european journal of histochemistry
a journal of functional cytology

ISSN 1121-760X
volume 61/supplement 3
2017

XXXVII MEETING OF THE ITALIAN SOCIETY FOR THE STUDY OF CONNECTIVE TISSUES SISC 2017

Como, September 29-30, 2017
Cloister of S. Abbondio - University of Insubria

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Published by PAGEPress, Pavia, Italy

Editorial Office:
Via A. Cavagna Sangiuliani 5, 27100 Pavia, Italy
Phone: +39.0382.464340 - Fax: +39.0382.34872
E-mail: info@pagepress.org

Printed quarterly by:
Press Up s.r.l.
via La Spezia, 118/C 00055 - Ladispoli (RM)
Phone and Fax: +39.076.15.27.351

Annual Subscriptions
Europe: Euro 200
All other Countries: $ 250

Subscriptions, cancellations, business correspondence and any enquiries must be sent to PAGEPress Publications, Pavia, Italy.
Cancellations must be received before the end of September to take effect at the end of the same year.

Reg. Tribunale di Pavia n. 289/23.2.1984.

Recognized by the Ministero per i Beni e le Attività Culturali, Italy as a publication of high cultural value.

Associato all'USPI
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European Journal of Histochemistry
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The *European Journal of Histochemistry* is the official organ of the Italian Society of Histochemistry and a member of the journal subcommittee of the International Federation of Societies for Histochemistry and Cytochemistry (IFSHC).

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european journal
of histochemistry
ISSN 1121-760X
volume 61/supplement 3
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TARGETING CELLULAR STRESS IN OI AMELIORATES BONE PHENOTYPE

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The brittle bone disease osteogenesis imperfecta (OI), is a heritable skeletal dysplasia characterized by bone fragility and deformity, frequent fractures and short stature. Classical OI is caused by dominant mutations in the collagen type I coding genes, *COL1A1* and *COL1A2*, but also dominant, recessive or X-linked defects in other proteins involved in collagen type I synthesis, post-translational modification, maturation and secretion as well as in osteoblasts differentiation had been described as causative for the disease. The bone phenotype of OI patients was traditionally attributed to the presence of altered collagen type I in the bone extracellular matrix. More recently, it became clear that a cellular function impairment, due to mutant protein retention, may have an effect on patients' outcome and could be a target for the disease treatment.
By using the OI murine model *Brtl* and the OI zebrafish model *Chihuahua*, carrying a typical glycine substitution in one α1 chain of collagen type I, we demonstrated that the severity of the disease could be modulated by a different ability of bone to cope with the stress caused by mutant collagen retained in the endoplasmic reticulum. The chemical chaperone 4PBA was used to ameliorate the cellular stress using a short-term treatment for zebrafish larvae and a long-term treatment for adult fish. 4PBA ameliorated bone mineralization in larvae and skeletal deformities in adult mainly acting on reducing ER cisternae size and favoring collagen secretion. Furthermore using patients primary fibroblasts we were able to better dissect the metabolic pathways involved in the OI cellular stress caused by intracellular collagen retention. We demonstrated that the unfolded protein response, the autophagy and the apoptosis are activated and that they can be modulated by the use of chemical chaperone drugs. Many evidences suggest that intracellular events contribute to the OI phenotype and cellular stress seems to be an appealing new pharmacological target for OI.
Fondazione Cariplo 2013-0612, Telethon GGP13098 and the European Community n. 602300.

ROLE OF THE LONG NON-CODING RNA HAS2-AS1 IN BREAST CANCER CELLS

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Hyaluronan is an ubiquitous glycosaminoglycan of extracellular matrix important for tissue homeostasis and development that can regulate different cellular behaviors like adhesion, motility, growth and inflammation. Its presence is critical in tumor microenvironment, where the up-regulation of HA synthase 2 (HAS2) and the overproduction of HA are often associated with tumor progression and metastasis.[1] Recently, it has been discovered that the natural antisense transcript for hyaluronan synthase 2 (HAS2-AS1) can modulate the expression of HAS2 and the production of (HA) in different pathologies.[2,3] HAS2-AS1 is a long-non coding RNA (lncRNA) transcribed in the opposite strand of HAS2 gene on chromosome 8. It has an alternative splicing site which generates two RNA isoforms of different lengths (HAS2-AS1 long and HAS2-AS1 short), that have 257 or 174 nucleotides of perfect complementary sequence to the first exon of HAS2, respectively. LncRNAs play important roles in cancer, like chromatin remodeling, as well as transcriptional and post-transcriptional regulation, through a variety of chromatin-based mechanisms and the interaction with other RNA species as sponge for microRNA.[4] Here we show that the knockdown of HAS2-AS1 in the aggressive triple negative breast cancer cells (MDA-MB-231) increases proliferation respect to the estrogen receptor (ER) positive breast cancer cell line MCF-7. Furthermore, we report that the silencing of HAS2-AS1 in MDA-MB-231 is linked to a higher migration and invasion rate. Moreover, quantitative PCR analysis reveals that the abrogation of HAS2-AS1 brings to higher levels of HAS2, HAS3, CD44 and hyaluronidase 2 (HYAL2) mRNA, suggesting a possible role of HAS2 antisense in tumor progression. Microarray analyses revealed that HAS2-AS1 is able to regulate different pathways probably interacting with several miRNA and altering the stability of different transcript coding for crucial proteins involved in tumor biology.

