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table of contents

PROCEEDINGS OF THE XXXIV NATIONAL MEETING OF THE ITALIAN SOCIETY FOR THE STUDY OF CONNECTIVE TISSUES (SISC)

Invited Lectures 1

Presentations...3

Index of authors...13

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INVITED LECTURES

EPITHELIAL STEM CELL IN CELL AND GENE THERAPY

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Adult stem cells are cells with a high capacity for self-renewal that can produce terminally differentiated progeny. Stem cells generate an intermediate population of committed progenitors, often referred to as transit amplifying (TA) cells, that terminally differentiate after a limited number of cell divisions. Human keratinocyte stem cells are clonogenic and are known as holoclones. Human corneal stem cells are segregated in the limbus while limbal-derived TA cells form the corneal epithelium[1]. Self-renewal, proliferation and differentiation of limbal stem cells are regulated by the ΔNp63 (α, β and γ), C/EBP and Bmi1 transcription factors[2-4]. Cultivated limbal stem cells generate sheets of corneal epithelium suitable for clinical application[5]. We report long-term clinical results obtained in an homogeneous group of 154 patients presenting with corneal opacification and visual loss due to chemical and thermal burn-dependent limbal stem cell deficiency. The corneal epithelium and the visual acuity of these patients have been restored by grafts of autologous cultured limbal keratinocytes[6-7]. In post hoc analyses, success was associated with the percentage of p63-bright holoclone-forming stem cells in culture[6-7]. Graft failure was also associated with the type of initial ocular damage and postoperative complications. Mutations in genes encoding the basement membrane component laminin5 (LAM5) cause junctional epidermolysis bullosa (JEB), a devastating and often fatal skin adhesion disorder. Epidermal stem cells transduced with a retroviral vector expressing the 3 cDNA can generate genetically corrected cultured epidermal grafts able to permanently restore the skin of patients affected by LAM5- 3-deficient JEB[8-9]. The implication of these results for the gene therapy of different genetic skin diseases will be discussed.

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MECHANISMS AND MEANING OF ECTOPIC VASCULAR CALCIFICATION IN DIABETES

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Diabetes mellitus is typically associated with vascular calcifications, but how this contributes to cardiovascular disease (CVD) is unclear. Medial calcification raises arterial stiffness, increases cardiac overload, and compromises vasodilatory responses. On the other hand, the clinical significance of intimal calcifications is debated. While large calcified area may stabilize atherosclerotic plaques, spotty sub-endothelial microcalcifications increase plaque instability and promote rupture and thrombosis.[1]The mechanisms underlying vascular calcification are manifold, including humoral and cellular processes. Several cell types are involved in the promotion, initiation and propagation of calcification in the arterial wall, including smooth muscle cells (SMC), pericytes, fibroblasts, mesenchymal cells, and endothelial cells.[3] In addition, several types of circulating cells, including mesenchymal stem cells, osteoblasts, endothelial progenitor cells and monocytes have been shown to be involved in ectopic vascular calcification.[2] We have identified a subpopulation of monocytes expressing osteocalcin and bone alkaline phosphatase that are able to calcify *in vitro* and *in vivo*. These so-called myeloid calcifying cells (MCCs) are increased in the blood, bone marrow, and atherosclerotic plaques of diabetic patients.[4] In animal models, MCCs promote atherosclerotic calcification by a paracrine stimulation of SMC.[5] Furthermore, MCCs are provided with anti-angiogenic activities, which potentially worsen CVD in diabetes.[6] The physiological integration of calcification and angiostasis by the same cell type points to a possible role of MCCs in the resolution of inflammation in response to persistent stimuli, such as foreign bodies, chronic infections, and cancer.

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THREE-DIMENSIONAL MODELS FOR THE STUDY OF NORMAL AND DISEASED SKIN

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In vitro skin models are based on a three-dimensional (3D) scaffold colonized by dermal cells (fibroblasts) covered by epidermal cells (keratinocytes) to form the 2 outer layers of the skin, the dermis and the epidermis. The great advantage over the 2-D *in vitro* culture is the ability of skin equivalents to recapitulate the three-dimensional stratified space where skin cells normally live. We have been using skin equivalents for many years mostly for the study of epidermal homeostasis and its alterations, such as psoriasis. In addition, we succeeded in generating skin equivalents starting from different keratinocyte subpopulations, including stem cells and transit amplifying (TA) cells. This has allowed to gain insight into the different properties of cell subpopulations and to better understand epidermal differentiation. Furthermore, organ culture models from skin biopsies, treated with tape stripping or detergents, are used to study skin barrier abnormalities typical of several inflammatory dermatoses. By modulating the cellular components (*e.g.* fibroblasts) we were able to create a skin equivalent displaying a psoriatic phenotype. Moreover, by inoculating squamous cell carcinoma or melanoma cell lines into the skin equivalents we have been able to reproduce the tumor in 3-D. Recently, we have started the study of melanoma development and progression by using cellular spheroids. Tumor spheroids have been developed with the purpose to better mimic the 3D architecture and heterogeneity of melanoma and to recreate the oxygen/nutrients gradient observed *in vivo*. In addition, spheroids display features of drug and radiation resistance that are closely correlated to those detected in patients, thus representing an excellent model to test drug efficacy *in vitro*. The use of spheroids has recently allowed to distinguish different melanoma cell populations with diverse aggressive behaviour and to detect genes involved in melanoma progression. We are currently testing the efficacy of traditional and novel drugs using both skin equivalents and spheroids.

ADULT MESENCHYMAL PROGENITORS AND THEIR POTENTIALS BETWEEN CONNECTIVE TISSUE REGENERATION AND CONNECTIVE NEOPLASMS REGRESSION

M. Dominici, G. Grisendi, C. Spano, V. Rasini, E. Veronesi, A. Murgia, O. Candini, S. Piccinno, M. Pignatti, P. Loschi, G. De Santis, A. Giorgini, F. Catani, P. Paolucci

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Our group has been involved in understanding the regenerative potential of several adult progenitors, such as the multipotent mesenchymal stromal/stem cells (MSC). In this paper, several findings will be presented with a specific focus on connective tissue regeneration. Issues such as animal models, *ex-vivo* cells amplifications, delivery routes, engraftment assessment and interaction with biomaterials will be shared with the purpose to dissect all the essential steps needed for clinical translation. Next to these intrinsic regenerative potentials, MSC can also secrete molecules capable to stimulate or inhibit other cell types. Based on these findings, MSC role has been also widen in oncology by the possibility to genetically modify them empowering their secretory potential against tumors. Based on this background, we here share data on the use MSC as vehicle to deliver potent anti-cancer agents in the context of connective tumors, such as sarcomas. Armed MSC targeted a variety of tumor cell lines *in vitro* and, when injected into a tumor NOD/SCID model, localized into tumors and mediated apoptosis without significant toxicities to normal tissues. While several aspects of these researches shall require further investigations, these pre-clinical data collectively indicate how MSC represent valid tools in cellular therapies targeting connective tissues.

