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Pisa, June 7-10, 2015
Università degli Studi di Pisa
Scuola Superiore Sant'Anna and
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SARCOGLYCAN SUB-COMPLEX, SATELLITE CELLS AND EXTRACELLULAR MATRIX PROTEINS IN MASSETER MUSCLE AFFECTED BY UNILATERAL POSTERIOR CROSSBITE

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Unilateral posterior crossbite disease is defined as any abnormal buccal-lingual relation between opposing molars, premolars, or both in centric occlusion. It is one of the most prevalent malocclusions in the primary and early mixed dentition and is reported to occur in 8% to 22% of the cases. The most common form is a unilateral presentation with a functional shift of the mandible toward the crossbite side, resulting in an increased frequency of reverse chewing cycles. Moreover, the masseter of the affected side results less active than the counterpart[1]. Our previous immunofluorescence study on masseter muscle of patients affected by crossbite has shown that in the affected side there is a lower expression of muscle specific integrins than the counterpart[2], demonstrating that integrins could be correlated with contraction activity in mastication, maybe promoting the production of new muscle fibers. Then, considering the existence of a known bidirectional signaling between integrins and sarcoglycans and considering that changes in masticatory musculature may be in relationship with developmental factors and remodeling processes of muscle fibers and connective tissue, here we performed an immunofluorescence analysis using antibodies against sarcoglycans, against Pax-7, in order to study the satellite cells, and against Myf5, MyoD to study myogenic differentiation; moreover, we performed immunofluorescence reactions using fibronectin, collagen type I, III, IV and laminin in order to study the behaviour of extracellular matrix during the crossbite condition. Our results have shown that the staining pattern of tested proteins for extracellular matrix increased in crossbite side whereas the staining pattern of sarcoglycans, Myf5, MyoD and Pax-7 decreased. In side non-affected by crossbite it was shown a lower staining pattern of extracellular matrix proteins and an increased sarcoglycans, Myf5, MyoD and Pax-7 positive cells. In our opinion, the increase of sarcoglycans, Myf5, MyoD and Pax-7 in side non-affected by crossbite, could be due to high workload of healthy muscle with a consequent hypertrophic response of muscle fibers leading to increased turnover of extracellular matrix slightly represented. Contrarily, the increase of extracellular matrix proteins in crossbite side could be important for remodeling and healing processes in malocclusion diseases.

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STUDY OF THE MIXTURE EFFECTS OF FLUCONAZOLE AND ETHANOL BY USING *CIONA INTESTINALIS* AS A NEW ALTERNATIVE TERATOLOGICAL MODEL

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The aim of the present work is to evaluate the effects of the co-exposure to sub-teratogenic levels of ethanol (Eth) and to the clinically used antimycotic fluconazole (FLUCO) by using a new alternative model for teratological screening: *Ciona intestinalis*. The simple development of ascidians (Chordata, Tunicata) and their key phylogenetic position within the sister group of Vertebrates, suggests the ascidian model as a potential good alternative experimental system for teratological purposes. *C. intestinalis* embryos were exposed to FLUCO (31.5-62.5-125-250-500 μM), to Eth at its not effect level (0.1%) or to the mixture of FLUCO and Eth from 2-cell to the larval stage. At the end of the colture period, larvae were morphologically examined. Eth alone was unable to affect embryo development. Larvae exposed to FLUCO showed a typical phenotype characterized by malformations at the trunk region comparable to those elicited by retinoic acid (RA). The effects were FLUCO concentration-related. Interestingly, a significant increase of larvae with severe malformations was observed in groups co-exposed to FLUCO and Eth: the larvae showed a severely affected phenotype characterized by absence of sensory vesicle cavity, by absence of pigment in the sensory organs and presence of a short, curled tail. The anterior end was round in shape, the palps were not elongated, and larvae failed the hatching. The obtained data point the attention to the teratogenic risk of co-exposure to FLUCO and Eth. The results are comparable to those previously obtained in postimplantation rat embryo cultured *in vitro* and could be related to RA increase. Considering that FLUCO and Eth do not share the same mode of action (MOA), our data support the need of a cumulative risk assessment not only for chemicals grouped on the base of similarities in chemical structure but also for chemicals differently acting on the same biological pathway. The evidence that different subphyla (Tunicates and Vertebrates) are susceptible to azole fungicides and that the observed effects are quite similar, suggests the hypothesis that these molecules alter the expression of ancestral conservative genes, starter of a cascades of events, which model the whole embryonic body plan. Finally, ascidian embryo seems to be a good experimental system for a comparative screening of the teratogenic potential of azole fungicide mixtures, pointing the attention to a possible environmental impact of azole fungicides.