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HYALURONAN OLIGOSACCHARIDES INDUCE INFLAMMATORY RESPONSE IN HUMAN THYROCYTES CULTURES BY AFFECTING TOLL-LIKE RECEPTORS

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Autoimmune Thyroid diseases, such as Hashimoto's thyroidite, result in characteristic lymphocytic infiltration and altered function of the thyroid. During inflammation, cytokines increase hyaluronan (HA) production that accumulates in the thyroid.[1] Hyaluronan (HA) fragments produced in different pathological states can modulate gene expression in a variety of cell types and may prime inflammatory response by interacting with the Toll-like receptor 2 (TLR-2), Toll-like receptor 4 (TLR-4) and CD44.[2,3] Particularly, TLRs activation induces a signaling complex mediated by adapter molecules, such as myeloid differentiation primary response (MyD88) and tumor necrosis factor receptor associated factor 6 (TRAF-6), that culminates in the nuclear factor kappaB (NF-kB) activation. NF-kB in turn modulates the expression of inflammation mediators, as inteleukin-1beta (IL-1beta) and inteleukin-6 (IL-6). The aim of this study was to investigate the effect of 6-mer HA oligosaccharides on human thyrocytes. HA treatment induced up-regulation of TLR-2, TLR-4,

MyD88 and TRAF-6 mRNA expression and related protein levels, and NF-kB activation that in turn increased IL-1beta and IL-6 concentrations. The exposure of thyrocytes to two specific blocking antibodies for TLR-2 and TLR-4 abolished up-regulation of MyD88 and TRAF-6, and significantly reduced NF-κB activation and pro-inflammatory cytokine production. This data suggest that HA depolymerization occurring during inflammation may contribute to prime the inflammatory response in thyrocytes through activation of TLR-2 and TLR-4 signalling cascade.

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EXTRACELLULAR MATRIX COMPONENTS IN THE TUMOR MICROENVIRONMENT INFLUENCE PANCREATIC DUCTAL ADENOCARCINOMA CELL PHENOTYPE

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Pancreatic ductal adenocarcinoma (PDAC) is characterized by an intense desmoplastic reaction and extracellular matrix (ECM) components in the tumor microenvironment interact in a cross-talk involving tumor cells and stromal fibroblasts, influencing tumor cell behavior. To better understand the role of ECM proteins occurring in the desmoplastic tissue in determining cancer cell phenotype, we aimed at analyzing *in vitro* the effect of the interplay between PDAC cells and ECM by culturing PDAC cell lines on different ECM proteins as substrate. For this purpose, we analyzed some epithelial-to-mesenchymal transition (EMT) markers and the migration and invasive potential in human HPAF-II, HPAC and PL45 PDAC cells cultured on collagen type I (COL), laminin (LAM) and fibronectin (FN) or without coating (NC). The expression of E-cadherin was not significantly affected in HPAF-II and HPAC cells, while resulted up-regulated in PL45 cells cultured on COL. The wound healing assay revealed that the effect of ECM components on cell migration was dependent on cell type, possibly related to the differentiation grade, but in all the three cell types COL increased the migration potential. SDS-zymography showed that COL induced a strong upregulation of MMP-2 activity in HPAF-II and HPAC cells, and of MMP-9 in HPAF-II and PL45 cells, compared to NC. These preliminary results suggest that ECM components could differently affect PDAC migration and invasion, possibly depending on the differentiation grade. The characterization of the mutual effects elicited by the tumor-stroma interaction on the cancer cell will contribute to better understand the influence of the stroma on PDAC cancer cell phenotype, in order to develop new therapeutic strategies.

