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European Journal of Histochemistry
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- cellular basis of diseases.

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INVITED LECTURES

EPITHELIUM/STROMA CROSS-TALK IN BREAST CANCER

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During tumorigenesis stroma initially plays a suppressing role, but overtime evolves to promote tumor growth, as demonstrated in many experimental studies. Normal tissuearchitecture becomes disordered; the extracellular matrix (ECM) is remodeled by mesenchymal cells and the relationships between epithelium and basement membrane are disrupted.

Tumor stroma is the microenvironment which surrounds the malignant cells and makes up about a half of malignant tumors. The cells found in the stroma include blood and lymph endothelial cells, pericytes, leukocytes, macrophages and fibroblasts. In particular, fibroblasts termed "cancer associated fibroblasts" (CAFs) have received increased attention because of their involvement in tumor development, including invasion and metastasis. In the tumor stroma, a great number of CAFs and myofibroblasts, high capillary density, deposition of mature type I collagen and other ECM components are observed.

The growing role of the reciprocal interaction between epithelial and stromal cells in the development and progression of breast cancer has been recognized. To verify if this cross-talk may affect some aspects of cell biology (i.e. proliferation, adhesion molecule expression, membrane fluidity, migration), we co-cultured estrogen receptor (ER)-positive, poorly invasive and low metastasizing (MCF-7) or ER-negative, highly invasive and metastatic (MDA-MB-231) breast cancer cells with fibroblasts isolated from breast healthy skin (normal fibroblasts, NFs) or from breast tumor stroma (CAFs) in monolayer or in a three-dimensional system (nodules).In terms of proliferation, we demonstrated that CAFs exerted a mitogenic effect on both ER+ and ER- breast cancer cells, while mammary cancer cells had no influence on the growth of both kinds of fibroblasts. NFs and CAFs respectively induced or inhibited E-cadherin expression in MCF-7 cells; CAFs promoted N-cadherin up-regulation in MDA-MB-231 cells and its *de novo* expression in MCF-7 cells.An increase in N-cadherin was observed in CAFs but not in NFs, as a result of the interaction with both kinds of cancer cells. Plasma membrane labeling of monolayer cultures with the fluorescent probe Laurdan showed an enhancement of the membrane fluidity in cancer cells co-cultured with NFs or CAFs. An increase in lipid packing density of fibroblast membranes was promoted by MCF-7 cells. Time-lapsed cell tracking analysis of mammary cancer cells co-cultured with NFs or CAFs revealed an enhancement of tumor cell migration velocity and even a marked increase in the directness induced by CAFs.

A dramatic increase in Stearoyl-CoA desaturase 1 (SCD1), the main enzyme regulating the fatty acid membrane composition, has been observed in both MCF-7 and MDA-MB-231 cells as the result of the interaction with CAFs. In addition, the presence of fibroblasts, in particular CAFs, stimulated binding of SREBP1, a transcription factor which specifically binds to the SREBP response element on the SCD1 promoter, in tumor cells.

Finally, performing co-culture assays in the presence neutralizing antibodies against hepatocyte growth factor, transforming growth factor-beta and fibroblast growth factor-2, which are released by CAFs, we demonstrated that both the speed and the directness increases induced by CAFs in cancer cell migration were strongly reduced or completely abolished.

All these results provide new insights in the understanding the role of CAFs in promoting the tumor cell invasiveness and may help in defining new therapeutic targets in breast cancer.

MOLECULAR AND CELLULAR DISSECTION OF THE "BONE MARBLE" DISEASE

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Osteopetrosis (OP) is a genetic inherited disorder caused by defects in osteoclast formation and function. On the basis of mode of inheritance, we distinguish the Autosomal Dominant Osteopetrosis (ADO, also known as Albers-Schonberg disease) usually considered an adult-onset and benign form characterized by multiple spontaneous fractures and frequent osteomyelitis, and the Autosomal Recessive Osteopetrosis (ARO), also named malignant infantile osteopetrosis because of the severity of the disease leading to death early in life.[1]

The first form was identified in 1983, when Carbonic Anhydrase 2 (CA2) gene was found to be mutated in a peculiar form of OP characterized by renal tubular acidosis and cerebral calcification.[2] More recently, the molecular analysis has revealed the genetic heterogeneity of the disease[1]. To date, seven genes have been identified in human ARO forms. The majority of them cause defects in osteoclast function and can be cured by hematopoietic stem cell transplantation (HSCT) since osteoclast originate from myeloid progenitors.[3] However, we have recently identified an OP form caused by defects in TNFS11 (RANKL), a gene expressed by mesenchymal cells (including osteoblasts and stromal cells). The product of the TNFS11 is a cytokine RANKL, which acts as a membrane bound factor and soluble molecule and it is responsible of fusion of osteoclast progenitors. Our group has demonstrated that RANKL replacement in the *Rankl* knockout mouse, might represent a valid treatment for patients carrying defects in RANKL gene. Indeed, the administration of appropriate doses of RANKL (1 mg/kg very 48 h for 1 month) for a finite period results in dramatic improvement of the trabecular space within bone and in parallel ameliorates the morphology of hematopoietic organs[4] (and unpublished observation). Thus these preclinical data indicate the need of a pilot clinical trail to confirm the efficacy of this treatment in the human disease. Conversely, for patients with ARO forms, the search for a bone marrow donor should be started early in life since HSCT should be performed before the development of irreversible secondary complications, such as visual impairment. To this end, prenatal diagnosis and/ or collaboration among multiple clinical disciplines is crucial to define the molecular diagnosis and establish the appropriate therapy. Finally, recent data in literature indicate the feasibility and partial efficacy of gene therapy approach using gammaretroviral vectors in the *Tcirg1*(*oc/oc*) mouse model and $CD34^+$ cells obtained from osteopetrotic patients [5,6].