This work was made possible by grants from Ministero Università (PRIN 2008) Ministero Salute, ASEOP, EU-FP7 Project, Fondazione CRM Modena, Fondazione G. Berlucchi and AIRC.

PRESENTATIONS

INHIBITION OF SMALL HA FRAGMENT ACTIVITY AND STIMULATION OF A2A RECEPTOR PATHWAY REDUCE CARTILAGE DAMAGE AND LIMIT APOPTOSIS ACTIVATION IN EXPERIMENTAL ARTHRITIS

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Recent findings showed that the block of small hyaluronan (HA) fragments, produced during inflammatory conditions, by native HA, reduced the inflammatory response in several model of experimental arthritis. At the same time the stimulation of A_2A receptors ameliorated inflammatory outcomes by inhibiting activated NF-kB. Synergistic actuation of both treatment resulted in a more significant reduction of the inflammatory response compared with the single treatment. Aim of this study was to investigate the effect of the combined treatment using the HA inhibitor Pep-1 and a selective $A_{2A}R$ agonist (CV-1808) in collagen-induced arthritis (CIA) in mice, on the structure and ultrastructure of the articular cartilage and on the role of apoptosis in untreated/treated animals. Arthritis was induced in mice via intradermal injection of an emulsion containing bovine type II collagen in complete Freund's adjuvant. Mice were treated with Pep-1 plus (and/or) CV-1808 intraperitoneally daily for 20 days. At the end of the experiments (day 35), mice were killed and the hind limbs were processed for light microscopy and for transmission and scanning electron microscopy. A morphometric exam was also performed on the thickness of the articular cartilage. CIA increased IL-6, caspase-3, and caspase-7 mRNA expression and the related proteins in the articular cartilage of arthritic mice. Significantly increased concentrations were also observed for BCL2-associated X protein (BAX), while B-cell-lymphoma-2 protein (BCL2) was markedly reduced in the articular cartilage. The treatment with Pep-1 together with CV-1808, particularly at the higher doses, significantly reduced CIA injury as shown by the presence of Safranin-O positive cartilage, whose surface was smooth with the SEM and which showed normal chondrocytes either in the superficial or in the intermediate/deep zones. The morphometric analysis provided confirmation to the morphological data. The structural and ultrastructural exam of the articular cartilage further supported the role of HA degradation and A_2A receptors during arthritis.

QUANTITATIVE AND STRUCTURAL ANALYSES OF URINARY TRYPSIN INHIBITOR IN TYPE 1 AND TYPE 2 DIABETES

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Urinary trypsin inhibitor (UTI) is a small proteoglycan consisting of a 147 amino acids long polypeptide that carries an N-linked oligosaccharide at Asn^{45} and a O-linked low-charge chondroitin sulfate (CS) chain at Ser^{10}. UTI levels are usually low in healthy individuals but they increase up to 10 fold in both acute and chronic inflammatory diseases. Recently, we set up a method for UTI purification and quantitation useful for its structural characterization[1]. This report describes the structural characterization of both protein and chondroitin sulfate moieties of UTI in type 1 and type 2 diabetic patients. UTI purification was performed by anion exchange chromatography starting from a volume of urine corresponding to 2 mg of creatinine. The eluate was splitted in two aliquots for structural and quantitative analyses. The first aliquot was subjected to chondroitin lyase ABC treatment and the obtained disaccharide units were analysed by Fluorophore-Assisted Carbohydrate Electrophoresis after derivatization with 2-aminoacridone. The second one was resolved by SDS-PAGE. A calibration curve for protein quantitation was set up by using a highly purified UTI fraction. Furthermore, UTI band was subjected to trypsin digestion and structural characterization by Nano-LC-MS/MS analysis. The method was applied to urine samples from 29 patients with type 1 diabetes, 22 patients with type 2 diabetes and 42 healthy controls, matched for age and sex with patients, evidencing higher UTI levels in both groups of patients (2.5 and 1.8 fold increase, respectively) in respect to controls ($p<0.0001$ and $p<0.05$, respectively). UTI structural analysis evidenced the presence of several sites prone to oxidative modifications. Interestingly, one of them (residues 107-121) may represent a potential marker of oxidation in both type 1 and type 2 diabetic patients. Preliminary results suggest that both UTI levels and structure could be modified representing a useful marker of chronic inflammatory condition in type 1 and 2 diabetes. The effects of such quantitative and structural alterations on UTI localization, function and pathophysiological activities deserve further studies.

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GLYCOSAMINOGLYCANS MODULATION IN ENDOTHELIAL CELLS DURING INFLAMMATION

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During inflammation, the extracellular matrix (ECM) undergoes several modifications in order to adapt the tissue responses to the injury, developing several diseases, *e.g.* vascular pathology, atherosclerosis etc. Noteworthy ECM modifications caused by cytokines (*e.g.* TNF-alpha or IL-1) and/or altered molecules such as oxidized LDL are also involved in the recruitment/activation of inflammatory cells. In the arterial vessel wall, endothelial cells regulate the vascular tone, the thrombosis/fibrinolysis balance, and the recruitment of inflammatory cells; most of these abilities are due to ECM forming the glycocalyx that is enriched with: i) hyaluronan (HA) that promote monocyte/macrophage adhesion through interactions with the hyaluronan receptor CD44 present on inflammatory cell surfaces; ii) heparin sulfate proteoglycans (HS/HEPGs) such as syndecans. Human Umbilical Vein Endothelial Cells (HUVEC) were used as a model to study the mechanism that regulates gly-

cosaminoglycans (GAGs) synthesis after treatment with the cytokine TNF-alfa. The HUVECs response to TNF-alpha was modulated within the 48 hours: HS/HE synthetic enzymes EXT1, EXT2, NDST1 increased their expression at 24 hours and EXT1 and EXT2 were back to control levels at 48. In parallel, the proteoglycans core proteins had an increase in syndecans -3 and -4 expression at 24 hour that were changed by syndecans -1 and 4 at 48 hours. Preliminary data regarding disaccharide composition of HS/HE chains demonstrate an higher amount of N-sulfated modification, according to the synthetic enzymes expressions. Moreover, also HA synthetic enzymes HAS2 and -3 are upregulated by TNF-alpha but the GAG is not increased in the medium. Even though the higher number of monocytes adherent to the inflammatory ECM, was mainly due to the HA accumulation, the HAS involved and the HA localization needs to be better investigated.The treated cells in particular have an high expression of HAS3 that can synthesize a pericellular coat rich in HA. Finally, also the HS/HE GASs have role in the monocytes adhesion since the use of hyaluronidase do not complete abrogate it.