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AN *IN VITRO* PORTRAIT OF POTENTIALLY INVASIVE BREAST CANCER CELLS

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The extracellular matrix (ECM) performs a primary role in the maintenance of cellular polarity and homeostasis and it represents a dynamic structure, due to a regulated remodelling of its components. Major responsible of matrix degradation are the matrix metalloproteases (MMPs), which belong to a large family of zinc-dependent endopeptidases. Some members of this family, named as MMP-2 and MMP-9, have received great attention due to their ability to degrade type IV collagen, a major constituent of basement membranes. A deregulated proteolysis of ECM molecules in the tumours causes the alteration of extracellular structure. These events, together with the loss of cell polarity, may lead the neoplastic cells to elude cell-cell and cell-ECM adhesions, promoting cancer progression. Literature data show that these alterations are correlated with poor prognosis in many tumour histories, where a positive relationship between the increase of MMPs and the malignancy grade has been reported.[1,2] The aim of this work was to prosecute and extend previous research on the differential properties of two prototypes of breast cancer, the 8701-BC cells derived from a ductal infiltrating carcinoma[3] and the SKBR-3 cells isolated from pleural effusion of an adenocarcinoma.[4] The comparative assays were based on their morphological properties, gelatinolytic activities, motility capability, integrated with proteomic and immune-cytochemical assays. The comparative morphology of the two cell lines shows that the 8701-BC cells lack cellular polarity in favour of an irregular cell shape characterized by the emission of several long protrusions, contrary to the SKBR-3 cells that grow as a rather regular island. The motility assay performed by the "scratch" test highlighted high performance of the 8701-BC cells compared with the SKBR-3 cells. Interestingly, when the SKBR-3 cells were exposed to the conditioned medium of the 8701-BC cells, their attitude to translocate through the scratch was significantly increased. This phenomenon may be attributable to the secretory molecules of soluble nature or released by vesicles.[5] In parallel, the gelatin-zymographic assay showed a relatively higher activity for MMP-2 and -9 released by the 8701-BC cells. In addition, the proteomic analysis highlighted several functional clusters involved in the cytoskeleton organization and cell motility, higher expressed in the 8701-BC cells.
Current results confirm the potentially aggressive nature of the 8701-BC cells, in comparison to SKBR-3 showing a low mobility capacity, a property which is the basis of tumor invasiveness by epithelial cells, which in normal tissues are typically stationary. These results also highlight a more general property of *in vitro* cultured cells: they appear to retain and remember their source phenotype. Therefore, this allows using these *in vitro* models to carry forward important molecular insights into the invasiveness of infiltrating carcinomas.

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BIOGENERATED SILVER NANOPARTICLES INDUCED APOPTOSIS AND AUTOPHAGY IN BREAST CANCER CELL LINE SKBR3

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Nanotechnology offers many advances in various fields and especially in medicine. Silver nanoparticles (Ag-NPs) are commonly used in consumer products and numerous studies have shown different biological effects, include antimicrobic and anticancer effects through the increase of reactive oxygen species (ROS) levels, DNA damage and cell cycle arrest. The aim of the present study was to study the effects of Silver nanoparticles (AgNPs), embedded into a specific polysaccharide (EPS) and biogenerated by Klebsiella oxytoca DSM 29614 under aerobic (AgNPs-EPSaer) and anaerobic conditions (AgNPs-EPSanaer). The potential cytotoxic effect of two types of AgNPs-EPS was firstly investigated after 24 h of treatment on two human breast cancer cell lines (SKBR3 and 8701-BC) and three human colon cancer cell lines (HT-29, HCT 116 and Caco-2) by using the MTT assay.[1] The results showed that AgNPs-EPSaer is most active than AgNPs-EPSanaer, and breast cancer cell lines (SKBR3 and 8701-BC) are more sensitive to the treatment compared to colon cancer cell lines (HT-29, HCT 116 and Caco-2). Based on these results, the SKBR3 cells[2] were further used to study the mechanisms of cytotoxicity associated with the AgNPs-EPS treatments. Morphological changes, generation of reactive oxygen substances (ROS) and induction of apoptosis and autophagy were assessed. Results showed that only AgNPs-EPSaer treatment induced ROS generation, which in turn induced cell death through apoptosis and autophagy. Moreover, the effect of the AgNPs-EPS was studied at proteomic level by applying two-dimensional difference in gel electrophoresis (2D-DIGE) followed by Matrix Assisted Laser Desorption Ionization-Time Of Flight/Time Of Flight (MALDI-TOF/TOF) mass spectrometry (MS) technique. Proteomic analysis revealed key proteins and pathways involved in AgNPs- EPSaer cytotoxicity, including endoplasmic reticulum stress, oxidative stress and mitochondrial disfunction.