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ABSTRACTS

AN *IN VITRO* MODEL TO STUDY BONE METASTASIS

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Bone metastasis is a frequent complication of advanced breast cancer. Fortunately, not all the breast cancers, even with the same diagnostic profiles, will metastasize to bone. This is an indirect evidence of the heterogeneous profile of breast cancer cells of the same origin, among and within the primary tumors. Indeed some cancer cell subpopulations should preferentially express adequate properties that enable them to migrate and grow into the bone matrix, which reciprocally provides a suitable microenvironment for the survival and growth of these cells. The mechanisms underlying the osteotropism of certain cancer cells are complex and involve a number of signals and factors, still not completely clarified. With this aim, we have undertaken the implementation of an *in vitro* system for the study of the differential proteomic profiling of the bone seeding breast cancer cells.

Viable bone fragments, deriving from surgery on traumatic lesions of young subjects were provided by the orthopaedic team at the DICHIRONS Department, after informed consent of the donor.

Bone fragments, not larger than 0.5 cm, were washed from bio-contaminants under sterile conditions and placed into cell culture capsules with DMEM medium supplemented with foetal bovine serum (10%), L-glutamine (1%), L-ascorbic acid (50 µg/mL) and penicillin/streptomycin (1%). The explants were kept in the humidified incubator under CO_2 at 37°C and maintained for 4 weeks in order to promote the delivery of the osteoblasts and other resident cells, according to the current protocols. After this period, the bone cells were collected and further characterized. The fragments were then washed under sterile conditions, exposed to UV irradiation for 20min and placed in co-culture with under-confluent SKBR3 breast cancer cells, for one week. The bone fragments were then recovered, washed, placed again in culture dishes and monitored daily. After one week, we observed the outgrowth of colony-forming cells. These were collected and processed for immunological characterization and proteomic profiling. The proteomic profiles of the bone-seeded cells were compared with the original SKBR3 culture, revealing an interesting differential pattern. Among the differentially expressed proteins were several proteins belonging to the cytoskeleton remodelling and proteins of the class of calcium-binding cluster. In parallel, bone fragments with seeded cancer cells, were submitted to scanning electron microscopy, which showed the peculiar relationship between cancer cells and the bone matrix. These results represent an innovative proteomic-based contribution to the study of the bone metastasis, which will be further developed.

IDENTIFICATION OF THE "UNCHARACTERIZED PROTEIN C10ORF118" IN BREAST CANCER CELLS AND ITS ROLE ON THE HYALURONAN METABOLISM

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The association of hyaluronan (HA) with tumourigenesis has been known for a long time. HA-rich matrices around tumours favour the cancer cells migration and infiltration of newly formed blood vessels (Savani *et al.* 2001). In fact high level of HA is often associated with malignant progression in many cancers, such as breast cancer, colorectal cancer, and glioma (Itano et al. 2004). At all stages of tumourigenesis, stromal cells become *activated* and release growth factors and cytokines that further increase HA synthesis by both stromal and tumour cell.

Recently in our laboratory in co-cultures (Transwell system) of human dermal fibroblasts with the breast cancer cell line BC 8701, we identified at the conditioned media the presence of a protein named "Uncharacterized protein C10orf118" or "CTCL-tumour associated antigen" whose accession number is Q7z3e2. Using the Real-time PCR, we observed that this protein was highly expressed in BC8701 cells and less in fibroblasts. Therefore, the aim of the present project is to explore the functional properties of the Q7z3e2 protein and the role it may have in the control of HA synthesis and in cross-talk between the two breast cancer cell lines: MCF-7 (ER positive, low metastatic) and MDA-MB-231 (ER negative, high metastatic) and stromal cells.

The Q7z3e2 protein and gene expressions were higher in MCF-7 than MDA-MB-231 cells and mainly localized around or within the nucleus, even though the protein was identified also in the conditioned media. Western blot analysis of cell cultures showed higher amount of protein in both tumor cells respect to fibroblasts. Fibroblasts co-cultured with each tumour cell line using the Transwell system showed that MCF-7 cells induced the HAS2, HYAL2 and Q7z3e2 expression in fibroblast, demonstrating a cross-talk between these cells. However, silencing of Q7z3e2 in breast cancer cells and co-culture with fibroblasts demonstrated that suppression of HAS2 and HYAL2 was observed only with MDA-MB-231. These data suggest that Q7z3e2 is possibly able to trigger some particular signal pathways inside the cells to regulate the expression of different genes involved in the metabolism of hyaluronan.

