefield microscope ([www.filippopiccinini.altervista.org/Mosaicing/Mosaicing.html](http://www.filippopiccinini.altervista.org/Mosaicing/Mosaicing.html)). In particular, to build mosaics of fluorescent images, besides correcting the vignetting effect using a calibration slide, we proposed a new method for dynamically correcting the photo-bleaching meanwhile the mosaic growths. Then, starting from these wide images, we developed specific image processing techniques to automatically segment the cells adherent on the granules, thus achieving a semi-quantitative analysis of the percentage of the surface of the biomaterial covered by cells.

The obtained results prove the precision of the segmentation method we conceived to estimate the area of the granules covered by cells. In particular, 60% of the granules’ surface is covered by MSC in a week only. Furthermore, we showed that the proliferation of the MSC in contact with HA-TTCP yields large solid aggregates in few days, capable to effectively fill cavities. Finally, we proved how achieving these results has been possible thanks to the availability of large mosaics of images, where separate images would provide unreliable statistics.

## **GridMos: a fully-automatic mosaicing method for improving precision and repeatability of manual cell counting.**

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Since the origin of biology, cell counting is one of the basic needs in most of the biological experiments. In particular, precision and reproducibility of counting strongly influence the assessment of treatments, the amount of drugs to be administered to the patients and next steps of biological procedures. For instance, in Regeneration Medicine cell counting is fundamental to assess proliferation and cells adherence to a scaffold, in order to evaluate biocompatibility and, in general, biomaterials behaviour. In Oncology, live and dead cells counting is performed to monitor the vitality status of cancer cells after different chemical or radiobiological treatments, also influencing care protocols.

Many methods and systems have been studied for cells counting. The solutions range from fully automated specific commercial instruments (e.g., ChemoMetec NucleoCounter® and Bio-Rad TC20TM Automated Cell Counter) to image processing techniques, also provided as facilities in automated microscopes. Nevertheless, at present the manual cell counting performed with a hemocytometer still is the gold standard.

Several problems limit precision and repeatability of counts. First of all, the counts manually performed are operator-dependent. Then, when a live and dead cells analysis is performed shortening the reading time of the sample becomes an important issue, since the sample dries, the cells die and Trypan Blue diffuses. Theoretically, this prevents the count to be performed more than once. In practice, the count is not repeatable and no report documentation can be provided. Furthermore, if many samples have to be counted, the microscope remains busy for a long time, this limiting its use by other operators. Finally, precision and reproducibility of the manual counts decay with the number of cells and the number of samples to be counted, strongly jeopardizing the accuracy of the obtained results.

In this work we propose an approach relying on an image processing method to improve precision and reproducibility of the counts. In particular, we introduce GridMos, a fully-automated mosaicing method to obtain a single large-image (namely, a mosaic) representing the whole hemocytometer’s grid. The method does not require prior information and it can be employed to obtain image mosaics of any hemocytometer and widefield microscope, even without a motorized x-y stage.

The obtained mosaic, combined with an application for image labelling, contributed to improve counts precision and repeatability, also providing a report documentation of the cells number and their status. Furthermore, the mosaic also enables the analysis by more operators, even being in several places and at different times, without problems of cell modifications or colour decay.

The statistical experiments, performed by asking 7 biologists to count the cells of different lines, confirm the improvements brought by our counting approach in both precision and repeatability measurements.

## Implication of Calcium-Sensing Receptor (CaSR) and the Calcimimetic R-568 in the Osteogenic Differentiation of Ovine and Human Amniotic Fluid Mesenchymal Stem Cells (AFMSCs)

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AFMSCs have been recognized as a promising source for cell therapy used in bone traumatic and degenerative damage. Calcium Sensing Receptor (CaSR), a G protein-coupled receptor able to bind calcium ions, plays a physiological role in regulating bone metabolism. It is expressed in different kinds of cells, as well as in some stem cells. Then, the bone CaSR could potentially be targeted by allosteric modulators, in particular by agonists such as the calcimimetic R-568, and these may be potentially helpful for the treatment of bone disease.