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14-3-3 F PROTEIN OCCURRENCE IN BREAST CANCER TISSUES

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The advancement of the knowledge on the molecular mechanisms driving the cancer progression through the interactions between cancer cells and stroma is one of the primary objectives of the current anticancer research. Here we report additional results of a large-scale proteomic study involving one-hundred tumor tissue samples, collected at the La Maddalena Hospital and histopathologically characterized for the clinical and molecular parameters.[1] Routinely, the surgical fragments were washed several times in PBS and then carefully prepared for the homogenization and the extraction of the total protein amount. The protein extracts were quantified by applying the Bradford colorimetric assay, and aliquots of 45µg (for the analytical gels) and 1.2 mg (for the preparative gels) were subjected to the 2D-IPG electrophoresis. The spots on each gel were revealed by the ammoniacal silver stain (analytical gels), and Coomassie blue stain (preparative gels); the spots of interest were identified by mass spectrometry utilizing a MALDI-TOF instrument (Voyager DE-PRO. ABSciex). At present we have identified and classified into functional groups 458 protein spots. Among the identified proteins, noteworthy was the detection of the overexpression of seven members of the 14-3-3 protein family, namely: 14-3-3B, -E, -F, -G, -S, -T, -Z. These proteins are specific phospho-serine and threonine-binding adaptors involved in several biochemical pathways closely related to the regulation of the cell cycle, the apoptosis and the cellular metabolism, both in physiological and pathological conditions.[2] Our investigations about the occurrence of 14-3-3 proteins in non tumoral tissues revealed the 14-3-3G as ubiquitously expressed and the other six (B, E, F, S, T, Z) as sporadically expressed. On the contrary, the comparative proteomic screening of the 100 selected breast cancer tissues highlighted that six members of the family (B, E, G, S, T, Z) resulted ubiquitously expressed, while only the 14-3-3 F form resulted sporadically expressed, among patients. We then analyzed the statistical relationship between the sporadic protein 14-3-3 F and the other 457 identified proteins by applying the Pearson's correlation coefficient in tumoral and non-tumoral proteomic profiles: the proteins showing a significant linear correlation with the 14-3-3 F were selected for the bioinformatic approach. The results derived from the integration of the proteomic, biostatistic and bioinformatic approaches revealed an intriguing evidence: the 14-3-3 F protein was significantly correlated with proteins playing pivotal roles in key processes for the cell cycle progression, the resistance to the apoptotic stimulus and the response to the drug-induced-DNA damage. On the basis of our evidence, we conclude that the 14-3-3 F and other overexpressed 14-3-3 members may be differentially involved in the molecular mechanisms of the breast cancer progression and strenghten their candidacy as biomarkers useful for the clinical applications of the breast cancer.

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EPIREGULIN: A NEW BIOTECHNOLOGICAL WEAPON TO HELP WOUND HEALING

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Wound healing is a complex and dynamic process resulting in the replacement of devitalized or missing cellular structures and tissue layers. Impaired wound healing process could affect both acute and chronic wounds and generally results from a failure in the normal stages of healing progression. Moreover, difficult to heal wounds frequently enter a state of pathologic inflammation due to a delayed, incomplete or uncoordinated healing process. Chronic wounds are, by definition, hard to heal wounds, taking substantial time to heal and being expensive to treat. Moreover, these wounds are often associated to major symptoms, negatively affecting patient's quality of life. One of the key players in maintaining skin integrity and homeostasis is epiregulin, a EGF-related growth factor that is known to regulate keratinocytes proliferation under subconfluent and confluent culture conditions as well as to influence their migration and differentiation.[1,2] According to these evidences, the development of a drug carrier for the targeted delivery of the growth factor could help wound closure in pathological conditions. *In vitro* studies demonstrated that such scaffold is able to actively sustain *in vitro* keratinocytes proliferation and stratification[3] thanks to the prolonged release of epiregulin from the scaffold directly near the cells. Such interesting results fostered the development of an *in vivo* model to verify the effectiveness of such drug delivery system also in a more physiological condition. Animal experimentation has been performed using a rat open wound model in which the proposed carrier bioactivity has been compared to the natural (control, only saline treated) wound healing process. *In vivo* results demonstrated that epiregulin enriched hydrogel treated lesions displayed a better quality of the newly formed skin, both in terms of epidermal layer thickness and stratification and extracellular matrix organization. Such results support the key role of epiregulin in skin repair and regeneration and suggest its use through biotechnological modifications to create new tools for wound management.

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IN VIVO MODELS OF CHONDRODYSPLASIAS CAUSED BY DEFECTS IN GLYCOSAMINOGLYCAN BIOSYNTHESIS: PHENOTYPING AND PHARMACOLOGICAL APPROACHES