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PROTEINS INVOLVED IN THE INTERACTION OF BREAST CANCER CELLS WITH TYPE V COLLAGEN

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In the complex scenario of the ECM remodeling at the tumour-stroma interface, the collagens play a crucial role, acting not only as a supporting scaffold for various cell types, but also interacting with the cancer cell surface which may participate to different signaling pathways (cell motility, cytoskeletal remodeling, cell cycle progression, metabolism, etc). Previous researches in our group have focused on the study of interac-

tions between the cancer cells and the components of the tumoral stroma, in order to improve the knowledge regarding the microenvironment influences on the cancer progression. In this context, we have reported the contrasting effects exerted by different collagen types, used as substrates for cancer cells in culture. In particular a restrictive effects on the cancer cell proliferation and motility was observed in the cell cultures seeded on type V collagen, contrary to the effects exerted by other collagen types. Type V collagen, belonging to the subgroup of the fibrillar collagens, is a minor component of the normal adult tissues, while its tissue concentration appears higher in fetal tissues and in several tumor tissues, including breast. To evaluate the cellular mechanisms responsible for the cellular responses to type V collagen, the 8701-BC breast cancer cells were seeded on type V collagen until confluent and then subjected to subcellular fractionation. The cell membrane fractions were isolated and solubilized to extract membrane proteins. These were then subjected to affinity chromatography and the bound fraction was quantified by Bradford assay. Aliquots of the sample were separated by 1D SDS-PAGE electrophoresis and the resulting bands were submitted to MALDI-Tof mass spectrometry. To date we have identified 20 proteins associated with Type V collagen, among which integrin 1, annexins, heat shock proteins, and other of interest, such as PLOD1 and -3 involved in the fibrillogenesis of collagen. The results of the present study confirm the occurring of multiple molecular interactions between type V collagen and the 8701-BC cancer cells. These interactions include some ECM receptors and a number of co-receptors and partners, probably acting in synergy and involving multiple cellular pathways, which are still in course of study.

PROTEOMIC EFFECTS INDUCED BY MICROENVIRONMENT COMPONENTS ON 8701-BC BREAST CANCER CELLS: AN UPDATED REPORT

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Many solid tumors, including breast cancer, are composed of heterogeneous cell populations, namely neoplastic cell subpopulations and host cells of various origin, which may interact between each others in complex networks. Tumor cells can modify the epigenetic program of stromal cells that in turn may produce epigenetic changes in tumor cells. The activated stroma, mainly composed of cancer-associated fibroblasts (CAFs), macrophages (TAMs), myofibroblasts and endothelial cells support the aberrant epithelial-mesenchymal cross-talk. At the same time, during tumor progression, the extracellular matrix (ECM) around and within the primary tumor undergoes a remodeling process where several components of the matrix are deposited, degraded, or otherwise modified. In addition, intratumoral hypoxia, deriving from high cell density, induce cellular adaptation to low oxygen tension and nutrient deprivation. The new and dynamic microenvironment generates signals that may influence the invasive behaviour of tumor cells through the expression of undefined set of genes, difficult to predict *a priori* and to search individually. Here we report an updating of our researches on the possible epigenetic influences exerted by selected microenvironment components and hypoxia on cancer cell growth and spreading, evaluated by the proteomics approach. We have used in an *vitro* model of human breast cancer cells (8701-BC) exposed to different collagen substrates[1], decorin[2], fibroblasts[3], endothelial cells and to a low O_2 tension[4]. Interestingly, while the hypoxia *per se* did not alter significantly the proteomic expression of 8701-BC cells, the other microenvironment components induced detectable changes in cell morphology and in cell proliferation rate, as well as in some protein clusters (cytoskeleton, metabolic enzymes, apoptosis), suggesting that the host environment influence positively and/or negatively the processes of viability/proliferation and motility, which may contribute in directing the neoplastic cells towards a more or less aggressive phenotype.

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DIFFERENTIALLY EXPRESSED PROTEINS IN FIBROBLASTS FROM A*bcc6*$^{-/-}$ AND *Abcc6*$^{+/+}$ MICE HIGHLIGHT THE ROLE OF A LOCALLY ALTERED PHOSPHATE METABOLISM IN THE OCCURRENCE OF ECTOPIC CALCIFICATION