The aim of our study was first to investigate the presence of CaSR in ovine and human AFMSCs and then the potential role of calcimimetics in *in vitro* osteogenesis.

AFMSCs were isolated, characterized and analyzed to examine the possible presence of CaSR by western blotting and flow cytometry analysis. Once we had demonstrated CaSR expression in both AFMSCs, we worked out that 1  $\mu$ M R-568 was the optimal and effective concentration by cell viability test (MTT), cell number, Alkaline Phosphatase (ALP) and Alizarin Red S (ARS) assays. Interestingly, we observed that basal diffuse CaSR expression in AFMSCs increased at the membrane when cells were treated with R-568 (1  $\mu$ M), potentially resulting in activation of the receptor. This was associated with significantly increased AFMSC mineralization (ALP and ARS staining), thus demonstrating a potential role of calcimimetics during osteogenic differentiation. Calhex-231, a CaSR allosteric inhibitor, totally reversed R-568 induced mineralization.

Taken together, our results demonstrate for the first time that CaSR is expressed in both human and ovine AFMSCs and that calcimimetic R-568, possibly through CaSR activation, can significantly improve the osteogenic process. Hence, our study may provide useful information on the mechanisms regulating osteogenesis in AFMSCs, prompting the use of calcimimetics in bone regenerative medicine.

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## Bone marrow mesenchymal stromal cells drive protective M2 microglia polarization after brain trauma

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Microglia/macrophages (M/M) are major contributors to post-injury inflammation but they may also promote brain repair in response to specific environmental signals that drive classic (M1) or alternative (M2) polarization.

We investigated the activation and functional changes of M/M in TBI mice receiving human bone marrow mesenchymal stromal cells (MSCs) or saline infusion. MSCs up-regulated *Ym1* and *Arginase-1* mRNA ( $p < 0.001$ ), two M2 markers of protective M/M polarization, at 3 and 7d post-injury and increased the number of Ym1+ cells at 7d post-injury ( $p < 0.05$ ). MSCs reduced the presence of the lysosomal activity marker CD68 on the membrane surface of CD11b positive M/M ( $p < 0.05$ ), indicating a reduced phagocytosis. MSC-mediated induction of the M2 phenotype in M/M was associated with recovery of neurological functions 7d post-injury ( $p < 0.01$ ) and reparative changes of the lesioned microenvironment.

*In vitro*, MSCs directly counteracted the pro-inflammatory response of primary murine microglia stimulated by TNF $\alpha$ +IL17 or by TNF $\alpha$ +INF $\gamma$  and induced M2 pro-regenerative traits, as indicated by the down-regulation of *iNOS* and up-regulation of *Ym1* and *CD206* ( $p < 0.01$ ) mRNA.

In conclusion we found evidence that MSCs can drive the M/M transcriptional environment and induce the acquisition of an early, persistent M2 beneficial phenotype both *in vivo* and *in vitro*. Increased *Ym1* expression together with reduced *in vivo* phagocytosis indicates M/M selection by MSCs towards the M2a subpopulation, which is involved in growth stimulation and tissue repair.

## **Exosomes mediate a paracrine interplay between Chronic Myelogenous Leukaemia and stromal cells: a role for interleukin 8**