FIBROTIC AND VASCULAR REMODELLING OF COLONIC WALL IN EXPERIMENTAL COLITIS

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Inflammatory bowel diseases progress often towards fibrosis of intestinal wall, which might account for abdominal pain and dysfunctions of intestinal transit. Of note, despite the relevance

of this condition, there is currently a scarcity of animal models of intestinal fibrosis and reliable parameters to evaluate fibrotic remodelling in the gut wall are highly expected (Jurjus AR *et al.*, J Pharmacol Toxicol Methods 2004). Accordingly, the aim of this study has been to assess colonic wall remodelling, with a focus on the neuromuscular compartment, in a rat model of colitis elicited by 2,4-dinitrobenzenesulfonic acid (DNBS). After 6 and 21 days, the following parameters were assessed on colonic samples by enzymatic assay, immunoblotting, histology, histochemistry, immunohistochemistry and confocal microscopy: tissue myeloperoxidase; tissue injury and inflammatory infiltration; collagen and elastic fibers; neuronal HuC/D, glial fibrillar acidic protein (GFAP), proliferating cell nuclear antigen (PCNA), nestin, c-Kit and transmembrane 16A/Anoctamin1 (TMEM16A/ANO1), which was also examined in isolated inflamed colonic smooth muscle cells (ICSMCs). DNBS-treated rats showed inflammatory fibrotic lesions with colonic wall thickening and fibrotic remodeling, which was evident on day 21. Colitis was associated with both an increase in collagen fibers and a decrease in elastic fibers. Moreover, the inflamed colon displayed a significant decrease in HuC/D-positive myenteric neurons and increase in GFAP/PCNA-positive glia, remodelling and activation of blood vessels, reduced c-Kit- and TMEM16A/ANO1-positive interstitial cells of Cajal (ICCs), as well as an increase in TMEM16A/ANO1 expression in muscle tissues and ICSMCs. The variety of markers and cells examined in the present study provide an integrated view of the impact of inflammatory and fibrotic processes on colonic neuromuscular compartment. According to our findings, the rat model of DNBS-induced colitis displays significant colonic remodelling, consisting not only in collagen deposition and wall thickening, but also in specific cellular alterations of the neuromuscular units, such as myenteric neurons, glial cells, ICCs, SMCs and vessels.

EFFECTS OF UREA ON ZEBRAFISH OLFACTORY ORGAN AFTER ENVIRONMENTAL EXPOSURE

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Chronic renal disease is known to alter olfactory function. Of the uraemic toxins, high urea blood levels have been suspected to induce a hyposmic condition. However, with the exception of olfactometric analysis on human subjects, no other examinations have been conducted, particularly histomorphological observations of the olfactory organ. Even if hematic administration could better mimic pathologic conditions, as first approach we opted for direct nasal exposure, to minimize potential systemic interference. In this study, we describe the effects of environmental exposure to elevated concentrations of urea (7 g/L, 13.5 g/L and 20 g/L) on the sensory mucosa of zebrafish (considered a good predictive model of mammalian toxicity) in acute (48h and 96h) and chronic (30 days) toxicity tests. We observed that lamellae maintained structural integrity and epithelial thickness was slightly reduced only after 30 days at highest urea concentration. However the ratio between the volumes of sensory and non-sensory epithelial regions sensibly decreased during exposure. Pan-neuronal labelling with anti-Hu was negatively correlated with high doses of urea (13.5 g/L and 20 g/L), thus, we investigated whether distinct neuron subtypes were equally sensitive to the toxicant. Using densitometric analysis we evaluated and compared the immunolabelling of G_{olf} -, TRPC2- and S100-expressing cells, as representatives of ciliated, microvillous and crypt neurons, respectively. The three subpopulations responded differently to urea. In particular, crypt cells were more severely affected than the other cell types, and G_{olf} -immunoreactivity increased when fish were exposed to low doses of urea. The moderate sensory toxicity of urea seems to be in accordance with the olfactometric measurements reported in the literature. We counted also the dividing $PCNA^+$-cells, whose density remained constant: it is possible that other non-neuronal cells substituted olfactory neurons preserving epithelial integrity.