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Progressive calcification of elastic fibers is typical of Pseudoxanthoma elasticum (PXE), a rare genetic disease due to mutations in the *ABCC6* gene. The pathogenesis of mineralization is only partially known.[1] Previous studies on dermal fibroblasts from PXE patients demonstrated that the calcification process is associated to impaired carboxylation of matrix-gla-protein that, in its active form, binds to calcium, therefore inhibiting mineralization.[2] However, the recent observation that PXE fibroblasts exhibit also a significant upregulation of alkaline phosphatase (TNAP) activity suggested that an abnormal phosphate metabolism may take place within soft connective tissues, thus contributing to ectopic calcification.[3] To improve the understanding of PXE it was developed a transgenic mouse model by specifically inactivating the *Abcc6* gene.[4] Consistently, *Abcc6*$^{-/-}$ mice recapitulate several histopathological findings typical of PXE, including the slow progression of the disease.[5] Aim of the present study was to isolate fibroblasts from Abcc6$^{+/+}$ and Abcc6$^{-/-}$ mice of different ages (*i.e.* before and after the development of ectopic calcification) and to investigate proteins controlling phosphate levels in the extracellular matrix. Results demonstrate a down-regulation of pyrophosphatase/phosphodiesterase 1, of progressive ankylosis protein and of osteopontin, whereas bone morphogenetic protein 2 and TNAP activity were up-regulated in fibroblasts from *Abcc6*$^{-/-}$ animals. These data support the hypothesis that in PXE the unbalanced ratio between factors locally controlling both calcium and phosphate homeostasis are crucial in triggering tissue calcification.

Work supported by grant from FCRM-EctoCal.

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BIOCOMPATIBILITY FEATURES OF HEART VALVE BIOPROSTHETIC DEVICES

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With respect to the limited lifespan of glutaraldehyde(GA)-treated bioprostheses to date there is almost no alternative when heart valve replacement surgery is required and most advanced current research attempts to develop tissue engineered valve scaffolds to be implanted *in vivo* or after *in vitro* preconditioning and dynamic seeding with host cells. However the clinical outcomes of detergent-based cell-depleted tissue engineered xenogeneic constructs are still controversial. Therefore, we investigated whether the clinical drawbacks of GA-treated and decellularized bioprosthetic devices might be related to residual xenogenic epitope and/or to biocompatibility problems associated with by-produts of the detergent-based decellularizing process. Accordingly we determined the residual content of detergents and that of residual xenogenic antigens in both GA-treated and detergent-based preparations (five different procedures). The presence of residual detergents was detected in all the resulting scaffolds albeit in different concentration, the content of Taurodeoxycholate (TDOC) (novel method) being the lowest (0.06%) with respect to Deoxycholate (8.13%) as the highest. Conversely relevant amount of xenoantigen was also detected in almost all the current GA-treated as well as in most detergent-based cell-depleted preparations with the exception of the procedure based on Triton associated with Cholate (COL) or Taurodeoxycholate (TDOC). The results of this investigation indicate that the clinical outcome of both current bioprosthetic devices as well as of putative tissue engineering substitutes might be significantly affected by the presence non-negligible amounts of remnant xenogenic epitopes and of residual detergent, respectively.

METHODS FOR CHEMOSELECTIVE COLLAGEN MATRICES MODIFICATIONS

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Collagen is widely used as a biomaterial for several tissue engineering applications.[1] Carbohydrates are interesting biomolecules for material surface functionalisation, since they can imprint the surface with double achievements at once. The first one is given by their polyhydroxylated nature, that confers hydrophylic features to the surface they are linked to. The second one, is due to their ability to convey biological information.[2] In fact, it is well known that carbohydrates are directly implicated in recognition processes, including adhesion between cells, adhesion of cells to the extracellular matrix, and in specific recognition of cells by one another. In addition, carbohydrates are recognized as differentiation markers.

Synthetic approaches toward the design of functionalised collagen patches will be presented. This topic will outline recent efforts in material functionalisation (''biodecoration'') with signalling and relevant glycidic structures for Tissue Engineering (TE) applications.[3,4] Chemoselective ligation approaches will be particularly emphasised. Due to the relevant role played by carbohydrates in recognition phenomena, this approach may present an attractive target for rational design of smart biomaterials.

Acknowledgments. We gratefully acknowledge Fondazione Cariplo, grant n° 2010-0378, n° 2011-0270 and MIUR, under project PRIN 2010/L9SH3K.

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CARTILAGE REGENERATION WITH NEOGLYCOSYLATED COLLAGEN PATCHES

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Collagen is the most abundant protein in the body and a major component of the extracellular matrix (ECM), it has been widely used both *in vitro* and *in vivo* and has been shown to have suitable properties for tissue engineering applications. One emerging feature is collagen glycosylation, reported as relevant in determining physiological and pathological states. In fact, glycosylated collagen is deeply involved in remodelling and breakdown of ECM; furthermore *O*-glycosylation events provide a novel regulatory level in the dynamic balance between collagen deposition and turnover with several implications in healthy and pathological processes.[1] In addition, it has recently been shown that lectin domains interacts directly with glycosylated collagens,[1] modulating the endocytic efficiency of the receptor toward highly glycosylated collagens such as basement membrane collagen IV. Despite the relevance of glycosylation in eliciting specific biological responses, the chemoselective surface modification of collagen-based scaffolds by carbohydrate epitopes has never been proposed to date.

We have approached the immobilization of simple carbohydrate structures to collagen through the thiol–ene click chemistry, planning the reaction between alkene-derived monosaccharides and thiol-terminated collagen patches, generated via functionalysation of the lysine residues.