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Heritable skeletal disorders are connective tissue diseases that primarily affect the development and homeostasis of the skeleton; unfortunately, in general no resolutive treatments are available for these painful conditions. Thus, the goal in this research area is the deep phenotyping of relevant *in vivo* and *in vitro* models in order to identify and test novel targets for innovative therapies.
In this respect among the skeletal disorders that are being studied in our group, Diastrophic Dysplasia (DTD) and Desbuquois Dysplasia (DBQD) are paradigmatic. Both dysplasias, with similar clinical features, are caused by defects in the biosynthesis of glycosaminoglycan (GAG) chains of proteoglycans (PGs).
DBQD type 1 is caused by mutations in the Calcium-Activated Nucleotidase 1 (CANT1) a Golgi/ER resident enzyme. To define the role of CANT1 in the etiology of DBQD, we generated a Cant1 knock-out mouse. Morphological and clinical observations in the murine strain confirmed the skeletal defects described in the patients. PG synthesis was studied in rib knock-out chondrocytes; mutant cells showed GAG chains with reduced hydrodynamic size, GAG oversulfation, reduced PG synthesis and impaired secretion. This latter observation was confirmed by transmission electron microscopy of mutant vs. wild type cartilage showing the presence of dilated vacuoli suggesting a role of CANT1 in protein secretion. Nowadays animal models are useful tools to elucidate the molecular mechanisms underlying genetic diseases as described above, but also to develop therapeutic strategies. The dtd mouse is a murine model of Diastrophic Dysplasia (DTD) a skeletal dysplasia caused by mutations in the sulfate-chloride antiporter (SLC26A2), crucial for sulfate uptake and GAG sulfation. Deep phenotyping of the model suggested that N-acetyl-L-cysteine (NAC) might play a role as an intracellular sulfate source for macromolecular sulfation. We demonstrated an increase of cartilage PG sulfation and an amelioration of the skeletal phenotype in dtd newborns after NAC treatment of pregnant females. At present, we are investigating whether NAC treatment might ameliorate the skeletal phenotype in dtd mice after birth. In conclusion, these different mouse models have recapitulated key aspects of disease pathology and identified new fundamental mechanisms paving the way for developing potential therapeutic approaches.
Work supported by MIUR-PRIN2015 and the European Community (FP7, "Sybil" project, grant n. 602300).

CANCER CELL ULTRASTRUCTURE AND ECM

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In general, cell shape and cell surface morphology are stricktly related to cell function. Similarly cancer invasion is related to changes of morphology and structural asimmetry of cancer cells involving mechanical and molecular mechanisms, organization of the cytoskeleton, capability to remodel the tissue structures, cell-substrate adhesions, cell-cell adhesions and intercellular comunications.[1] Analysis of cell morphology and cytoplasmic ultrastructural changes in cancer cell cultures may help in understanding and confirming different biochemical data concerning cellular signaling pathways. Cancer local invasion in extracellular matrix is a prerequisite for migration of cancer cells in blood and limph vessels and then colonization of distant organs. Extracellular matrix is a dynamic network of macromolecules contributing to cell behaviour, gene expression and diverse functional properties.[2] The main component of extracellular matrix is collagen which is organized in collagen fibrils forming collagen fibres. Cancer cell invasion in extracellular matrix is a three dimensional event where cancer cells have to adapt their shape and size to penetrate and migrate into the guiding scaffold of collagen network. It is reported that mammary tumors are related to an increase of collagen deposition, straightening of collagen fibres firstly parallel and then perpendicular aligned to the tumor boundary.[3] Epithelial low invasive ERa-positive MCF-7 breast cancer cells as well as highly migrating and invasive ERa-knockdown cells (MCF-7/SP10+) were cultured in 2D and 3D cultures (millipore filter, millipore filter with Matrigel, type I and III collagen membrane). Invasivity test and scanning electron microscope analysis demonstrated that breast cancer cells have different behaviour and show different shape and different cytoplasmic surface depending on the diverse substrate. Data from this study confirm that collagen fibres of extracelluar matrix may influence the phenotype of breast cancer cells and suggest to study cancer cell migration in 3D cultures with natural extracellular matrix which can mimic the *in vivo* microenvironment.

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HEPARANASE AND FIBROSIS

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The organ fibrosis is a response to chronic injury characterized by the progressive accumulation and decreased remodelling of ECM. Persistent injury to parenchymal cells leads to chronic inflammation which, in turn, stimulates the activation of effector cells into fibrogenic myofibroblasts. Myofibroblasts express α-SMA and secrete large amount of ECM proteins (primarily collagen, fibronectin and laminin), which are responsible for tissue scarring and organ architecture deformation and failure. The source of the myofibroblast pool is variable and includes resident fibroblasts, fibrocytes, pericytes and epithelial cells undergoing epithelial to mesenchymal transition (EMT). In the last years, increasing interest has been paid to Heparanase in this field since its involvement in the EMT of renal tubular cells in kidney fibrosis was discovered. Heparanase (HPSE) is the only mammalian endo-glucuronidase capable of cleaving heparan sulfate (HS) chains of HS proteoglycans. Through its enzymatic and other non-enzymatic functions, HPSE contributes to extracellular matrix (ECM) turnover and regulates several physiological and pathological processes. Once secreted, HPSE is a key regulator of FGF-2 and TGF-β activity, the main pro-fibrotic factors and EMT inducers in the kidney. Indeed, HPSE knock-down in a proximal tubular cell line prevents the EMT induced by FGF-2 and delays that induced by TGF-β.[1,2] Moreover, HPSE-ko mice show no increase in TGF-β in injured kidneys and do not develop fibrosis.[3] Recently, we demonstrated the involvement of HPSE in early phases of reaction to liver damage and inflammatory macrophages as an important source of HPSE. HPSE seems to play a key role in the macrophage-mediated activation of hepatic stellate cells (HSCs) into myofibroblasts thus determining a dramatic alteration of ECM.[4] These findings suggests the potential of HPSE as a pharmacological target in the treatment of organ fibrosis.