EXPRESSION AND FUNCTIONS OF THE TRANSCRIPTION FACTOR COUP-TFI IN NEUROGENIC REGIONS OF THE ADULT MOUSE BRAIN

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The chicken ovalbumin upstream promoter-transcription factor I (COUP-TFI) is a nuclear orphan receptor belonging to the superfamily of steroid/thyroid hormone receptors. COUP-TFI can act either as transcriptional activator or repressor, depending on the cell context, and plays key functions during embryonic development and organogenesis. A role for COUP-TFI has been demonstrated in multiple aspects of brain development, including the control of embryonic neural stem/progenitor cell (NSPC) fate/potential, neuronal migration, and cortical arealization. The expression of COUP-TFI persists in the adult brain as well, however its functions in the adult age are almost unknown and are just beginning to be investigated. In this context, we are particularly interested in exploring COUP-TFI action in highly plastic brain regions in which neurogenesis occurs throughout life, namely the olfactory bulb (OB) and the hippocampal dentate gyrus (DG). Our in situ hybridization and immunohistochemical data showed that COUP-TFI is highly expressed in both regions. In the OB, COUP-TFI is found in a subset of mature adult-born interneurons, including granule cells and TH-positive dopaminergic juxtaglomerular cells, while migrating neuroblasts and immature newborn neurons only rarely express COUP-TFI. By means of the Cre-lox system we conditionally deleted COUP-TFI in the Emx1 lineage demonstrating that COUP-TFI is involved in the maintenance of the identity of the dopaminergic phenotype in the OB and acts as key regulator in the control of activity-dependent tyrosine-hydroxylase expression in these cells. COUP-TFI expression in the DG was found in a large population of adult NSPCs, immature progenitors and mature granule neurons, suggesting that its role in this system could regulate different steps in the neurogenic process. We generated GLAST::CreERT2;Rosa26-YFP;COUP-TFIfl/fl inducible conditional ko mice. Two and 6.5 weeks following tamoxifen administration in these mice we obtained a highly efficient loss of COUP-TFI and expression of YFP selectively in Glast-positive adult NSPCs and their progeny in the hippocampal DG. Thus we established a model - which is currently under analysis- to dissect COUP-TFI functions on

the neurogenic process in the adult brain.
Funding: University of Turin [ex 60% 2013-2014]; Mobility grants: Campus France, Galileo project.
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AN INNOVATIVE STRATEGY IN SELECTING OOCYTES WITH HIGH IMPLANTATION POTENTIALITY FOR INTRACYTOPLASMIC SPERM INJECTION PROCEDURE

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The aim of the research was to investigate the apoptosis rate of individual cumulus cell-oocyte complexes (COC), associated to the level of pAKT, to verify the difference between oocytes who produce embryos able to reach the blastocyst stage compared with embryos arrested during the *in vitro* culture. It was demonstrated that DNA fragmentation in cumulus cells was remarkably lower in patients who achieved a pregnancy after ICSI cycles, related to the quality of oocytes and embryos[1,2]. AKT pathway plays a critical role in the regulation of cell survival, and most growth factors activate this pathway[3]. The study focused on 53 patients, involved after informed consent. In this prospective and randomized study, it has been measured the DNA fragmentation rate and the level of pAKT in cumulus cells of individual COC for each follicle containing a mature oocyte. Normo-responder patients have been selected. DNA fragmentation rate in cumulus cells has been examined with the use of a TUNEL assay *in situ*. pAKT has been examined by immunological assay *in situ*. Statistic of molecule expression and DNA fragmentation was tested through the repeated measures ANOVA test of log-transformed variables. Out of 255 MII oocytes, 197 were fertilized and the derived embryos had the following evolution: 117 completed the development to blastocyst (day 5or 6) and were transferred in uterus, 57 were vitrified at blastocyst stage and 23 were arrested during *in vitro* culture at different stage of cleavage. In conclusion we found a statistical difference between the DNA fragmentation rate of cumulus cells between the arrested embryos compared to the transferred and vitrified blastocysts (p=0.004), confirming that apoptotic rate of the cumulus cells could be considered as a marker of oocyte competence. Likewise we a found statistical significance between oocytes resulting in transferred blastocyst and arrested embryos in the ratio pAKT/TUNEL (p=0.043). Therefore, the ratio pAKT/TUNEL could be considered also a marker of oocyte quality. More studies are needed to confirm these data and to determine the how these molecular pathways are involved on the oocyte competence.