The presence of monosaccharides introduced by thiol-ene reaction on the patch surface was confirmed by synchroton analysis (SR-XPS). In order to assess if the exposed monosaccharide could also exploit its biological signaling functions upon recognition of its complementary receptor, preliminary Enzyme Linked Lectin Assays (ELLAs) were performed on the glycosylated collagen samples. Preliminary *in vivo* biological assays in osteo-arthritic mice clearly indicate that surface modification of collagen patches, even with small biomolecules such as biologically relevant monosaccharide (i.e. glucose and galactose), has a significant impact in the tissue repair.[2]

Acknowledgment. We gratefully acknowledge Fondazione Cariplo, grant n° 2010-0378, and MIUR, under project PRIN 2010/L9SH3K.

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AGE- AND DIABETES-RELATED NONENZYMATIC CROSSLINKS IN COLLAGEN FIBRILS: CANDIDATE AMINO ACIDS INVOLVED IN ADVANCED GLYCATION END-PRODUCTS

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Ageing and diabetes share a common deleterious phenomenon, the formation of Advanced Glycation Endproducts (AGEs), which accumulate predominantly in collagen due to its low turnover. Though the general picture of glycation has been identified, the detailed knowledge of which collagen amino acids are involved in AGEs is still missing. In this work we use an atomistic model of a collagen fibril to pinpoint, for the first time, the precise location of amino acids involved in the most relevant AGE, glucosepane[1]. The results show that there are 14 specific lysine-arginine pairs that, due to their relative position and configuration, are likely to form glucosepane. We find that several residues involved in AGE crosslinks are within key collagen domains, such as binding sites for integrins, proteoglycans and collagenase, hence providing molecular-level explanations of previous experimental results showing decreased collagen affinity for key molecules[2]. Altogether, these finding provide insights on the possible molecular mechanism by which glycation affects the biological properties of collagen tissues, which in turn contribute to age- and diabetes-related pathological states. Ongoing work includes the experimental validation of the model through the further evaluation of changes in intermolecular recognition and alteration of collagen mechanical properties at the fibril scale.

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TENDON SHEATHS AND WATER IN TENDON BIOMECHANIC

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In tendon the major tissue component is water representing the 55-70% of the total wet weight. Four groups of tissue water have been described: a) the structural water, b) the bound water, c) the free water and d) the water in transition. In the extracellular matrix of tendon insoluble fibrillar collagen mainly consists of type I collagen which represents the 80-90% of tissue dry weight. Small proteoglycans like decorin and a small amount of biglycan fill inter fibrillar spaces, whereas large proteoglycans like aggrecan and versican seem to be located in inter fibre spaces. Collagen fibers seem to retain the main part of interstitial bound water whereas proteoglycan aggregates are supposed to retain large amount of free water. Fluids move within tendon when it is stretched since it has been shown that in humans there is a marked negative tissue pressure around the Achilles tendon during exercise. We investigated the morphological aspects and biomechanical behavior of collagen fibrils/fibres under physiological stretching in Achilles tendon of rat. Relaxed and physiologically stretched tendons were analyzed at the PLM, TEM and SEM. Under stretching, fibre crimps straightened and disappeared and fibril diameter reduced. We believe that an increased molecular packing with a fluid displacement occurs in fibrils under tension. Both small (decorin and biglycan) and large proteoglycans (aggrecan and versican) binding large amount of water could play a role in favoring and regulating a fluid shift/water flow during stretching and recoil of tendon. The particular structure of tendon sheats suggests an involvement of these tissues in the mechanical behavior of tendon. Water is the major constituent of all connective tissues: altering the tissue water content may affect the tissue elastic and viscous behavior. An excess of water causes swelling of fibrils and changes the mechanical properties of collagen fibres in tendon. In tendinitis and after training the proteoglycan content and consequently water amount changes. The water content in tendon could assume an important role of healthy or pathological index for different clinical applications.

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INHIBITION OF NF-kB ACTIVATION BY β-ARRESTIN 2 IN IL-1 β-INDUCED INFLAMMATION IN MOUSE CHONDROCYTES

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Hyaluronan (HA) degradation occurring during pathological state produced small HA oligosaccharides that may induce an inflammatory response by interacting with the toll-like receptor 4 (TLR-4) and CD44. TLR-4 stimulation activates different pathways that culminate with the activation of the transcriptional nuclear factor kappaB (NF-kB). The translocation of NF-kB into the nucleus may induce a pathway that involve the transforming growth factor activated kinase-1, (TAK-1), that in turn primes the expression of different inflammatory mediators.

β-arrestin 2 is an adaptor protein that alter signalling of G protein and seems to be involved in the down-regulation of the inflammation response.

In this study we investigated the role of β-arrestin 2 in mouse chondrocytes exposed to interleukin-1β.

Chondrocyte stimulation with IL-1β enhanced HA levels and its degradation, up-regulated cyclic adenosine monophosphate (cAMP), β-arrestin 2, TAK-1, protein 38 mitogen-activated protein kinase (p38MAPK), and protein kinase A (PKA) mRNA expression and of the related protein levels.