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Chronic myeloid leukaemia (CML) is a clonal hematopoietic stem cell disorder in which leukemic cells display a reciprocal t(9:22) translocation that results in the formation of the chimeric Bcr-Abl oncoprotein with a constitutive tyrosine kinase activity. As a consequence, Bcr-Abl causes increased proliferation, inhibition of apoptosis and altered adhesion of leukemic blasts to bone marrow (BM) microenvironment. Exosomes are small vesicles of 40-100 nm diameters that are initially formed within the endosomal compartment and are secreted when a multivesicular body (MVB) fuses with the plasma membrane. These vesicles are released by many cell types including cancer cells and are considered messengers in intercellular communication. Recently, a considerable interest in the cancer field has focused on tumour microenvironment and how the interaction between malignant cells and normal host cells affects cancer progression. Several data indicate that during cancer progression, tumour stroma has not only a supportive role, but is also a leading player in the modulation of carcinoma development. A stimulating aspect in the field of CML biology is the role of the microenvironment in regulating the growth, survival and drug-resistance of leukemic cells. Bone marrow (BM) stromal cells are the source of signals such as cytokines, growth factors and ECM molecules that can modulate leukaemia cell growth and response to drug. Exosomes released by cancer cells constitute an important part of the tumour microenvironment as they can transfer various messages to target cells thus affecting disease, pointing out the role of vesicles as new actors in the crosstalk between cancer and normal cells in the tumour microenvironment. Data concerning the effect of exosomes in CML-stromal cell interactions are up to now missing. Our proposal is to better investigate if the release of exosomes from CML cells can modulate the tumour microenvironment (the secretion of both soluble and insoluble molecules) through a paracrine interplay among leukaemia cells and stromal cells. Our results showed that treatment of stromal cells, HS5, with CML-derived Exo induced a significant increase of IL8 as well as LAMA84 cell adhesion to stromal monolayer. Addition of recombinant IL8 to LAMA84 cells activated survival pathways and increased the adhesion of leukemic cells to stroma. The inhibition of IL8 receptors, CXCR1 and CXCR2, on LAMA84 cells, reverted the previously described effects. In conclusion, we show that leukemic and stromal cells establish a bi-directional crosstalk: CML-derived exosomes induce the production of the IL8 in stromal cells thus sustaining the growth and survival of CML cells in a paracrine way.

## Human amniotic stem cells transcriptome is affected after xenotransplantation in a sheep achilles tendon defect model

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Amniotic derived cells are ideal seed cells for regenerative medicine protocols since they conjugate a remarkable plasticity to safety properties. This study investigated the role exerted by human amniotic cells during the process of tendon healing. In particular, in the present research it has been evaluated, by microarray technique, the presence of transcriptome variations in human amniotic epithelial (hAECs) and mesenchymal (hAMCs) stem cells when xenotransplanted into the preclinical ovine model of tendon defect, compared to freshly isolated ones. This analysis allowed to understand in which way, after 28 days transplantation, hAECs and hAMCs transcripts have been affected. The gene expression profile was performed by employing a Human Whole Genome OneArray™ Microarray (30,968 total probe). Hierarchical clustering analysis was performed on the array data by using Cluster 3.0 and treeview software. Data were also analyzed with the Ingenuity Pathways Analysis (IPA) in order to achieve a classification of the results on the basis of their biological functions and disclose functional networks and/or pathways.

Clustering analysis of gene expression profiles allowed to detect 2 clusters containing differentially expressed genes in xenotransplanted hAECs and hAMCs respect to freshly isolated ones. In these cells 384 and 61 human transcripts resulted respectively down- and up-expressed, after xenotransplantation. IPA functional analysis of the affected transcripts revealed that the main biological functions associated with these are: Cell Death and Survival (as GFRA1, HNRNPUL1, HTRA1, CTCF, COX8A, ADIPOR1, SUN2), Cellular Growth and Proliferation (as C14orf169, MLXIPL, CREB3L3, CNOT6L, DUSP22, DAO, RNF139, MALAT1, LGALS3, KLF6, PRDM2), Inflammatory Response (as CCRL2, MREG, PDE5A), Cellular Function and Maintenance (as STMN2, VLDLR, S100A4, BAG3, RAB9A), Skeletal and Muscular System Development and Function (as NR2F2, CD81, HSPA1A/HSPA1B, NDUFA7, KERA, MITF, AQP1, LTK) and Connective Tissue Development and Function (as COL11A1, FGF4, HOXD12).

These results show that hAECs and hAMCs after xenotransplantation produce a modulation of the genes involved in their survival, and in the inflammatory response. Intriguingly, it has been observed also the up-expression of the transcripts related to the connective tissue development and function as COL11A1 which is involved in the synthesis of collagen type I, the most expressed protein in the tendon. In conclusion, the present study demonstrates that human amniotic derived cells have a direct involvement in new tendon matrix remodeling and tissue regeneration. Altogether these results strongly support the idea that human amniotic cells could be effectively proposed as a prompt stem cell-based therapy that does not require any preliminary in vitro differentiation or any genomic transfection.