PROTEOMIC SIGNATURE OF HUMAN PRIMARY METASTATIC COLON CANCER AND ITS METASTASES TO LIVER

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Colorectal cancer (CRC) is among the three leading causes of cancer related mortality worldwide. The develop of liver metastasis from colon cancer is considered to be the main cause of death. Although, the molecular changes that occur during CRC tumorigenesis and progression has been elucidated, it is crucial to identify new signatures that are able to predict liver metastasis and patient prognosis. In the present study we perform a comparative proteomic profile of colon cancer tissue paired with adjacent non-tumoral mucosa and with liver metastasis from the same patient, in order to identify putative proteomic signatures for CRC occurrence and metastasis. Using a three-step approach we selected unique and common proteins involved in tumorigenesis (normal *versus* tumoral) and metastasis (tumoral *versus* metastasis). A total of one hundred protein spots were found to be differentially expressed, among which some metabolic enzymes, molecular chaperones and proteolityc enzymes, such as Cathepsin D (CATD). CATD, a lysosomal aspartyl endopeptidase, has been suggested to act at multiple tumor progression steps, both in colon and breast cancer, affecting cell proliferation, angiogenesis and apoptosis. It has also been associated with the invasion and metastasis[1,2] and proposed as molecular biomarker for breast and colon cancer[3,4]. Interestingly, we identified two different isoforms of cathepsin D, differentially expressed between tumor and metastatic tissue. Next, we will explore the possible mechanism of action and the prognostic value of CATD in CRC.

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IDENTIFICATION OF POTENTIAL BIOMARKERS IN MYASTHENIA GRAVIS DISEASE BY PLASMA PROTEOMIC ANALYSIS

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Myasthenia gravis (MG) is a chronic autoimmune neuromuscular disease characterized by varying degrees of weakness of the skeletal muscles, caused by auto-antibodies against some proteins of the neuromuscular junction involved in nerve signal transduction. In particular, about 85% of MG patients have circulating auto-antibodies against the muscle acetylcholine receptor (AChR+), whereas about 6% of MG patients have autoantibodies against the muscle specific kinase (Musk+). In the remaining 9% of MG patients, known as Double-seronegative (DSN), the target of the autoimmune response is not known yet. The aim of this study is to identify differentially expressed plasma proteins in MG patients with different autoimmune responses with respect to a control group by applying two-dimensional electrophoresis analysis coupled with mass spectrometry.Briefly, 17 plasma samples from AChR+, 10 from Musk+, 15 from DSN, and 17 from healthy controls were subjected to a low-abundance proteins enrichment step using hexapeptide combinatorial ligand libraries (ProteoMiner™ enrichment kit, Bio-Rad Laboratories) followed by two-dimensional electrophoresis. Protein profiles were compared by using PD-Quest software. Differentially expressed proteins were subjected to tryptic digestion and identified by peptide mass fingerprinting analysis using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS). Preliminary results show a panel of proteins differentially expressed among MG subgroups and healthy controls. Complement C3, Fibrinogen beta chain and Vitronectin, which are well known proteins involved in inflammation, innate immune response and blood coagulation, were identified with high confidence, suggesting the potential of these circulating stress-related proteins as biomarkers useful in Myasthenia gravis diagnosis and treatment. Further studies will be addressed to the identification of the other differentially expressed protein spots and to understand the underlying biochemical mechanisms.

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NOVEL MUTATIONS INCREASE THE SPECTRUM OF ABCC6 GENE ALTERATIONS FOUND IN ITALIAN PATIENTS WITH PSEUDOXANTHOMA ELASTICUM

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Pseudoxanthoma elasticum (PXE) is a rare systemic heritable disorder characterized by progressive mineralization of elastic fibers, mainly affecting skin, eyes and the cardiovascular system.PXE is caused by mutations in the ABCC6 gene composed of 31 exons located on chromosome16p13.1.The most frequent sequence changes are missense and nonsense mutations, as well as small deletions, whereas less frequent alterations are repre-

sented by splicing errors, large deletions and insertions[1]. Along the last years, we have genotyped 254 Italian PXE patients belonging to 208 families spread in all Italian regions. By classical nucleotide sequencing of exons and of flanking intronic regions, we have identified 104 different mutations spread all over the ABCC6 gene with the exception of exon 3, 4, 20 and 31, whereas sequences in exons 24 and exon 12 are the most frequently involved (30.5% and 15.4%, respectively). In addition to mutations already described in the literature and/or present in databases, we have found 15 newmutations: c.1-139C>T (promoter); c.31_32insG (exon 1); c.613G>T (exon 6); c.794+1G>A (intron 7); c.956T>A (exon 8); c.1160G>T (exon 9); dup11-18 (exon 11-18); c.1526C>G (exon 12); c.2307_2308insA (exon 18); c.3398G>A (exon 24); c.3563C>G (exon 25); c.3677 T>C and c.3700G>A (exon 26); c.4159-171dup13bp (exon 29); c.4403G>A (exon 30). These novel variants contribute to increase the total number of PXE mutations (>300 already described). The severity of clinical manifestations is positively correlated with age, in agreement with the progression of mineralization, without differences between patients carrying nonsense or missense mutations. The PXE clinical phenotype is in fact heterogeneous also in patients with identical mutations and, even within the same family,there are differences regarding the severity of the disease as well as the number of organs involved. Preliminary data on a small number of PXE patients indicate the presence of polymorphism in genes (as ENPP1, GGCX, VKOR, MGP) considered as possible modifiers, since they may interfere with the pathologic phenotype acting in synergy with ABCC6 mutations. Further studies are required to demonstrate their role in the occurrence and severity of clinical manifestations in order to improve prognostic perspectives in PXE patients.

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NEW OBSERVATIONS ON THE MINERALIZED TURKEY TENDON

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The leg tendons of the turkey and of some other birds undergo a physiological gradual calcification which makes them a classic model for the study of biomineralization processes. Less attention, however, has been paid to the structural base of the functional modifications brought about by the calcification, which – among other effects - causes a twofold increase of the tensile strength and a tenfold increase of the stiffness[1]. In the present study calcifying turkey tendons were investigated by conventional light microscopy (LM) and scanning electron microscopy (SEM). In comparison with most other tendons, turkey tendons appear to be more finely subdivided in slender fascicles; they contain a greater amount of endotenon associated with a rich cell population, as well as occasional nodules of cartilage-like matrix. Crimps with the usual morphology[2] are easily observed by LM, under both bright field and polarized light; however, they completely disappear in the calcified portions, where fascicles and fibrils run perfectly straight and parallel. High-magnification electron microscopy reveals the distinctive nicks of the pre-existing crimps, which were clearly locked in the straightened-out position by the mineralization process. The inorganic phase itself appears composed of two different types of fine particles. A first, well known form is represented by tightly packed fine needles or small platelets, growing inside or around the collagen fibrils; the infiltrated fibrils swell and merge with the neighboring fibrils, forming a common matrix[3]. Other particles appear as long platelets or ribbons, orthogonal to the fibril axis and regularly arranged in relation with the D-period. This perifibrillar mineral could play a critical role in the mechanical coupling of adjoining fibrils and in the transmission of mechanical load along the tendon, a still controversial aspect of the biomechanics of unmineralized tensile tissues.