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IMMUNOLOCALIZATION OF COCAINE-AMPHETAMINE REGULATED TRANSCRIPT (CART) IN BILIARY EPITHELIUM

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Cholangiocytes proliferation can be modulated by several neuroendocrine factors[1]. The peptide cocaine-amphetamine-regulated transcript (CART) has various physiological functions and is widely expressed in many organs[2]. CART increases the survival of hippocampal neurons by up-regulating Brain Derived Neurotrophic Factor (BDNF)[3]. Recent study has detected the expression of BDNF and of its two receptors (TrKB and p75NT) in cholangiocytes of rat liver and their involvement in proliferation rate of biliary tree[4]. Aim of this study was to investigate the expression of CART in the rat biliary epithelium. Male Wistar rats were divided into normal (n=6) and BDL (n=6) group. All rats were sacrificed after 1 week. Liver samples from both BDL and normal group were collected to perform light microscopy and immunohistochemistry for CART, CK19 (cytokeratin-19) and PCNA (proliferating cell nuclear antigen). Our results show an increased expression of CART together with the growth of intrahepatic bile ductal mass (IBDM) and of cholangiocytes proliferation in BDL. CART may be implicated in the remodeling of biliary epithelium during cholestasis through key mediators of cell survival and proliferation, as well as through BDNF activated pathways.

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EFFECTS OF POLYSACCHARIDE A PRODUCT BY *BACTEROIDES FRAGILIS* ON ENDOTHELIAL PROGENITOR CELLS

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Endothelial progenitor cells are produced in the bone marrow and have the hemangioblast as common precursor to hematopoietic stem cells. They have been identified in 1997 in the peripheral blood stream. The EPC are able to migrate, proliferate and differentiate into mature endothelial cells and to determine the formation of new blood vessels even in the postnatal period. These cells can be infected by *Bartonella henselae*, a Gram-negative pathogen, aetiological agent of a series of human diseases known as bartonellosis. Polysaccharide A pro-

duced by *Bacteroides fragilis*, a human intestinal bacterium, has immunoregulatory properties, among which is proven in mice to provide protection from inflammation caused by *B. henselae*. We have infected the EPC with *B. henselae*, *B. fragilis* WT or *B. fragilis* PSA (not able to produce the polysaccharide A), or co-infected with *B. henselae* and *B. fragilis*, WT or PSA respectively, to assess whether the polysaccharide A had a role in the response of these cells to the infection. After the ultrastructural characterization of bacterial strains used in our experiments, *Bacteroides* ability to infect EPC was assessed by CLSM. Both bacterial strains are internalized already at 24h after infection and at a multiplicity of infection of 100. By TEM it was observed that *Bacteroides* in contrast to *Bartonella*, are internalized activating cytoplasmic lysosomes that digest them. *B. henselae* instead infects those cells forming invasomes in which they continues to proliferate. So polysaccharide A accentuates the macrophagic features that these cells have in their early differentiation stage. Analysis of genic expression using real time PCR from total RNA extracted by the cell cultures have shown the up-regulation of the gene coding for the IL-10 anti-inflammatory cytokine in cells co-infected with *B. fragilis* WT and *B. henselae* compared to the cells infected only with *B. henselae*. The main inflammatory cytokines secretion evaluated by ELISA has also evidenced an increase of IL-10 produced by cells co-infected with *B. fragilis* WT and *B. henselae*. These cells react to the co-infection in the same manner of macrophages. These data assert that the EPC does not play only a key role in vasculogenesis, but in an adult organism they could also play an essential role in the immune response. The EPC may collaborate with macrophages and T cells $CD4^+$, to the inflammatory process triggered by the polysaccharide A after infection.