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EFFECTS OF NANOSYSTEMS DOXORUBICIN-LOADED ON SKBR-3 BREAST CANCER CELLS: AN *IN VITRO* MODEL

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In the last years, the therapeutic approach to cancer underwent several re-modulations, due to the synthesis of new biotechnological drugs, specifically directed against tumoral targets and to the introduction of new concepts and modality of drug delivery based on the innovative application of nanomedicine. The present report describes the results obtained from a preliminary *in vitro* study on the effects of the treatment of SKBR-3 breast cancer cells with a nanosystem loaded with the doxorubicin, a drug used in the majority of the chemotherapeutic panels against the breast carcinoma and others. For the present research, we designed a folate-decorated PEGylated nano-graphene oxide (GO-PEG-Fol) capable of loading high amount of hydrophobic drugs such as doxorubicin, and endowed with a favorable combination of physico-chemical properties (*e.g.*, 30 nm diameter, stability in physiological fluids, 33% drug loading and proper amount of targeting agent) useful for the photothermal treatment of breast cancer. GO-PEG-Fol was characterized in terms of size (AFM and DLS), Z-potential and chemical composition (ATR-FTIR). To evaluate the

effects on the morphology and proliferation rate of the SKBR-3 cells, the proper culture dishes were separately treated with three concentrations (0.1, 0.5 and 1 μM) of doxorubicin-loaded GO-PEG-Fol nanosystem (N-Doxo) for seven days. The cell cultures treated with pure doxorubicin (Doxo) were used as positive control and the untreated cells as negative control. On the basis of the results obtained the $IC50_{48h}$ values for Doxo and N-Doxo were calculated. Hence, the SKBR-3 cells were treated for 48h with IC_{50} concentrations of Doxo and N-Doxo and the surviving cells were collected and properly prepared for the proteomic analysis. Briefly, protein extracts from treated and untreated SKBR-3 cells were quantified and aliquots of 45 μg were used for the 2D-IPG electrophoresis. The protein spots were revealed by silver stain and the obtained proteomic maps were analyzed with Image Master 2D Platinum software. The proteomic approach revealed that parallel treatments with Doxo and N-Doxo induce a marked modification on the expression of proteins involved in the cytoskeleton remodeling and of proteins belonging to the mitochondrial pathways and to the proteasomal machinery. In addition, the treatment with N-Doxo induces significant effects on the expression of several calcium-binding proteins (two tumor-related chaperones and other proteins belonging to the families of S100s and Annexins). Present results support the potential efficacy of the drug-delivery system based on the GO-PEG-Fol nanosystems and reveal the positive combinatory effect of doxorubicin and nanosystem targeting agents on the *in vitro* system.

PERSISTENCE OF LUNG INFLAMMATION IN MICE FOLLOWING SMOKING CESSATION IS ASSOCIATED WITH PROGRESSIVE LOSS OF ALVEOLAR SEPTA AND ACCUMULATION OF COLLAGEN IN THE SMALL AIRWAYS

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Chronic Obstructive Pulmonary Disease (COPD) is a collection of conditions, which include emphysema, chronic bronchitis with mucus hyper-secretion, bronchiolar and vascular remodelling, and sometimes the presence of fibrotic areas scattered throughout the parenchyma, in which emphysema and fibrosis may coexist. The most important risk factor for COPD is cigarette smoking. Until now, smoking cessation is the only treatment effective in slowing down the progression of the disease. However, in many cases smoking cessation may only relieve airflow obstruction and the inflammatory response. Consequently, a persistent lung inflammation is associated with a progressive deterioration of respiratory function (progressive decline in FEV1). This is an increasingly important clinical problem whose mechanistic basis remains poorly understood. Available therapies do not adequately suppress inflammation and are not able to stop the "vicious cycle" that is at the basis of persistent pulmonary inflammation. By using a "curative" experimental model of cigarette smoke (CS) induced pulmonary lesions[1] in a mouse able to replicate several features of human COPD[2] we studied the inflammation and the ongoing lung deterioration that follows smoking cessation. The role of formyl-peptide receptors (FPRs) signalling in the persistency of lung inflammation was also investigated. These receptors[3] and have been recently involved in lung inflammation and damage induced by CS.[4,5] Lungs of mice exposed for 4 months to CS showed mild emphysematous changes. However, inflammation was still present in lungs after smoking cessation and emphysema progressed during the following 6 months period of observation. Destruction of alveolar walls with elastin loss is associated with a progressive accumulation of collagen in peri-bronchiolar areas and with development of goblet cell metaplasia in bronchi and small airways. The modulation of FPRs signalling with FPRs antagonists, immediately after smoking cessation, mitigated persistent inflammation and prevented deterioration of lung structures during the whole observation period. This study indicates an important role of N-formylated peptides in the progression and exacerbation of COPD. Modulating FPR signal should be explored as potential new therapy for COPD.