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A LIGHT AND SCANNING ELECTRON MICROSCOPY STUDY ABOUT HEAT TREATMENT ON HUMAN CORTICAL BONE

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The bone tissue is a biological, composite material consisting of an organic matrix whose main component is collagen and a mineral phase. The development of bone structural models, particularly for the compact cortical bone, was based on the extraction of the mineral phase with chemical decalcification while the study of undecalcified specimens requires preparation with cutting-grinding or fracturing with chisels. Both these methods imply strong mechanical or chemical manipulation of the bone tissue with the inevitable bias of processing artifacts. The aim of this study is to show a novel approach to the investigation of the lamellar bone morphology, based on heat treatment. Human bone specimens were obtained from below the knee amputation in three young male subjects aged respectively 25, 30 and 32 years old, who underwent severe traumatic limb injuries. The specimens have been stored at 4°C in a 10% formaldehyde solution, then they were cut with a low speed, circular, diamond coated saw (Remet, Casalecchio di Reno, Bologna, Italy). After dehydratation in ascending concentrations of ethanol, 15 specimens were left to dry in air at room temperature for a week (control dehydrated specimens), while other 15 were submitted to heat treatment in a muffle furnace at 500 °C overnight (heat-deproteinized specimens). All cutting surfaces of both dehydrated and heat-deproteinized specimens were first manually ground on abrasive paper (grain 500 and 1000) to 2 mm of thickness under running tap water and then polished on a very fine-grained black slate. At each passage the specimens were repeatedly ultrasonicated to clean the surface from debris. The specimens used for SEM observations were again dehydrated in ascending grade of ethanol and subjected to critical point drying in CO2. They were secured on stubs with conducting tape, coated with a thin layer of gold or carbon in a vacuum sputter Emitech K550 and studied with a Philips XL30 scanning electron microscope either in the SE or BSE mode, coupled with an energy dispersive X-ray analyzer (Edax Genesis, 2000). The human bone samples, first heat treated and then ground, when observed with light microscope, show a morphology consistent with the one seen in SEM specimens and allows you to appreciate the lamellar organization of the osteon to the finer details. The thermal treatment of the specimens (500°C overnight) caused the total loss of the soft tissues and the organic component of the matrix: the samples obtained are brittle, easy to cut and to break. This study suggests the thermal treatment of bone specimens as a

simple, fast and inexpensive method to to obtain morphological information on the lamellar bone organization.

MORPHO-FUNCTIONAL CHANGES OF HCASMC CULTURED IN A PRO-OSTEOGENIC ENVIRONMENT

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Studies on human lesions and in mouse models of arterial calcification, as well as *in vitro* culture systems where calcification is induced by increased mineral availability, indicate that mesenchymal-derived vascular cells actively participate in hydroxyapatite deposition, thus mimicking bone formation and exhibiting several hallmarks of endochondral ossification[1]. However, beside alterations occurring in a number of hereditary conditions, ectopic calcification is more frequent in later life, being also associated to age-dependent disorders as atherosclerosis, diabetes, kidney diseases. It has been already demonstrated that the cardiovascular system undergoes progressive morpho-functional changes with age, as fragmentation of elastic laminae, increased collagen deposition and altered cell-matrix alterations contributing to modify elasticity and resilience of valves and blood vessels. Since the pro-osteogenic phenotype of vascular smooth muscle cells is associated to a shift from a contractive to an osteogenic signature, it could be suggested that changes in the environmental milieu and/or the contractile potential of cells could influence the susceptibility of a tissue to favour/inhibit pathologic mineral deposition. Within this context, we have investigated the *in vitro* behaviour of human coronary artery smooth muscle cells (HCASMC) as an excellent model to study cardiovascular function/disfunction including ectopic calcification. Cells were cultured in two different media: one supplemented with growth factorsspecifically supportingthe proliferative status (SMGS), and the other supplemented with factors inducing differentiation (SMDS). Moreover, in order to promote the *in vitro* deposition of mineral deposits, cells were also cultured in the presence of a calcifying environment (CM)[2,3]comprised of beta-glycerophosphate (a source of organic phosphate); dexamethasone (a glucocorticoid that up-regulates and down-regulates osteogenic or inhibitor molecules, respectively), and ascorbic acid (an essential cofactor for collagen synthesis). After 10, 20 and 30 days of culture, we have evaluated the presence of matrix mineralization and the enzyme activity of alkaline phosphatase (TNAP), a classical marker of calcification. Results demonstrate that HCASMC cultured in SMGS+CM are more elongated, regularly oriented with clear evidence of progressive deposition of mineral precipitates. By contrast, cells cultured in SMDS+CM appear enlarged, with a polyedric shape and negligible hydroxyapatite accumulation. Interestingly, TNAP activity undergoes a progressive increase with time in culture, even though levels are significantly higher in SMGS+CM than in SMDS+CM cultured cells. These data support the theory that differentiated contractile cells are more resistant to hydroxyapatite deposition and that, in the balance between pro- and anti-calcifying factors, a threshold levels of TNAP activity must be reached in order to promote ectopic calcification.

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ECTOPIC CALCIFICATION: FROM PATHOGENIC PATHWAYS TOWARDS THERAPEUTIC PERSPECTIVES

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Soft connective tissue mineralization is not a passive process, but the consequence of an active balance between pro- and anti-calcifying factors. Both genetic and environmental factors can modulate these pathways. Ectopic calcification can be classified into three major categories: metastatic, dystrophic and calcinosis. Moreover, there are several genetic disorders that, although associated to different genes, share phenotypic similarities with acquired forms of metastatic and dystrophic calcification. Pseudoxanthoma elasticum (PXE) is due to ABCC6 gene mutations[1,2]and it is characterized by progressive mineralization of the elastic component of soft connective tissues mainly affecting skin, eyes and the cardiovascular system with a great phenotypic heterogeneity even in the presence of the same mutation[1]. The pathogenic mechanisms of ectopic calcification in PXE are still elusive and no specific therapies are available. It has been suggested that elastic fibre calcification can be mediated or induced by factors that, released from the liver, can influence mesenchymal cells, which are responsible for the correct amount and quality of the extracellular matrix and can further control the mineralization process by producing anti- and pro-calcifying factors. To understand the role of fibroblasts in ectopic calcification, we have isolated these cells from the skin of *Abcc6*$^{-/-}$ mice[2] and from human dermal biopsies. Molecules related to the calcification process have been investigated by PCR and Western blot. Results demonstrate that: i) in fibroblasts, isolated from Abcc6 $^{-/-}$ and Abcc6$^{+/+}$ mice of 0.5 and 12 months age (absence and presence of mineral deposits), there are changes that can be related to the genotype and that are present well before the development of calcification; ii) PXE human fibroblasts, in comparison with control fibroblasts, are more susceptible to pro-calcifying stimuli present in the culture medium and exhibit an altered inorganic pyrophosphate (PPi) metabolism. Moreover, preliminary data indicate that *ex-vivo* or *in-vitro* aged fibroblasts are more efficient in inducing mineral crystal deposition.