## **Indirect co-culture with foetal tendons or tenocytes can program amniotic epithelial cells towards tenogenic differentiation**

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Amniotic epithelial cells (AECs) have potential applications in cell-based therapy. Thus far their ability to differentiate into tenocytes has not been investigated although a cell source providing a large supply of tenocytes remains a priority target of regenerative medicine to respond to the poor self-repair capability of adult tendons. Starting from this premise, the present research has been designed to verify whether the co-culture with ovine foetal derived tenocytes/tendons can induce AECs tenogenic differentiation. Since the co-culture system inducing cell differentiation takes advantage of specific soluble paracrine factors released by tenocytes, the research has been then addressed to study whether the co-culture could be improved by making use of the different cell populations present within the high regenerative properties of foetal tendon explants or foetal derived cells. To this aim, freshly isolated AECs, obtained from ovine fetuses at mid-gestation, were co-incubated with explanted tendons or primary tenocytes obtained from foetal calcaneal tendons. The morphological and functional analysis indicated that AECs possessed tenogenic differentiation potential. In particular, AECs exposed to foetal-derived cells/tissues developed in vitro tendon-like three dimensional structures with an expression profile of matrix (COL1 and THSB4) and tendon related genes (TNM, OCN and SCXB) similar to that recorded in native ovine tendons. The tendon-like structures displayed high levels of organization as documented by cell morphology, the newly deposited matrix enriched in COL1 and widespread expression of gap junction proteins (Connexin 32 and 43). In conclusion, the co-culture system improves its efficiency in promoting AECs differentiation taking advantage of the inductive tenogenic soluble factors released by foetal tendon cells or explants. The co-cultural system can be proposed as a low cost and easy technique to engineer tendons for biological study and cell therapy approach.

## Tissue dissociation and primary cell isolation using Recombinant collagenase class I and II

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Collagenases class I (CI) and class II (CII) currently available for tissue dissociation are produced by extraction technologies from *Clostridium histolyticum* (human pathogen) strains. They are produced by two separate and distinct genes and are metalloproteases belonging to the family M9. In the processes of extraction of the cells from the tissue, combined activity of both classes of enzymes is required. CI and CII are complementary in degrading collagen. ABIEL recently produced the collagenase class I and II using the recombinant DNA technologies (PCT WO 2011/073925 A9). The enzymes were produced in *E. coli* and purified by affinity chromatography. The method of production adopted allows absolute control of the final composition of these enzymes, as well as their stability, purity, activity, absence of toxicity and higher reproducibility of batches of collagenase (Salamone et al. *Chemical Engineering Transaction* VOL. 27, 2012). The activity of Col G and H has been characterized both *in vitro* and *in vivo*. We tested Collagenase for the extraction of cells from murine and bovine tissues, with the aim to obtain cell populations exploitable in the field of tissue engineering and transplants. The two collagenases produced separately have been used in conjunction according to precise proportions to dissociate pancreas, rib cage, calvaria and liver of the BALB/c mouse; bovine hoof. The cell types obtained, islands of Langerhans, chondrocytes and osteoblasts, hepatocytes were subjected to specific assays of vitality and functionality. These enzymes show no cytotoxicity. The analysis carried out on all isolated cell populations suggests that the cells maintain the structural and functional integrity of specific tissues/organs originating. Recombinant Col G-H enzymes are promising in the context of the dissociation of tissue.

## Pro-angiogenic potential of adipose-derived mesenchymal stem cells (AD-MSCs) in hypoxic condition

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Mesenchymal stem cells (MSCs) are multipotent cells found in bone marrow, adipose tissue and other adult tissues. Transplantation of MSCs is able to improve regeneration of injured tissue but it is still uncertain whether the morphological and functional benefits are determined via MSC differentiation or release of paracrine stimulating factors [1]. Current knowledge suggests that effective neovascularization could be mainly related to paracrine trophic effect of MSCs [2]. However, the fate of transplanted MSCs is determined by microenvironment and, hypoxia, a frequent condition associated to wound repair, has been described as able to modify MSC phenotype [3].