IL-1β treatment also induced NF-kB activation, with a consequent transcription of pro-inflammatory mediators, such as tumor necrosis factor-alpha (TNF-α), and inducible nitric oxide synthase (iNOS). The treatment of IL-1β-stimulated chondrocytes with β-arrestin 2 inhibitor, significantly increased the inflammatory response, while the treatment with a specific TAK-

1 inhibitor significantly reduced the inflammatory response. The anti-inflammatory action exerted by β-arrestin-2 was mediated both through a direct inhibition of TRAF-6, that prevent NF-kB activation, and in part through cAMP and PKA activation mediated by p38MAPK up-regulation. The treatment of IL-1β-stimulated chondrocytes with a p38MAPK and PKA specific inhibitors increased the inflammatory mediators levels, confirming the involvement of the p38MAPK pathways activation induced by β-arrestin 2. Taken together, these results could be useful for future anti-inflammatory strategies.

URINE BIKUNIN QUANTITATION AND STRUCTURAL CHARACTERIZATION IN TYPE 1 AND TYPE 2 DIABETES PATIENTS

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Bikunin is a plasma proteinase inhibitor often associated with inflammatory conditions. It has a half-life of few minutes and it is rapidly excreted into urine as urinary trypsin inhibitor (UTI). UTI levels are usually low in healthy individuals but they increase up to 10 fold in both acute and chronic inflammatory diseases. The report describes a sensitive direct method for UTI quantitation that does not require any antibody for protein detection. UTI purification was performed by anion exchange chromatography (DEAE-Sephacel resin) followed by SDS-PAGE. A calibration curve for protein quantitation was set up by using a highly purified UTI fraction. UTI identification and structural characterization was performed by Nano-LC-MS/MS analysis. The method was applied on urine samples from 9 patients with type 1 diabetes, 11 patients with type 2 diabetes and 28 healthy controls, matched for age and sex with patients, evidencing higher UTI levels in both groups of patients in respect to controls ($p<0.001$ and $p=0.001$, respectively). Spearman's correlation tests highlighted no association between UTI levels and age in each group tested. Owing to the elevated sensitivity and specificity, the described method seems to be suitable for large-scale clinical studies. Additionally, MS/MS analysis prospects the possibility of characterizing post-translational modification (PTM) sites potentially able to influence UTI localization, function and pathophysiological activity. Preliminary results suggest that UTI levels could represent a useful marker of chronic inflammatory condition in type 1 and 2 diabetes.

COLLAGEN MATRICES NEOGLYCOSYLATION VIA LYSINE SIDE-CHAINS AND ITS BIOLOGICAL EVALUATION

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Site-selective chemical modification of collagen matrices requires an efficient and chemoselective reaction. Different amino acid side-chains can be exploited toward this aim (cysteine, glutamine, serine, threonine, proline, hydroxyproline, lysine, and arginine). Performing residue-specific chemical reactions on collagen and maintaining its integrity is not an easy task. In the present work lysine amino groups have been functionalised via reductive amination[1] with different glycan structures,[2] obtaining a "neo-glycosylated collagen". In order to assess the effect of the "neoglycosylation" reaction on collagen structure, molecular dynamics simulations, AFM, water contact angle and FTIR have been performed. In order to assess if the exposed saccharide could also exploit its biological signaling functions upon recognition by its complementary receptor, preliminary Enzyme Linked Lectin Assays (ELLAs) was performed on the glycosylated collagen samples. Finally, preliminary *in vitro* biological assays on MG-63 cell lines showed that proliferation of MG63 cell lines on neoglycosylated collagen is favored if compared to pristine collagen.

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EFFECTS OF NEOGLYCOSYLATED COLLAGEN ON NEURONAL DIFFERENTIATION

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Glycans participate in neural cell migration, neurite outgrowth and synapse formation in both the developing and adult nervous system[1-5]. Understanding their functions would improve the knowledge in the mechanisms that determine nervous system differentiation and regeneration and could lead to the use of glycans as therapeutic strategies for neurological disorders.

For this reason we studied the effects of native and neoglycosylated collagen (glc-collagen) on neuronal differentiation by using a neuroblastoma, dorsal root ganglion-derived cell line (F11). The choice of cells was motivated by their capability to differentiate into neurons when exposed to differentiating agents[6,7]. We compared the behaviour of cells plated on native and glc-collagen with cells seeded on petri dishes. After 7 days, the electrophysiological properties of cells were analysed by the patch-clamp technique in the whole-cell configuration. Sodium current densities showed the tendency to increase from petri dishes to collagen and to glc-collagen and significantly higher

potassium current densities were calculated for cells plated on glc-collagen. Mean resting membrane potential was very depolarized in cells from the petri dishes but showed a trend to hyperpolarize in cells plated on collagen and was significantly more negative on glc-collagen. The electrical activity was tested by applying depolarizing current pulses by the patch-electrode; in petri dishes, the majority of the cells showed slow depolarisations which were not able to reach 0 mV, on the contrary, the fraction of cells able to generate action potentials was significantly higher on collagen and reached almost the totality on glc-collagen.