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MALDI-IMS: A PROMISING APPROACH FOR INVESTIGATING ECTOPIC CALCIFICATION

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Mineralization of soft connective tissues, also known as ectopic calcification, is a pathologic process that may occur in different contexts. During aging, for instance, it could be the consequence of atherosclerotic lesions or it may be associated to

osteoporosis that, through the calcification paradox, lead to increased levels of circulating ions. Similarly, ipercalcemia-and/or iperphosphatemia-mediated calcification is present in hormonal diseases and in patients suffering from chronic kidney disorders. Furthermore, ectopic calcification takes place also in a group of genetic diseases, as a consequence of defects in different genes, some related to bone metabolism (MGP, ENPP1, GCGX), some apparently unrelated to the calcification process (ABCC6, HB). Even though many key regulators have been found to be abnormally expressed in mineralized areas within soft connective tissues, pathogenic mechanisms are elusive and it is still puzzling whether calcification affects peculiar matrix components in specific organs/tissues, whereas other areas remain unaffected. Classical proteomic techniques do not allow to discriminate proteins on the basis of tissue localization, since extraction procedures usually blend data from affected and unaffected areas. Nowadays many informative data can be obtained by matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS) enabling the combined identification and localization of molecules directly on tissue section in a single experiment. As an experimental model to test the potential of this approach, we have used the skin from a patient affected by Pseudoxanthoma elasticum (PXE), a rare genetic disorder mainly affecting skin, eyes and the cardiovascular system due to progressive calcification[2]. Interestingly, calcification does not involve the whole tissue as in the skin, where mineral deposits specifically accumulate in the middle reticular dermis. IMS was therefore performed on mineralized and non-mineralized areas of the same PXE biopsy, in order to reveal differences in protein distribution and content abolishing inter-individual variability. Data have been also compared to those from healthy skin. Analysis of ion density maps demonstrates a different protein distribution between PXE and healthy skin but also among different regions within the same PXE sample. We have identified a number of proteins that have been never related to the disease, nor to the calcification process.

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CHIMERIC RESILIN-, ELASTIN-, AND COLLAGEN-LIKE ENGINEERED POLYPEPTIDES AS TISSUE ENGINEERING SCAFFOLDS

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Protein-inspired biomaterials have gained great interest as an alternative to synthetic polymers, in particular, for their potential use as biomedical devices. The potential inspiring models are mainly proteins able to confer mechanical properties to tissues and organs, such as elasticity (elastin, resilin, spider silk) and strength (collagen, silk). The proper combination of repetitive sequences, each of them derived from different proteins, represents a useful tool for obtaining biomaterials with tailored mechanical properties and biological functions. Herein, we describe the design, the production, and the preliminary characterization of chimeric polypeptides, based on sequences derived from the highly resilient proteins resilin and elastin and from collagen-like sequences. The results show that the obtained chimeric recombinant material exhibits promising self-assembling properties. Young's modulus of the fibers was determined by AFM image analysis and lies in the range of 0.1-3 MPa in agreement with the expectations for elastin-like and resilin-like materials[1]. In order to improve the mechanical properties the design and production of higher molecular weight polypeptides was carried out. The ideal scaffolding material should have the needed porosity and mechanical strength to allow a good integration with the surrounding tissues, but it should also assure high biocompatibility and full resorbability. Therefore, single-molecule mechanics, aggregation properties and compatibility with human mesenchymal stem cells were tested, showing that the engineered compound is a good candidate as a stem cell scaffold to be used in tissue engineering applications[2].

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ELASTIN-DERIVED PEPTIDES: COMPREHENDING THE ROLE OF HYDROXYPROLINE IN ELASTIN

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Tropoelastin undergoes several post-translational modification before maturation into elastin. The hydroxylation of some proline residues by prolyl-4-hydroxylase to form (2S,4R)-4-hydroxyproline (Hyp) is one of the post-translational modification of elastin. In order to define the role played by Hyp in the function and self-assembly of the mature protein, elastin model peptides containing proline and analogues, Hyp and (2S,4R)-4-methoxyproline (Mop), were analyzed. Mop amino acid was introduced in order to distinguish between stereoelectronic effect (present in Hyp as well as in Mop) and the role played by the H-bonds (present only in Hyp)[1]. Elastin peptides of different length and sequences were analyzed by Circular Dichroism, NMR, and FTIR spectroscopies. The self-assembly of the peptides was studied by Turbidimetry assay, Atomic Force and Transmission Electron Microscopies. At molecular level, the conformational studies show that the presence of proline analogues, Hyp and Mop, instead of proline present in the elastin model peptides, did not significantly change the secondary structures populating the conformational space. As a matter of fact, PPII, unordered conformations and beta turns are still present. On the contrary, at supramolecular level the self-assembling properties instead are very different. The presence of Hyp reduces the tendency to coacervate and alters the morphology of the aggregates.Finally, the susceptibility to protease digestion was evaluated[2]. The biological significance of these findings will be discussed.

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AVAILABLE DECELLULARIZED DISTRIBUTION AND QUANTIFICATION OF RESIDUAL NUCLEIC ACID AND ALPHA-GAL EPITOPE

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Commercially available decellularized xenogeneic scaffolds are

currently employed for the healing of diseased tissues. However their use is permitted even without the assessment of xenogeneic cell material elimination, like the alpha-Gal epitopes. Moreover, the decellularization procedures are not monitored to prove the elimination of the calcific potential associated to the nucleic acids remnants. The current treatment with glutaraldehyde is unable to grant a complete immuno-tolerance of implanted xenogeneic tissues by reducing but not eliminating the immunogenicity particularly for the alpha-Gal epitopes (the major hindrance for the success of xenotransplantation). Recent studies have extensively reported investigations focused on the biocompatibility evaluation of bioprosthetic devices based on xenogeneic tissues. Here we are reporting the results of our investigation concerning alpha-Gal epitopes and nucleic acid detection in novel xenogeneic bioprosthetic preparations that have shown promising preclinical/clinical results in different areas of application. The alpha-Gal quantification was carried out by an ELISA test previously developed and patentedby our research group which involves the use of the monoclonal anti alpha-Gal antibody M86. Immunofluorescence analysis was performed for the visual distribution of both xenogeneic epitopes and nucleic acids residues. Forthe total DNA quantification a commercially available kit was adopted. The amount and distribution of the alpha-Gal epitopes was found to be different between the investigated biomaterials, while the presence of nucleic acid remnants appeared as a common feature, even in those tissues indicated as free from cellular remnants by the manufacturer. Insufficient quantitative evaluations performed at preclinical level about the residual content of xenogeneic epitopes, detergents and nucleic acid materials have led to disappointing and disastrous results. The risk of these dramatic accidents reoccurring remains very high unless safety parameters, among which the complete removal of major xenogeneic determinants (alpha-Gal) and calcification-prone nucleic acid residues,are identified and introduced in the manufacturing practices.