The aim of our study was to investigate the effect of hypoxia on AD-MSC phenotype and in particular we examined the changes in the pattern of released pro-angiogenic growth factors.

Human AD-MSCs were isolated from adipose tissue obtained from obese (35-45 years old) donors during abdominal surgery. All donors gave their informed consent and the local ethics committee approved the study protocol. After isolation, cells were efficiently expanded for several passages showing "stemness" characteristics. In the presence of appropriate stimuli these cells were able to differentiate into cells of osteogenic, adipogenic and chondrogenic lineages. In addition, according to specific culture conditions, we were able to induce MSCs to differentiate also in endothelial lineage. MSCs differentiated in endothelial cells (endo-MSCs), respect to undifferentiated MSCs, formed tubule like-structures in Matrigel already after 24 h of incubation. Moreover, we established that conditioned medium from AD-MSCs and endo-MSCs harvested after hypoxia preconditioning (48h) significantly promoted tubule formation of HUVECs in comparison with conditioned medium from MSCs and endo-MSC cultured under normoxic conditions. In particular, hypoxia determined in AD-MSCs a significant increase of vascular endothelial growth factor (VEGF) production. An increase in VEGF secretion, even though less relevant, was also observed in hypoxic conditioned endo-MSCs.

In conclusion, our results suggest that hypoxia stimulates in AD-MSCs the release of VEGF leading to a paracrine enhancement of neovascularization/angiogenesis. This aspect is worth to be further investigated because its potential application in regenerative medicine.

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## **Mesenchymal Stem Cells potentiate the feasibility of pancreatic islets transplantation through a double action**

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Transplantation of pancreatic islets is an innovative and promising clinical option to treat patients with type 1 diabetes. This is a minimally invasive therapeutic approach, which allows a good metabolic control and a long-term insulin independence. The therapeutic feasibility of pancreatic islets transplantation is however limited by the poor yield of pancreatic islet explants and even more the immune graft rejection, which have as a consequence the very limited lifespan of transplanted tissue. To avoid these side effects besides the treatment with immunosuppressive drugs, promising results have been obtained in vivo with the use of Mesenchymal Stem cells (MSCs), already known in literature to be able to support the survival of many cell types.

In this in vitro study we shed light on the concealed molecular mechanisms of MSC positive action, by analyzing the effect of both direct and indirect co-cultures of rat MSCs with pancreatic islets. The survival, the insulin expression, and the ability to correctly adjust insulin release of rat pancreatic islets in co-culture with MSCs were evaluated for 4 weeks.

Our results demonstrated that MSCs can improve the therapeutic feasibility of pancreatic islets in a double manner: i) the direct co-culture drives a differentiation of MSCs into a “insulin-releasing” phenotype, thus cooperating with pancreatic islets; ii) the indirect co-culture prolongs the survival of pancreatic islets, probably by the release of some soluble factors. Both the mechanisms can be exploited to improve pancreatic islets therapeutic feasibility.

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## Human mesenchymal stromal cells can uptake and release ciprofloxacin acquiring “*in vitro*” anti bacterial activity