FUNCTIONAL MORPHOLOGY OF THE ALIMENTARY CANAL OF THE ASCIDIAN *BOTRYLLUS SCHLOSSERI:* A HISTOCHEMICAL CHARACTERISATION

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A variety of histochemical assays were carried out on the monolayered epithelium of the alimentary canal of the colonial ascidian *Botryllus schlosseri* with the aim of increasing the knowledge on the physiology of the filter-feeding nutrition of tunicates. The endostyle of the branchial pharynx, the oesophagus, the saccular stomach and the U-shaped intestine divided into proximal, mid- and distal (or rectum) intestine and associated with a pyloric gland in its mid-tract, were examined as regards the distribution of enzymatic activities for both digestion (amylase, acid phosphatase, 5′-nucleotidase, aminopeptidase M, lipase, non-specific esterase) and absorption (alkaline phosphatase, Ca^{2+}- and Mg^{2+} -ATPases), the presence of storage substances (glycogen, proteins, lipids) and the quality of mucous substances. The argentaffin reaction revealed the presence of endocrine cells scattered in all tracts with the exception of endostyle and mid-intestine. The alimentary particles enter the pharynx, where they are trapped and rolled up by the mucous net secreted by the glandular cells of the endostyle producing different types of mucins. Along this tract, a pre-digestion of polysaccharides occurs and the epithelium is protected by an antimicrobic peroxidase activity. The digestible string is transported towards the post-pharyngeal gut by the activity of the ciliated mucous cells. The oesophagus plays the role of food progression. The stomach, with its many folds in which are located various cell types, carries out functions of extracellular digestion (zymogenic cells), absorption and intracellular digestion (vacuolated cells), osmoregulation (plicated cells of the pyloric caecum), and storage of lipids and glycogen (ciliated mucous cells). These functions, with the exception of the extracellular digestion and osmoregulation, continue in both proximal and mid-intestine, where the peroxidase activity reappears. The pyloric gland reveals hydrolytic activities and storage of glycogen and proteins. In the distal intestine, weak digestive and absorption activities still occur; faecal pellets are formed and then ejected through the anal opening in the atrial cavity and then into the common cloacal chamber of the colonial system. *B. schlosseri* can represent a model species for the development of histochemical methods which will be extended to other species belonging to other classes of tunicates showing more or less tissue complexity due to different specialisations.

PHOSPHORYLATED TAU IN PURKINJE NEURONS. NEUROARCHITECTURAL ALTERATIONS OF CEREBELLAR CORTEX IN PROLIDASE DEFICIENT MICE

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Many enzymes differently contribute to extracellular matrix (ECM) remodeling, being implicated in various physiological and pathological processes. The ECM of the Central Nervous System (CNS) surrounds neurons and glial cells providing a unique microenvironment. ECM not only has a passive structural property, but also actively influences cell proliferation, migration, differentiation, axonal guidance, synaptogenesis and homeostatic plasticity, during both development and adulthood. One special family-member enzyme linked to ECM remodeling is prolidase. The absence of prolidase activity characterizes a rare human autosomal recessive disorder called Prolidase Deficiency (PD) (OMIM 170100). The PD phenotype varies widely, spanning from the principal clinical feature, ulcers mainly located on the lower part of legs and feet, to different degrees of mental retardation. In 2008, a PD mouse model was found, name dal mice. The heterozygous and homozygous mice had cerebral and cerebellar morphological defects with a disorganized ECM profile. In this study, we further investigated the neurodegenerative aspects manifested by dal mice, focusing on the cerebellum and considering as principal marker the phosphorylated form of Tau (p-$Ser^{519/202}$). The Tau protein belongs to the family of Microtubule Associated Proteins (MAPs) and in its phosphorylated form is present in a lot of neurodegenerative disorders, like Alzheimer's disease and in other tauopathies. In order to identify when the alterations start to arise, our study had taken in consideration some critical phases of cerebellar postnatal development (postnatal day 10, 21, 30 and 60). Some of the Purkinje neurons were positive for phosphorylated Tau in both principal branches and cell soma. In those neurons, using MAP2 antibody, we observed a less extended, disorganized and thicker dendritic tree branches, as well as a decreased immunopositivity for calbindin, marker of morphology/functionality of Purkinje neurons. The surrounding basket cells axons, highlighted with neurofilaments marker, were found less structured and had fewer contacts with Purkinje neurons. Since prolidase is involved in ECM remodeling and integrity, it is essential for the correct neu-