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HEPARAN SULFATE INTERACTS WITH AMYLOIDOGENIC ELASTIN-LIKE PEPTIDES: SUPRAMOLECULAR ASSEMBLIES AND BIOLOGICAL SIGNIFICANCE

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In the extracellular environment and *in vitro* heparan sulfates (HSs) are known to interact with several matrix components such as tropoelastin[1] and elastin-derived peptides (EDPs),[2] thus modulating tissue elasticity in (patho)physiological conditions. However, in age-related diseases, elastin degradation leads to loss of tissue elasticity either releasing EDPs or favouring the massive deposition of protein aggregates called 'elastotic material'. Although, these aggregates have not been fully characterized, it is known to contain also elastin fragments and amyloid-like fibers,[3] consistently with the hypothesis that elastin can be involved in the amyloidogenic process. *In vitro* studies performed on EDPs encoded by exons of the tropoelastin gene, and containing the XGGZG consensus motif, were shown to produce amyloid-like fibrils characterized by cross-β-sheet structures.[4] A similar amyloidogenic behaviour was recently demonstrated also for elastin-like peptides (ELPs) without any evidence of cytotoxic effects.[5] On these premises, it has been suggested that ELPs similarly to other amyloid-like peptides could be safely used as bio-inspired nanomaterials.[6] Interestingly, amyloid fibril formation is accelerated by various biomolecules, including HS, even if the specific interactions and structural requirements remain unknown. Therefore, within the frame of studies aiming to investigate if ELPs may interact with HS, we have explored the interactions between HS and amyloidogenic ELPs, based on the XGGVG motif (where X=V, L). Fluorimetric and spectroscopic experiments using a semisynthetic HS (ss-HS of 10,3 kDa, and SO_3^-/COO^- = 1,4) demonstrated that the ELPs aggregation kinetic in the presence of HS was favoured at a different extent depend-

ing on the type of ELPs. Ultrastructural (TEM) and atomic force (AFM) observations revealed that HS influences the supramolecular assemblies of ELPs depending on their sequence and length. Results indicate that the polymeric nature of HS is associated with a relatively high potential to promote β-transition and can facilitate fibril self-association in more packed assemblies. Moreover, ELP-HS fibrils did not exhibit cytotoxic effects on cultured 3T3 fibroblasts. In conclusion, we have provided evidence that ELP-HS interactions may play important roles in amyloid fibril formation, and that this experimental *in vitro* model can represent a suitable reductionist approach to better understand the alterations affecting the elastic component in aging and in pathological conditions and may add further insights for a better comprehension of the amyloidogenic process and of its biological significance.
Work supported by FCRMO

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THE PHYSIOLOGICAL ROLE OF SIALIDASE NEU3

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NEU3 sialidase is a glycohydrolytic enzyme often referred to as the "ganglioside sialidase", because it preferentially removes sialic acid residues from gangliosides. While the pathological role of the enzyme in cancerogenesis has been extensively studied, its physiological role has only recently been revealed. In particular, NEU3 has been shown to participate in various cellular processes, including the differentiation of skeletal myoblasts, where its activity is needed for myotube formation and apoptosis protection. Moreover, NEU3 was shown to be up-regulated under hypoxic conditions, and the overexpression of the enzyme greatly increases cell resistance to oxygen deprivation opposing cell death. Along this line, we recently reported a novel mechanism of HIF activation, the master regulator of cell response to hypoxia, mediated by NEU3. Interestingly, we discovered that NEU3 is up-regulated in the myocardium of chronic cyanotic congenital patients, as compared to acyanotic congenital hearts that were used as controls. We found that endogenous NEU3 expression and activity of NEU3 is up-regulated in the heart of cyanotic congenital heart pediatric patients, and that this caused an up-regulation of the EGFR signaling pathway. This caused the activation of a signaling cascade, down-stream of EGFR, ultimately leading to an activation of HIF-1alpha and down-stream pro-survival signaling pathways. These results support that NEU3 sialidase plays a critical role in the response of the myocardium to hypoxia and the preservation of cell viability. Pharmacologically mimicking NEU3 sialidase activation was also tested, and it may result in the development of new drug candidates for hypoxic diseases of the cardiovascular system.