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Traditional antibiotic therapy of deep infection (eg: osteomyelitis) by a single drug or a drug combination sometimes is ineffective in producing complete sterilization of infected sites. To face this problem, we investigated the possibility to use Bone Marrow- Mesenchymal Stem Cells (BM-MSC) as a delivery system for the treatment of fastidious infections. In particular we investigated if BM-MSCs are able to uptake and release ciprofloxacin (CPX) that is a fluoroquinolone considered the drug of choice for the treatment of chronic osteomyelitis due to its favorable penetration into poorly vascularized sites of infection. So, human bone marrow stromal cells (BM-MSCs), were primed with CPX (BM-MSCsCPX) according to a methodology previously standardized in our laboratory for paclitaxel (PTX). The antimicrobial activity of CPX released from BM-MSCs cells (BM-MSCsCPX-CM), or supernatant from cells lysate (BM-MSCsCPX-LYS) was evaluated by agar dilution and microdilution methods on three bacterial strains (*Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*). To investigate if primed cells (BM-MSCsCPX) were able to directly act on the bacterial growth, co-culture was performed by mixing *E. coli* suspension to an increasing number of BM-MSCsCPX. The antibacterial activity was determined as number of BM-MSCsCPX that completely inhibited bacterial growth. The results demonstrated that BM-MSCs are able to uptake and then release CPX in the conditioned medium. The loaded antibiotic maintains its active form throughout the process as tested on bacteria. There are evidences that infused MSCs have higher engraftment capacity within sites of inflammation or injury and it is demonstrated that CPX does not alter the function of hMSCs also in association with other drugs. Significant data on active arrest of MSCs within inflamed tissues and that local application is yet practicable for bone pathological condition. Our finding suggest that CPX loaded MSCs may represent an important device for carrying and delivering CPX (and perhaps other antibiotics) into infected deep microenvironments; they could be used for local application and also by systemic infusion when their homing capacity into the bone will be cleared.

## **Characterisation of the skeletal stem and progenitor populations using high resolution quantitative mass spectrometry, and characterisation of the Stro-1 epitope**

**Nunzia Sposito**, Dr Spiro Garbis, Professor Paul Townsend and Professor Richard O. C. Oreffo  
University of Southampton

Skeletal stem cells (SSCs) are a subset of cells from bone marrow (BM) with significant therapeutic potential in the field of regenerative medicine. SSCs have been utilised in bone tissue engineering strategies for almost 30 years and have been shown to exhibit exquisite multipotentiality to form fat, cartilage and bone tissue. However, the clinical potential of SSCs is hampered by the cell population that currently exist in the absence of specific isolation techniques. The use of specific cell surface markers to isolate a homogeneous SSCs population for clinical applications remains a significant unmet challenge.

In this study, the methodology employed seeks to identify cell surface markers or groups thereof which are selective for a homogeneous bone stem cell population. To address the issue a Multidimensional Protein Identification Technology (MudPIT) proteomic approach has been employed.

Bone marrow stromal, plastic adherent skeletal stem cells isolated from a female patient aged 85 were used in this study. Cells were sorted for the Stro-1 marker, and cultured in osteogenic and basal media. Changes in proteins expression in the Stro-1 sorted fraction and in the unsorted cell fraction under basal and osteogenic conditions were investigated. 3447 proteins were identified and quantified by nano-reverse phase-electrospray ionization-liquid chromatography-mass spectrometry/mass spectrometry (RP-ESI-LC-MS/MS) using isobaric tags (iTRAQ). The most upregulated protein was found to be biglycan by 2.9 fold differences and the most downregulated was found to be peptidase inhibitor 16 by 3.8 fold differences.

Overall the data indicate that the majority of the upregulated proteins were found in the Stro-1 sorted osteogenic group when compared to the Stro-1 sorted fraction in basal media. These observations suggest that both sorting and osteogenic induction play an important role in regulating protein expression.

Alongside to the mass spectrometry experiments, investigation aimed to characterise the Stro-1 antigen was undertaken. Stro-1 is an established SSCs markers, used worldwide as a potent isolation tool for the BM non-hematopoietic fraction of cells that is capable of osteogenic differentiation. Stro-1 antigen characterisation experiments using western blotting, immunoprecipitation and protein fractionation technique showed that the Stro-1 antibody reacts with an epitope which appeared to be of 50 kDa. In this study HK and MG63 cells were used as a positive control whilst Raji and 721.221 cells were used as a negative control. Furthermore it was observed that the Stro-1 antigen can be enriched by cell compartments fractionation, where a band appeared in the membrane fraction at 50 kDa. Immunoprecipitation was successfully performed using a resin binding the purified Stro-1 antibody; a band at 50 kDa was observed for the immunoprecipitated antigen.