ronal differentiation and synaptogenesis; absence or decrease in prolidase could trigger defects in Purkinje neurons differentiation. Further study to link morphological alterations with functional consequences are ongoing.

EVOLUTION OF VERTEBRATE GLYCINE TRANSPORTERS: INSIGHTS FROM AMPHIOXUS

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Glycinergic neurotransmission among invertebrates is not completely understood, and many more phyla must be studied before a complete picture of the evolution of this neurotransmitter can be reconstructed. The glycinergic neurotransmitter phenotype requires the expression of the glycine transporters (GLYTs) and vesicular transporter (VGAT) for proper neurotransmission. In vertebrates, GLYT1 and GLYT2 are the glycine transporters in CNS where they act as endogenous regulators of the dual functions of glycine: as a classical inhibitory neurotransmitter at glycinergic synapses and as a modulator of neuronal excitation mediated by NMDA receptors at glutamatergic synapses. Moreover, GLYT1 seems to be expressed both by glial and non-glycinergic neurons while GLYT2 is largely expressed in glycinergic neurons and in very few cases by astroglial cells in the spinal cord. In order to add more interesting data to the evolution of glycinergic neurotransmission, we have isolated the glycine transporters from the invertebrate chordate amphioxus and have analyzed their expression patterns throughout development. Amphioxus possesses three different genes encoding GLYT; such sequences show a high sequence identity with GLYT1 (AmphiGLYT1) or GLYT2 (AmphiGLYT2a and AmphiGLYT2b) of vertebrates. Moreover, two alternative transcripts of AmphiGLYT2a have been detected (AmphiGLYT2d and c). Phylogenetic reconstruction points out that the GLYT1 sequence from protochordates does not represent true orthologous to vertebrate GLYT1 but is an ancestral deuterostome glycine transporter from which both GLYT2 and vertebrate GLYT1 genes were derived. Such relationship seems to be confirmed by the expression patterns of AmphiGLYT1 and AmphiGLYT2a-d that resembles the expression domains of both vertebrates GLYT1 and GLYT2. Such functional differentiation suggests that glycine transporter evolution was driven by gene duplication and functional divergence. In amphioxus GLYTs transporters are likely expressed by different neurons types as well as glial-like cells. Finally, our data seems to support the dual role for glycine as inhibitory or excitatory neurotransmitters because amphioxus GLYTs are expressed by both VGAT and VGLUT positive neurons. Moreover, the presence of AmphiGLYT1 and AmphiGLYT2a before neuronal differentiation takes place, suggests an early pre-neural function of glycine in regulating neuronal differentiation, neuronal proliferation and synaptic modeling.