A PROTEOMIC CLUSTER FOR OSTEOTROPIC BREAST CANCER CELLS

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Bone metastasis is the dramatic culmination of the breast cancer progression for many patients. To date the metastatic process is still partially shrouded in molecular "mystery" with regard to several highlights: the signaling pathways, the cancer cell-microenvironment crosstalk and the other mechanisms involved in the cancer cells ability to recognize, implant and proliferate in the metastatic sites. The present work is the prosecution of a project previously reported in this society, having the aim of studying the ultrastructural and molecular properties of breast cancer cells (SKBR-3) with propensity for bone colonization, through an appropriate *in vitro* model. Here we report the proteomic characterization of the osteotropic breast cancer cells, recovered after *in vitro* bone colonization. Viable surgical bone fragments, derived from traumatic lesions of young subjects after informed consent. The bone fragments were washed under sterile conditions and placed into culture capsules with enriched DMEM medium. The explants were kept in the humidified incubator under CO2 at 37°C and then co-cultured with confluent SKBR-3 breast cancer cells for 7 days. The co-cultured fragments were then recovered, washed, placed again in culture dishes and monitored daily. After one week, the outgrowth of colony-forming cells was observed. These cells, named SKBR-3 B1, were collected, immunologically and ultrastructurally assayed, and processed for the proteomic profiling. The 370 identified proteins, were grouped into functional clusters and then compared with the profiles of other selected normal (MCF10A, HB2) and tumoral (8701-BC) cell lines, showing no evidence of osteotropism *in vitro*, in order to identify differentially expressed proteins. Finally we compared the SKBR-3 B1 proteome with the profiles of several breast cancer surgical tissues, preliminarily subdivided into two cohorts (from patients with and without clinical evidence of bone metastases). The *in vitro* and *in vivo* results highlighted the prevalence of three cluster of proteins in the bone-conditioned breast cancer cells with respect to the other cells and tissues studied. These clusters belong to the following functional categories: calcium-binding, cytoskeleton remodeling and cell motility, and response to environmental stresses. We suggest that useful clinical markers for patient with propensity to develop bone metastasis, may be identified.

BIOLOGICAL EFFECTS OF THE AMYLOIDOGENIC ELASTIN-LIKE PEPTIDE (VGGVG)$_3$ INTERACTING WITH HEPARAN SULFATE

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Elastin-glycosaminoglycan interactions have been demonstrated since many years, when it was suggested they may play a role in elastic fiber assembly[1]. More recently, it has been shown that heparan sulfate (HS) can positively interfere with the coacervation temperature of tropoelastin and of elastin-like peptides (ELP)[2]. Interestingly, these peptides adopt different supramolecular organization, from filaments to amyloid-like fibers, depending on the aminoacid sequence[3]. Amyloid fibers have been involved in many age-related pathological conditions, including neurodegenerative disorders, although their pathogenic role is actually becoming question of debate[4]. Therefore, a better understanding of the biological effects of amyloid fibrillar structures and of their toxic potential will be of great importance. To address these issues we have developed an *in vitro* assay using VGGVG containing peptides, *i.e.* (VGGVG)$_3$ forming amyloid fibrils and poly(VGGVG) forming bundles of elongated fibrillar structures[5], in the presence of heparan sulfate of 10.3 kDa and a SO_3^- / COO^- ratio of 1.4. Interactions between HS and ELPs have been evaluated by transmission electron microscopy, assessment of cell adhesion and viability on fibril-coated surfaces and determination of reactive oxygen species (ROS) accumulation. Data demonstrate that HS favor the formation of larger aggregates of fibrils maintaining their original amyloid or elongated structure. In all experimental conditions cells show similar proliferative capabilities. Assessment of ROS accumulation indicates that amyloid fibrils, even in the presence of HS, do not show evidence of altered redox balance, at least at the concentrations tested (10 g/ml of ELP and of HS). Finally, when cells were cultured on fibrillar coated surfaces, adhesion was not significantly affected, being only slightly reduced in the presence of ELP plus HS compared to tissue culture plates, possibly due to charges effects. Data indicate that HS favors ELP assembly, independently from the supramolecu-

lar organization of peptides and that the amyloid fibrillar structures *per se* are not responsible for cytotoxic effects. These findings are in agreement with the large body of data indicating that amyloid-like fibrils can be used as potential nanostructures for their stability, weaker or absent toxicity, ability to efficiently interact with cells and low cost of production[6].

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A PUTATIVE PROTEOMIC SIGNATURE FOR STROMAL FIBROBLAST-LIKE STEM CELLS

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The human limbus is a highly specialized region of the eye that includes several folded niches. Each niche hosts two major classes of limbal stem cells, surrounded by a rich extracellular matrix that provides the favorable microenvironment for the maintenanceof the undifferentiated phenotypes. The two classes of stem cells are: the Epithelial Stem Cells (LESCs) that sustain the corneal renewal, and the Stromal fibroblast-like Stem Cells (f-LSCs) which posses a remarkable cellular plasticity. The f-LSCs are able to differentiate into several cytotypes of mesenchymal origin. Moreover they are scarcely immunogenic, as they do not express HLA-DR molecules. For their peculiar features, the f-LSCs could be employed in the treatment of several diseases[1]. In order to outline a possible limbal stemness signature, we have performed a comparative proteomic analysis between the undifferentiated stem cell line (f-LSCs) and a differentiated human fibroblast cell line. The fibroblastic phenotype, indeed, represents one of the natural end-points of the f-LSCs differentiation process.Both cell cultures were individually processed and submitted to 2D-IPG electrophoresis (IPG strips 18 cm long with pH range 3.0–10). Protein spots were identified by MALDI-TOF mass spectrometry and grouped into functional categories, as previously reported[2-4]. Qualitative and quantitative comparison of the proteomic profiles of the two cell lines showed a very high percentage of similarity between staminal and differentiated fibroblasts. The quantitative comparison showed that about 40% of the identified proteins are differentially expressed in terms of relative abundance (vol%). The majority of these proteins display higher expression levels in the stem cells with respect to the differentiated fibroblasts. Collectively these proteins belong to the following functional clusters: cell motility, cell proliferation and molecular folding. In consideration of the pivotal role played by these classes of proteins in the maintenance of stemness,we suggest that latters may be helpful to draw the limbal stem cell proteomic signature.

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CHARACTERIZATION OF PAR6 INTERACTORS IN 8701-BC BREAST CANCER CELL LINE

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Junctional systems are crucial for epithelial biogenesis and maintenance of polarized phenotype[1]. The acquisition and maintenance of epithelia cell polarity is governed by a set of evolutionarily conserved proteins, PAR-aPKC system[2]. Par6 is a key element of this system, since it acts as a scaffold that binds the CDC42/Rac1 GTPase activity with aPKC[3]. Polarity and junctions are closely interrelated and required for regulation of different biological processes[4]. Loss of polarity is considered a prerequisite for tumor formation and progression, infact the expression of polarity proteins is frequently altered in tumor cells. A growing body of evidence involves polarity proteins in EMT, cell migration and invasion, but the underlying mechanisms are not been investigated. In the present study the expression of Par6 was evaluated both in normal (HB2) and in breast cancer (8701-BC) cell lines by Western Blot assays. In normal cells, Par6 was detected only in membrane fraction whereas, in tumor cells, only in citoplasmatic lysate suggesting that, in tumor cells, Par6 delocalizes from membrane. In order to identify new interactors for Par-6, the total lysate of 8701-BC cells was coimmunoprecipitated with anti-Par6 antibody. Coimmunoprecipitated proteins were separated by 2D-IPG and the gel stained with silver nitrate. The revealed proteins were then identified by MALDI-TOF mass spectrometry. The study of Par6 interactome resulted in the identification of 13 proteins, apparently not correlated with the polarity. These proteins may be grouped into two main clusters: the cluster that connects Par-6 with the cytoskeleton and the cluster of proteins sharing the possibility to localize Par6 in the nucleus. Conclusively, we believe that present study will contribute significantly to increase the knowledge on the involvement of Par-6 protein in breast cancer. Further investigation will be necessary to verify the alternative role proposed.