## Skin substitutes based on keratinocytes and human adipose-derived stem cell feeder layers for clinical use

**Marta Cecilia Tosca**<sup>1,2</sup>, Theodora Chlapanidas<sup>2</sup>, Marta Galuzzi<sup>1,2</sup>, Barbara Antonioli<sup>1</sup>, Sara Perteghella<sup>2</sup>, Melissa Mantelli<sup>3</sup>, Daniela Maria Ingo<sup>4</sup>, Mario Marazzi<sup>1</sup> and Maria Luisa Torre<sup>2</sup>

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The most common methods for keratinocyte (KC) culture are based on the use of a cell substrate, called feeder layer, consisting in a culture of mouse embryo immortalized fibroblasts (3T3 cells). Although 3T3 cells, and also other immortalized animal cell lines, are commonly used for skin substitutes, doubts about their safety are emerging. In this work the use of irradiated adipose-derived mesenchymal stromal cells (ADSCs) has been proposed as human feeder layer replacing murine cells, according to current regulation.

Cutaneous and adipose tissues were obtained from informed donors during abdominoplasty surgery. Each adipose tissue was digested with collagenase and the recovered stromal vascular fraction was seeded in T75 flasks. At passage 3 (P3) ADSCs were splitted into three groups: two groups were X-rays irradiated at two different doses, 60 or 120 Gy; the third one was not irradiated. Cells were then seeded in 6-well plates, and cell growth kinetics and percentage of vitality were monitored until 38 days. Skin biopsies were treated with Dispase II for dermis-epidermis separation and epidermis layer was incubated with trypsin and EDTA. Keratinocytes were seeded on murine feeder cells (3T3-J2) irradiated at 60 Gy. Keratinocytes were tested at P3. ADSCs irradiated at 120 Gy at 2 different densities (1,250 and 625 cell/cm<sup>2</sup>) were used as feeder layer. To evaluate the persistence of feeder cell layer during the time, 3T3 and ADSCs in co-culture with KC were phenotypically characterized by flow cytometry at day 3, 7 and 14, using Pe anti-rat CD49e and FITC anti-human CD105.

Results indicated that X-ray irradiation deeply altered the growth ability and reduced cell vitality ( $p < 0.001$ ) of ADSCs; only irradiated ADSCs at 120 Gy presented a behavior similar to that of irradiated 3T3. The seeding density (650 or 1,250 cells/cm<sup>2</sup>) did not influence either the number of total living cells ( $p > 0.05$ ), or vitality ( $p > 0.05$ ) of ADSCs. Flow cytometric analysis at day 3 of the co-culture showed a high percentage of 3T3 and very low percentage of ADSCs. At the end of the culture, the percentage of both feeder layers was very low. Keratinocytes in co-culture with ADSCs, reach confluence in 5.5 days and, the number of live cells increases to form multilayered cell structure. This suggests that keratinocytes keep the ability to give stratified epidermis.

In conclusion, ADSCs represent a viable alternative to murine fibroblasts for skin substitutes production. The use of human cells as feeder layers can prevent animal contaminations. Moreover, autologous ADSCs may be applied in clinical practice, reducing the risk of cells rejection.

## Leiomyogenic potential of vessel derived porcine mesenchymal stromal cells

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Blood vessels were recognized as a reservoir of mesenchymal stromal cells (MSCs). In particular, pericytes were isolated from micro- and macro- vasculature and can differentiate toward adipo-, osteo- and chondrocytes phenotype (Crisan M et al., 2008, *Cell Stem Cell*). Recently, we isolated and characterized a population of precursor cells from porcine aorta media layer that share similar features with pericytes. As media layer is composed of several layers of smooth muscle cells, we investigated if the isolated cells were able to differentiate toward the leiomyogenic phenotype.