MONITORING CYTOTOXICITY AND INTRACELLULAR DISTRIBUTION OF MANGANESE-CONTAINING NANOPARTICLES BY CYTOCHEMICAL TECHNIQUES

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Magnetic resonance imaging (MRI) is a current non-invasive tool in daily clinics that exploits the properties of atom nuclei inside a living body[1]. However, MRI is characterised by a low sensitivity and different types of contrast agents have been developed to overcome this disadvantage[2]. At present, gadolinium (Gd) is the most commonly used MRI contrast agent but it must be administered in chelated form due to the high toxicity of its free form[3]. Manganese (Mn) is increasingly regarded as a promising substitute due to its T1 contrast ability comparable to Gd[4], and several Mn-containing nanoparticles (NPs) have been developed to be used as contrast agents adding them functionalities such as a targeting moiety, a second imaging component, or a therapeutic agent[5]. In the present work, we have synthesized MnO NPs encapsulated in micelles and we have tested them in human HeLa cells. To examine the cellular uptake, fluorescent micelles were prepared by adding rhodamine B dye. Two days after seeding, the cells were incubated with fresh medium containing MnO NPs at different concentrations; cell samples were observed after 1 to 24 h incubation. Cell viability was assessed by the Trypan blue exclusion test. Cell internalization was assessed by fluorescence microscopy and flow cytometry as well as by transmission electron microscopy after rodamine B photooxidation. Our results demonstrated that MnO NPs did not increase cell death at any of the concentrations used, even after long (24 h) incubation time. The MnO NPs were rapidly and massively internalised by HeLa cells already after 1 h incubation and their concentration progressively increases with time. The NPs were ubiquitously distributed in the cytoplasm but were never observed to make contact with any organelle or occur inside the cell nuclei. Moreover, the ultrastructural observations excluded any cellular damage. These promising data suggest that MnO NPs are worth of further investigations as suitable contrast agent for MRI.

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PLURIPOTENT STEM CELLS AS A MODEL OF *IN VITRO* NEUROGENESIS

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Embryonic stem (ES) cells are becoming a popular model of *in vitro* neurogenesis, as they display intrinsic capability to generate neural progenitors that undergo the known steps of *in vivo* neural development. These include the acquisition of distinct regional fates, which depend on the growth factors and signals

that are present in the culture medium. The control of intracellular signaling acting at different steps of ES cell neuralization, even when cells are cultured in chemically defined medium, is complicated by the endogenous production of growth factors. We found that during their neuralization, ES cells increased the expression of members of Wnt, FGF and BMP families. Conversely, the expression of Activin/Nodal and Shh ligands was low in early steps of neuralization. In this experimental condition, neural progenitors and neurons generated by ES cells expressed a gene expression profile that was consistent with a mid-brain identity. We found that endogenous BMP and Wnt signaling, but not FGF signaling, synergistically affected ES cell neural patterning, by turning off a profile of dorsal/telencephalic gene expression. Double BMP and Wnt inhibition allowed neuralized ES cells to sequentially activate key genes of cortical arealization and of cortical layers differentiation, whose expression was modulated by known signals acting *in vivo* such as FGF8 and Notch signaling. Moreover, in Wnt-inhibited cultures, Activin treatment during a narrow time window induced retinal fate, whereas in the same conditions Shh signaling promoted ventral diencephalic identity. We have recently focused our interests on the expression of mRNAs and miRNAs controlling cortical layering. Preliminary analysis of miRNA global gene expression highlights sets of miRNAs which might be crucial for regulating the sequential production of distinct pyramidal neurons during embryonic cortical development. All in all, our findings show that ES cells allow to investigate *in vitro* the molecular mechanisms controlling the acquisition of distinct neuronal cell fates.

PACAP MODULATES EXPRESSION OF HYPOXIA INDUCIBLE FACTORS IN THE EARLY PHASE OF DIABETIC RETINOPATHY

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The pathogenesis of diabetic retinopathy is not been completely elucidated, however retinal hypoxia seems to have a central role. This latter event is mediated by the Hypoxia Inducible Factors (HIFs), including HIF-1, HIF-2 and HIF-3. In a previous study, we observed the protective role of pituitary adenylate cyclase-activating peptide (PACAP) in early phase of diabetic retinopathy[1]. In the present work, we investigated whether PACAP effect in hyperglycemic retina is mediated through modulation of HIFs expression. The rats were made diabetic using a single injection of streptozotocin (STZ: 60 mg/Kg), whereas vehicle injected animals were used as control. Subsequently, a group of diabetic rats was subjected to either a single intravitreal injection of PACAP peptide (1 pM/L) or saline solution after 1 week from STZ injection. Changes in HIFs expression levels were then evaluated in the retinas after 3 weeks. The expression of HIF-1 and HIF-2 were significantly increased in diabetic rats as compared to controls. Instead, their expression levels were significantly decreased after PACAP intraocular administration, as detected by western blot analysis. Conversely, the expression of HIF-3 was significantly downregulated in retinas of STZ-injected rats as compared to the controls. However, its level was significantly increased in diabetic group after PACAP treatment. These data were supported by the immunohistochemical analysis. HIFs were present either in inner and outer retinal layers. Diabetes interferes with their distribution which is changed following intravitreal injection of PACAP. The present results suggest that the protective effect of this peptide in diabetic retina might also be mediated through modulation in the HIFs expression.