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EFFECTS OF CANCER AND STROMAL CELLS CROSS-TALK ON TUMOUR MICROENVIRONMENT

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It is well known that the cellular microenvironment dramatically influences cell behaviour and is critical in many physiological functions. Hyaluronan (HA) is a widely distributed extracellular matrix macromolecule that plays a crucial role in tissue development and is highly expressed in cancers. HA is up-regulated in breast cancer, generating a microenvironment that promotes tumour progression and metastasis. Moreover, breast tumour cells express proteins, either secreted or presented on cell surface, that interact with surrounding stromal cell and induce tumour progression. Recently, in our lab using proteomic techniques we found a new protein called "Uncharacterized protein of c10orf118 or

Q7z3e2" in the extracellular matrix of the co-culture of the breast tumour cell line BC-8701 and the normal human skin fibroblasts. The c10orf118 mRNA is highly expressed in breast tumour cells, even though it was found expressed by stromal cells as well. Interestingly, the estrogen receptor (ER) positive breast cancer cell line MCF-7 showed an increased amount of this protein in cell lysates, whereas a part was found also secreted in the conditioned. Thus, we hypothesize that this protein has a role in cellular mechanisms but its extraction, probably through exosomes, is implicated in a cross-talk of cells. Indeed, in this study we also demonstrate that the increased protein level of Q7z3e2 in breast cancer cell lines is correlated to an up-regulation of HAS2 mRNA, whereas a co-culture of normal fibroblasts with MCF-7 cells transfected with a plasmid containing the Q7z3e2 gene showed an increase of peritumoural HA. Considering the important role of Q7z3e2 in HA alterations, in our future studies we are willing to investigate the regulatory mechanism of Q7z3e2 and elucidate the cellular and molecular events that lead to the increase of HA.

PROLIDASE DEFICIENCY: ALSO A BONE DISEASE

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The degradation of extracellular matrix (ECM) molecules is a crucial process for bone development. Considering the amino acid sequences in the ECM collagens, the most abundant dipeptides generated from their catabolism contain proline and hydroxyproline. In humans, prolidase is the only enzyme able to hydrolyze dipeptides containing these residues at the C-term end, thus being a key player in collagen turnover[1]. Mutations in the prolidase gene cause prolidase deficiency (PD), a rare recessive disorder. Here we demonstrate that 13 of the 14 PD patients molecularly characterized in our lab show a bone phenotype revealing a correlation between the disease and bone health. The bone outcome mainly revealed dismorphism features, microcephaly and short stature. Recently, a mouse model for PD, the *dal/dal* mouse, has been identified and, as human patients, has a pleiotropic phenotype[2]. We exploited the murine model to investigate the bone phenotype that arises from the lack of prolidase. First the bone/cartilage ratio and the bone structural and mechanical properties were investigated. No differences were detected in bone/cartilage content but *dal/dal* femurs were significantly shorter than control and pQCT and CT analyses revealed compromised bone properties both at the cortical and at the trabecular level. Material elastic properties were also reduced in mutant mice suggesting the presence of more heterogeneous composition of mutant *versus* WT bone. A delay in the formation of the second ossification center was evident at P10 in *dal/dal* mice suggesting a delay in chondrocytes differentiation with a consequent delay in the substitution of the cartilaginous tissue with the bone tissue. Anhistological analyses of the cartilage growth plate revealed a decrease in the hypertrophic zone height, in the number of hypertrophic cells columns and in the number of cells for each column together with an increase of the apoptosis rate in the proliferative zone. These data suggested that the increased apoptosis of the proliferative cells could be responsible of the hypertrophic zone impairment and can explain the compromised bone properties in *dal/dal* mice. The involvement of bone in human and murine phenotypes demonstrates for the first time the crucial role of prolidase on the architecture and mechanical properties of bone as well as on skeletogenesis.

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HEPARANASE AND FIBROSIS

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Fibrosis can be defined as an excessive accumulation of extracellular matrix (ECM). Exposition to noxious stimuli and growth factors (TGF- 1, FGF-2 ...) induces the epithelial to mesenchymal transition (EMT) of renal tubular cells, a process that sustains fibrosis[1]. These events are common also to other organs. A body of literature supports the involvement of heparanase (HPSE) in the pathogenesis of proteinuric disorders. HPSE is an endoglycosidase that cleaves heparan sulfate (HS) thus participating in ECM remodeling and degradation[2]. We proved that HPSE is increased in plasma and/or urine of cancer patients[3], and in plasma of end stage renal disease subjects[4] suggesting a possible role for HPSE activity in body fluids as a diagnostic marker. Experimental findings show that a tight functional connection among HPSE, FGF-2 and syndecan-1 exists. Moreover, our recent data have demonstrated that HPSE is up regulated in tubular cells in response to several injuries and that HPSE regulates syndecan-1, a transmembrane HS proteoglycan (HSPG) that controls FGF-2 signaling[5]. Overall, HPSE is necessary to FGF-2 to induce EMT in tubular cells and to sustain its signaling[6]. Moreover we proved that the presence of HPSE is necessary to pro-fibrotic factors to up-regulate the expression of TGF- and that the lack of HPSE delays the onset of EMT inducedby TGF- even if it does not completely prevent the transdifferentiation of tubular cells[7]. These data suggests that strategies aimed to inhibit HPSE can be useful to control renal fibrosis[8].