Vascular precursor cells were isolated through enzymatic digestion with Collagenase I, characterized for the positivity to some pericytes/MSCs markers, the negativity to some endothelial and smooth muscle cells markers and tested for the canonical tri-lineage differentiation potential (Zaniboni et al., 2013, *Proceeding of IV SCR Italy Society Meeting*). Cells were grown at confluence in Pericytes Growth Medium (PGM – Promocell) and cultured for 21 days in high glucose – DMEM + 10% Foetal Bovine Serum (DMEM + 10% FBS) in order to assess if cells spontaneously differentiate to smooth muscle cells, as showed by Tang and colleagues (2012, *Nature Communication*). Control cells were cultured for 21 days with PGM.

After the long term culture, cells were analysed for the expression of three smooth muscle cells genes ( $\alpha$  smooth muscle actin –  $\alpha$ SMA, smooth muscle myosin heavy chain – MYH11 and smooth muscle calponin1 – CNN1) with qPCR and immunocytochemistry.

Statistical differences were observed between cells cultured with PGM and DMEM + 10% FBS in term of gene expression; in particular,  $\alpha$ SMA, MYH11 and CNN1 expression was respectively about 3.5, 10 and 5 times higher if compared to control.

Immunofluorescence protein detection data showed that  $\alpha$ SMA was expressed in control cells too, and that MYH11 was strictly expressed in cells cultured with DMEM + 10% FBS.

In conclusion, in this work we demonstrate that precursor cells derived from aorta tunica media, spontaneously differentiate toward the smooth muscle cells phenotype.

## **Aquaporin 1 and CXCR4 expression in human and sheep mesenchymal stem cells**

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Mesenchymal stem cells (MSCs) are adult stem cells with a self-renewal and multipotent capability and are expressed extensively in multiple tissues. Their possible use in the treatment of many degenerative diseases such as Alzheimer, multiple sclerosis, diabetes, etc., has increased the research for their biochemical characterization. Further, recent evidences show that bone marrow derived mesenchymal stem cells (BM-MSCs) are recruited into the stroma of developing tumors where they contribute to progression by enhancing tumor growth and metastasis, or by inducing anticancer-drug resistance. In particular, BM-MSCs are able to selectively migrate and home to sites of tissue damage as well as to tumors.

Therefore, the aim of this work was to determine whether chemokine receptor type 4 (CXCR4) and water channel molecule, aquaporin 1 (AQP1), known be involved in cell migration, could be expressed in human and sheep BM-MSCs to mediate their behavior in the target tissue microenvironment.

We used human and sheep mesenchymal stem cell isolated from bone marrow aspirates by gradient centrifugation and seeded in standard cell culture conditions. Both human and sheep BM-MSCs were tested for CXCR4 and AQP1 expression by western blot and FACS analysis. Furthermore, human hepatocarcinoma (SNU-398) and osteosarcoma (U2OS) cells were used to test the cross-talk between BM-MSCs and tumor cells. BM-MSCs and tumor cells were grown for 48 h in medium without fetal bovine serum to obtain conditioned medium (CM) that was collected, filtered and stored at -20 °C. BM-MSCs and tumor cells were cultured in complete medium and allowed to attach for 24 h. BM-MSCs were exposed to CM obtained by tumor cells whereas SNU-398 and U2OS were exposed to CM obtained by BM-MSCs for 72 h. Morphological changes of cells exposed to CM were observed by light microscope. Then, whole protein extracts were prepared and analyzed for CXCR4 and AQP1 expression.

Our results showed that CXCR4 and AQP1 are overexpressed in human and sheep mesenchymal stem cells as well as in human hepatocarcinoma (SNU-398) and osteosarcoma (U2OS) cells. BM-MSCs grown in presence of conditioned medium obtained by tumor cells showed an increase of CXCR4 and AQP1 expression and morphological changes of cells were observed.

In conclusion these findings suggest that both mesenchymal stem cells and tumor cells express high level of CXCR4 and AQP1 that play a key role in cell migration. Furthermore, cross-talk between mesenchymal stem cells and tumor cells causes an enhancement of CXCR4 and AQP1 expression in mesenchymal stem cells thus increasing their capacity to migrate.

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Roma, 14 giugno 2013 Il Presidente della ONLUS-AICC  
Carlo Leonetti

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