Our data show for the first time F11 cells differentiated without the use of chemical and differentiating agents and suggest that glc-collagen is an efficient material to be employed for neuronal differentiation.

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OXIDIZED LDL REGULATE HYALURONAN SYNTHASES ACTIVITY IN HUMAN AORTIC SMOOTH MUSCLE CELLS

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Atherosclerosis, usually classified as a chronic inflammatory disease of the blood vessel wall, is the most common cause of cardiovascular diseases. The localized inflammation causes the thickening of the arterial wall due to the increased deposition of extracellular matrix (ECM) and native LDL trapped within the arterial wall undergo modifications like oxidation (oxLDL). Thickening of the vessel in response to high LDL levels is a hallmark of atherosclerosis, characterized by increased hyaluronan (HA) deposition in the neointima. We investigated the link between internalization of oxLDL and HA production *in vitro*, using human Aortic Smooth Muscle Cells (AoSMC), showing the effects on scavenger receptor LOX-1 up-regulation and Hyaluronan synthases (HASs) expression[1]. OxLDL treatments enhanced transcripts of HA synthase HAS2 and HAS3 1.9 and 3.8 folds. Accumulated HA stimulated AoSMC migration and monocyte adhesiveness to ECM. The effects induced by oxLDL were inhibited by blocking LOX-1 scavenger receptor with a specific antibody (10 µg/mL). To unravel the mechanism of HASs transcription and activation, we depleted oxLDL of cholesterol, abolishing the HA synthesis and HASs overexpression, highlighting the role of sterols in the pro-atherosclerotic transformation. Nevertheless, free cholesterol (20 µg/mL) alone or oxysterols delivered to AoSMC did induce increased HAS expression but not HA synthesis. In oxLDL treatments, HA deposition was associated with higher expression if endoplasmic reticulum stress markers (CHOP and GRP78), condition present also with the use of the only free cholesterol or oxysterols, indicating that the overexpression of the synthetic enzymes is a necessary but not sufficient condition for the production of HA. The ER stress can indicate a regulatory point during the enzyme transport trough the internal compartment to the plasma membrane, even though is possible a finest regulation at transcriptional level involving microRNA or antisense RNA[2].

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SEA URCHINS AND MECHANICALLY ADAPTABLE CONNECTIVE TISSUES: ALTERNATIVE SOURCES FOR BIOMATERIAL DESIGN

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Sea urchins, like all echinoderms (starfish, sea cucumber, etc.), possess connective tissues that undergo drastic changes in their mechanical properties (Mutable Collagenous Tissues: MCTs)[1]. Mammalian connective tissues rarely undergo significant changes within a physiological timescale, the only major exception being the destiffening then restiffening of the mammalian uterine cervix at the end of pregnancy. In contrast, MCT can switch reversibly between stiff and compliant conditions in timescales of less than a second to minutes following nervous stimulation. In view of this behaviour, MCT could be an inspiration for new matrices capable of changing their molecular and structural conformation in response to external stimuli. Furthermore, elucidating the molecular mechanism underlying MCT mutability could have implications for veterinary and biomedical science, particularly regarding the pathological plasticization or stiffening of connective tissue structures. The MIMESIS (Marine Invertebrate Models & Engineered Substrates for Innovative bio-Scaffolds) project has being developed within this scientific context[2]. This contribution presents a review of the distinctive features of MCT together with the first results regarding the production of MCT-derived matrices as cell culture /tissue regeneration substrates.

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CHARACTERIZATION OF ZEBRAFISH COLLAGEN TYPE I

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Procollagen type I is the major constituent of connective tissues. In higher vertebrates it is a heterotrimer formed by two proα1 and one proα2 chains folded in a triple helical superstructure characterized by the presence of N- and C- terminal random coiled propeptides. In the last years the bony fish *D.*

rerio (Zebrafish) imposed itself as animal model for several human diseases, including skeletal disorders. Thus, being collagen I the main bone protein, it is mandatory to better investigate Zebrafish collagen I composition, which is still poorly known. In many Teleosts a collagen I α3 chain was identified both at gene and protein level and its presence seemed to be dependent on species, developmental stage and tissue type1. In Zebrafish a *Col1a3* gene was characterized as duplication of the Col1a1 locus, but its expression analysis at protein level is still missing. To this aim we purified acid and pepsin soluble collagen I from bone, skin and scales of adult fishes and whole embryos. On SDS-PAGE adult collagen I from all the examined tissues showed the classical collagen bands namely, from higher to lower molecular weight, the γ trimers, β dimers and the single α1(I) and α2(I) chains. Densitometric analysis of the α bands allowed to calculate the α1/α2 ratio, that is near 2, as expected. By 2D electrophoresis and mass spectrometry the presence of the α3(I) chain in the γ trimer and β dimer bands, as well as in the band corresponding to the α1(I) chain, was demonstrated. The 1D SDS-PAGE analysis of collagen I extracted from embryos allowed to identify an α1 band distinct from a band corresponding to α1/α3 mixture, suggesting a different post translational modification for the heterotrimers [α1(I)]2α2(I) and α1(I)α2(I)α3(I). By differential scanning calorimetry (DSC) a skin collagen Tm of 35°C was measured. In conclusion, we demonstrated for the first time also at protein level the existence of α3(I) chain in Zebrafish, even if we have not been able to evaluate the α1(I) and α3(I) chains stoichiometry in collagen I yet. Based on α1 and α3 high sequence similarity and on adult and embryos collagen I behavior on 1D and 2D PAGE, we can speculate the existence of [α1(I)]2α2(I) and α1(I)α2(I)α3(I) heterotrimers in embryos and of α1(I)α2(I)α3(I) in adults, although we cannot exclude the existence of the [α3(I)]2α2(I) form in both developmental stages.