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ALTERED ECM EXPRESSION AND COMPOSITION BY HUMAN OSTEOBLASTS IN MYELOPROLIFERATIVE NEOPLASMS

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Philadelphia negative Myeloproliferative neoplasms (Ph-MPNs) are a heterogeneous group of related diseases including polycythemiavera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF)[1]. Ph- MPNs are defined as clonal malignant hemopathies that arise from the transformation of hematopoietic stem cells/progenitors.The principal cell thought to be responsible of the occurrence of fibrosis of PMF patients is the megakaryocyte. Indeed, the megakaryocytic lineage appears altered, showing hyperplasia and dysplasia resulting in an excessive production of mitogenic and fibrogenic factors[2]. The increased release of these growth factors would stimulate myelofibrosis through the activation of endothelial and stromal cells. Myelofibrosis is defined as an increase in bone marrow fiber content without referring to the exact quantity or quality of extracellular matrix (ECM) proteins, suggesting that myelofibrosis is a process that involves the accumulation and assembly of collagenous (reticulin) and non-collagenous ECM components. In this context, we have analyzed the differentiation in osteoblasts (hOSTs) of mesenchymal stem cells from PMF or ET patients, and from healthy control. *In vitro* differentiation was demonstrated through the up-regulation of the bone related genes alkaline phosphatase and collagen I and through the analysis of calcium deposition. Interestingly, after 21 days of culture, hOSTs derived from PMF patients showed an increase in total amount of deposed ECM proteins as compared to ET derived cells and control. Specifically, PMF derived hOSTs produced and deposited higher amount of collagens and of fibronectin with respect to normal subjects and to patients affected by ET. We further investigated the organization of the ECM deposited by hOSTs by immunofluorescence staining. hOSTs from controls and ET patients deposited fibronectin regularly along cell body. On the contrary fibronectin, from PMF derived hOSTs, was irregularly dispersed and more abundant around cells. All together these data demonstrate several alterations in ECM protein expression, deposition and organization in hOSTs from PMF patients. Further investigations are necessary to understand the eventual stromal epigenetic modifications or chromosomal alterations that occur *in vivo* when the bone marrow environment is in contact with the pathological hemopoietic precursors.

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ANIMAL MODELS TO EVALUATE ER STRESS EFFECTS ON OSTEOGENESIS IMPERFECTA (OI)

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Osteogenesis Imperfecta is a genetic disorder characterized by bone fragility and deformity mainly due to dominant mutations in the genes coding for the α chains of collagen type I.The Brtl mouse is a murine model for classical OI carrying the G349C substitution in the α1(I) chain[1]. Brtl fibroblasts show a partial retention of collagen containing mutant chains leading to the swelling of ER cisternae[2]. Also, a modulation of the phenotype of mutant miceis linked to the status of the ER, with lethal mice overexpressing the ER stress-related pro-apoptotic protein Gadd153 and the non-lethal ones showing upregulation of the heat shock protein αB crystallin[3]. Proteomic analysis on skin and calvarial bone of Brtl mice with different outcome highlighted differences in the expression of chaperones and proteasome components, stressing the occurrence of aberrant protein folding and degradation. Mutant MSCs following osteo-differentiation showed the overexpression of the specific collagen I chaperone HSP47 and the activation of an autophagic pathway related to collagen ER retention, that cause impaired osteogenesis.Since OI phenotype is suggested to be modulated by ER response, ER stress seems to be an appealing new pharmacological target. For drug testing, we decided to use a more convenient OI animal model, thezebrafish Chihuahua (Chi+/-)[4]. Heterozygous Chi+/- fishes carry the G574D substitution in the α1(I) chain. X-ray and histological analyses of Chi+/- bone and cartilage showed severe skeletal alteration, with bone deformity and fractures, while μCT revealed a reduced bone mineral density. We demonstrated that zebrafish collagen composition varies during the development from $(\alpha1)_2\alpha2$ in embryos to $\alpha1\alpha3\alpha2$ in adults. A slight delay in electrophoretic migration of $Chi^{+/-}$ collagen and larger bands size suggested collagen overmodification. Calorimetry analysis revealed the presence of molecules with a Tm lower than WT, suggesting an altered collagen structure. Mutant collagen retention and ER enlargement in $Chi^{+/-}$was demonstrated in adults and embryos by TEM and ER labeling. These data validate the Chihuahua as a good model for testing ER stress targeting pharmacological compound for OI treatment.

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Index of authors

A
Abbonante V. 10
Aguiari P. 7
Albanase N.N. 4
Albanese N.N. 8,9
Avenoso A. 3

B
Baldinu P. 4
Balduini A. 10
Barosi G. 10
Bartolomeo A. 6
Basso P. 5
Besio R. 10
Bianchi L. 11
Biggiogera M. 11
Bini L. 11
Bishop N. 10
Bochicchio B. 7,8
Boraldi F. 6,8
Boscarino A. 9
Bracalello A. 1,7
Buttacavoli M. 4

C
Calatroni A. 3
Campo G.M. 3
Campo S. 3
Cancemi P. 4,8,9
Candini O. 2
Caprioli M.R. 6
Caravà E. 5
Carina V. 9
Carra S. 11
Catani F. 2
Cillino G. 9
Congiu T. 5
Costa S. 4
Cotelli F. 11
Crudele M.A. 7

D
D'Angelo M.L. 3,9
D'Arienzo M. 8
D'Ascola A. 3
De Luca G. 3
De Luca M. 1
De Muro P. 3,4
De Santis G. 2
Deiana G. 4
Di Cara G. 4,8,9
Dominici M. 2

F
Fadini G.P. 1
Fancellu L. 4
Fisher S. 11
Forlino A. 10,11
Formato M. 3,4
Foster S. 10
Fresu P. 3

G
Gagliardi A. 11
Gallagher O. 10
Gambaro G. 10
Gerosa G. 7
Gioia R. 10,11
Giordano C. 9
Giorgini A. 2
Grabowski P. 10
Grisendi G. 2
Gruppi C. 10
Gunn T.M. 10

H
Heinz A. 7

I
Idini M. 3,4
Iop L. 7

J
James P. 10

K
Karousou E. 3

L
Leikin S. 11
Lepedda A.J. 3,4
Lobina O. 3,4
Loschi P. 2
Lotti R. 2
Lupo A. 10
Lupo C. 4

M
Marconi A. 2
Martini D. 8
Maruelli S. 10
Masola V. 10
Micali A. 3
Minafra S. 8
Moscarelli P. 8
Murgia A. 2
Musso R. 4,8,9

N
Naso F. 7
Nieddu G. 3,4

O
Onisto M. 10

P
Paolucci P. 2
Passi A. 3
Pazzaglia U.E. 5
Pellegrini G. 1
Penza V. 11
Pepe A. 7,8
Peri G. 8
Piccinno S. 2
Pignatti M. 2
Pincelli C. 2
Pisani A. 3
Pizzolanti G. 9
Pucci-Minafra I. 4,8,9
Puzzolo D. 3

Q
Quaglino D. 4,6,8

R
Rabacchi C. 4
Rasini V. 2
Raspanti M. 5,8
Reguzzoni M. 5
Rocchiccioli S. 3
Rossi A. 11
Rosti V. 10
Roz E. 4

S
Saltari A. 2
Salvi A.M. 8
Sbrana F. 7
Schmelzer C.E.H. 7
Scuruchi M. 3
Secchi M.F. 10
Sechi G.P. 4
Sindona G. 6
Spano C. 2

Spina M. 7

T

Taverna D. 6

Tenni R. 10,11

Tira M.E. 10

Tomasello L. 9

Tonelli F. 11

U

Uitto J. 6

V

Valentino B. 8

Vassalli M. 7

Veronesi E. 2

Vigetti D. 3

Villa I. 10

Viola M. 3

Z

Zaza G. 10