ANALYSIS OF MINERAL DEPOSITION IN CARTILAGE MATRIX AND EARLY PERIOSTEAL BONE. A MODEL FOR THE STUDY OF ENDOCHONDRAL OSSIFICATION

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The growth plate cartilage has been the most widely-used model of the endochondral ossification because in the restricted environment of the intercolumnar septa all the basic phenomena which characterize endochondral ossification (cartilage matrix calcification, vascular invasion, osteoblasts differentiation and osteoid apposition) can be followed in their morphological evolution. The study presented here was carried out in human metacarpals primary ossification centres before vascular invasion began, thus favouring a comparative study of mineral deposition in cartilage matrix and periosteal osteoid. Thin, sequential sections from the same paraffin inclusions of metacarpal anlagen from between the 20thand 22nd week of gestational age were examined with light microscopy and SEM/EDAX, either stained or heat-deproteinated. This enabled analysis of corresponding fields with the different methods. From the initial $CaPO_4$ nucleation in cartilage matrix, calcification progressed increasing the size of focal, globular, randomly distributed deposits (size range 0.5-5 μm), aggregation into polycyclic clusters and finally compacting into a dense mass of calcified cartilage. The early osteoid calcification showed a fine granular pattern (size range 0.1-0.5 μm), which soon merged to form the compact layer of the first periosteal lamella. Scanning electron microscopy of heat-deproteinated sections revealed a rod-like hydroxyapatite crystallite pattern, with only size differences between the early globular deposits of the two calcifying matrices. A similar morphology of the early calcium deposits in cartilage and osteoid was documented, but with differences of the globular size. The reported observations can be integrated to form part of the actual knowledge of the cell mechanisms controlling calcium/phosphate concentration, the ions transport pathways and the specificity of the collagen layout where the mineral deposits are settled. The different morphology and dynamics of the calcification process in cartilage and bone matrix can find an explanation in the anatomical and environmental condition where the two phases of endochondral ossification develop.

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VASCULAR INFLAMMATION AND EXTRACELLULAR MATRIX MODIFICATIONS

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Atherosclerosis is an inflammatory disease of the wall of large- and medium-sized arteries. It's an extremely complex series of events that causes the modification of tissue architecture and of extracellular matrix (ECM). The accumulation of oxidized-LDL (oxLDL) in the tunica intima and the endothelium damage are early stages of vessel inflammation[1]. Our research is focused on the study of the effect of different type of inflammation stimuli, such as oxLDL and TNFα, on ECM metabolism of endothelial cell (HUVEC). In particular, we aimed in understand the changes of the ECM components like hyaluronan (HA), glycosaminoglycans (GAGs) and HE/HS proteoglycans family. HUVEC treated with TNFα for 24 hours show an increase of Syndecan4 core protein both mRNA transcription and protein synthesis, an increase of transcription levels of HS/HE polymerization enzymes (EXT1, EXT2, NDST1) and, accordingly to that, HS/HE disaccharide composition shows a higher amount of N-sulfated modification. HA metabolism was investigated by the expression of the synthetic enzymes HAS2 and HAS3: HAS2 increases its expression while HAS3 decreases. Summarizing, endothelial glycocalyx is highly modified during inflammatory condition, in both GAGs and proteoglycans. As reported in several studios, Syndecans4 expression is related to NO production and release from endothelial cells. NO synthases (NOSs) have a central role in modulating vascular tone and can be altered by various forms of endothelial cells injury[2]. In our model, we observed a decrease of endothelial NOS (eNOS) expression in TNFα stimulated HUVEC, in according with changes in permeability and adhesiveness *in vivo* due to endothelial lesion. HUVEC treated with oxLDL respond in a different way since HAS3 decreases like in TNFα treatment, while HAS2 remains at control level. This difference ought to be compared with SMC because in the tunica intima the SMC are subjected to the action of oxLDL. We treated AoSMC with oxLDL to compare their effect on HAS2 and HAS3. HAS2 expression with oxLDL increases while HAS3 expression remains at control level, showing a different regulation way. LDLR, LOX1 and PCSK9 are involved in regulation of LDL uptake[3]. To know how they behave in our cells model after LDL stimulation, we treated HUVEC and SMC with LDL (both n and ox). In HUVEC and SMC treated with nLDL, LDLR expression is down-regulated as expected. oxLDL did not seem to influence LDLR expression in both cells line. In HUVEC treated with nLDL we found a down-regulation of LOX1 but no effect on LOX1 expression after oxLDL treatment. PCSK9 conduct in HUVEC and SMC after LDL stimulation is still under investigation.

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