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FIBRONECTIN AND MEGAKARYOCYTE DEVELOPMENT

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Fibronectin is a 440-kd dimeric major ECM glycoprotein that includes 3 different types of repeating segments (types I, II, and III) and plays a regulatory role during embryogenesis, wound healing, and maintenance of tissue integrity[1]. The fibronectin gene encodes 15 type III repeats that are constitutively expressed plus 2 that are alternatively spliced: extra domain A (EDA) and extra domain B (EDB).[2] EDA is a single exon that is included or excluded from the fibronectin mRNA by exon skipping.[2,3] Two major forms of fibronectin exist: plasma fibronectin, a soluble form found in plasma secreted by hepatocytes, which lacks both EDA and EDB segments, and cellular fibronectin, a form found mainly as fibrills in the ECM and secreted by activated fibroblasts that contains variable proportions of EDA segments, EDB segments, or both. Recently we demonstrated the expression of the Extra Domain A (EDA) fibronectin isoform in human megakaryocytes.[4] However, little information exist about the expression of fibronectin containing the EDA domain in adult human hematopoiesis. Here we performed a comprehensive *in vivo* study of the hematopoietic stem cell (HSC) compartment in adult mice constitutively including or excluding the EDA domain exon and show that the production of mature blood cells, lineage distribution within hematopoietic organs, frequencies of the most primitive HSC populations are comparable to those of wild-type littermate controls. Finally, EDA inclusion/exclusion result in equal megakaryocytes bone marrow content, ploidy and platelet release *in vitro* as well as *in vivo* after lipopolysaccharide-induced thrombocytopenia. Therefore, although expressed by hematopoietic cells, EDA fibronectin is not a critical regulator of steady state or stressed hematopoiesis.

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N-ACETYLCYSTEINE INCREASES PROTEOGLYCAN SULFATION IN A MOUSE MODEL OF DIASTROPHIC DYSPLASIA

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Diastrophic Dysplasia (DTD) is a severe recessively inherited chondrodysplasia caused by mutations in the diastrophic dysplasia sulfate transporter (DTDST) gene, leading to impaired uptake and intracellular depletion of sulfate. DTD patients show growth retardation, skeletal dysplasia, joint contractures and reduced viability. Functional impairment in the sulfate transporter causing low levels of intracellular sulfate determine glycosaminoglycan (GAG) undersulfation in newly synthesized cartilage proteoglycans (PGs).

Previous data have pointed out that sulfur-containing amino acids could have a potential role as intracellular sulfate sources when extracellular sulfate concentration is low or when its transport is impaired such as in DTD. On the basis of this observation we considered N-acetyl-L-cysteine (NAC) as a potential sulfation source. In this study we used our mouse model of diastrophic dysplasia (dtd mouse) to evaluate the contribution of NAC to cartilage PG sulfation in the fetal period. Since cartilage and bone growth and remodelling are processes that occur also in the early stages of development we administrate in the drinking water a solution 30 g/L NAC to pregnant mice from the first day of pregnancy to delivery. Then wild-type and mutant animals were sacrificed at P1 and cartilage PG sulfation was evaluated. GAGs were recovered from cartilage of the femoral head by digestion with papain and subsequent precipitation with cetylpyridinium chloride. Purified GAGs were digested with both chondroitinase ABC and ACII and disaccharide sulfation was evaluated by HPLC. A significative increase in PG sulfation was observed in mutant mice treated with NAC compared to the ones treated with a placebo. The amount of mono-sulfated disaccharides (ΔDi-4S and ΔDi-6S) relative to the total amount of disaccharides (ΔDi-0S, ΔDi-4S and ΔDi-6S) observed in mutant treated mice was about 78% vs 63% in untreated dtd mice (normal value 83%). Furthermore the skeleton of newborn mice, studied by alcian blue and alizarin red staining, indicated an amelioration towards the normal bone morphology in the tibia and hip of mutant mice treated with NAC compared to dtd pups treated with a placebo.

These data confirm that sulphur-containing amino acids such as NAC can be a source of sulfation for PG *in vivo* and suggest potential therapies of DTD with thiol compounds.

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