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INTERFERENCE OF NONYLPHENOL ON HUMAN ENDOMETRIAL CELL PHYSIOLOGY

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Nonylphenol (NP) is an industrial compound belonging to Endocrine Disrupting Chemicals (EDCs), a large group of synthetic or natural substances that can act as agonists or antagonists of endogenous hormones and represent a risk for human health. NP is a well known xenoestrogen found ubiquitously in the environment and its xenoestrogenic activity was demonstrated both by *in vitro* and *in vivo* studies. Since NP mimics endogenous estrogens, in this study we examined the effects of NP and 17-estradiol (E2) on the proliferation of endometrial adenocarcinoma cell line Ishikawa and their interaction with estrogen receptor ER alpha (ER) in order to highlight relationships between NP and endometrial pathologies. We assessed the effects of NP and E2 on Ishikawa proliferation after 24h of exposure through MTT assay. NP stimulated cells proliferation in a dose dependent manner and it had significant effect at concentration of 10^{-7} M. In contrast, E2 did not appear to affect significantly the cell viability, except for a slight increase observed at the concentration of 10^{-7} M. In order to study estrogen receptors involvement, we studied the cellular localization of ER after the treatment with NP and E2 for 30 min, 2h, 6h and 24h with immunofluorescence technique. Interestingly both NP and E2 act on ER cytoplasm-nucleus translocation but with different times. In fact, nuclear localization of ER was already shown after 30 min of treatment with E2 whereas NP induced ER translocation only after 2h and 6h. After 24h of treatment both NP and E2 seem to lose their action on the nuclear translocation of ER receptor. Finally through qPCR analysis we studied changing in gene expression of genes involved in proliferation pathways. We observed that only treatment with E2 induced upregulation of cyclin D1, cyclin E, p53, and ki-67 and it also induced an upregulation of pro-inflammatory cytochine IL-8. These results showed that NP stimulates cell proliferation but differently from E2, probably it may interfere with other molecular pathways activated in human endometrial cells.

ANALYSIS OF THE EXPRESSION OF CHOLINERGIC MARKERS IN THE CENTRAL NERVOUS SYSTEM OF EAE MICE BY *IN SITU* HYBRIDIZATION AND ENZYMATIC HISTOCHEMISTRY

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Experimental autoimmune encephalomyelitis (EAE) is an animal model of multiple sclerosis (MS) presenting neuroinflammation, axonal damage and demyelination, and T- and B-cell responses to myelin oligodendrocyte glycoprotein which produce a wide range of pro- and anti-inflammatory cytokines. Acetylcholine (ACh) is a neurotransmitter that shows neuroprotective effects and participates in the modulation of inflammatory response. EAE mice choline acetyltransferase (ChAT) levels are altered along the disease and are associated to the glial activation. EAE mice treated with acetylcholinesterase inhibitors generally present an amelioration of the clinical symptoms. The goal of this work was to determine the levels of mRNAs coding for cholinergic biosynthetic and hydrolyzing enzymes in the spinal cord and brain of EAE mice in remitting phase. EAE was induced in C57BL/6J mice by immunization with MOG_{35-55} in complete Freund`s adjuvant (CFA). Clinical signs and score were monitored up to the day of sacrifice which took place at day 29 after immunization. We used in situ hybridization histochemistry to determine expression levels of the mRNAs coding for the enzymes acetylcholinesterase (AChE) and ChAT as well as 4 and 7 nicotinic acetylcholine receptors (nAChRs), and compared them with mice injected with CFA. ChAT mRNA significantly increased and AChE mRNA generally decreased in some cholinergic nuclei. In parallel we measured AChE activity in tissue sections finding reduced levels, similar to the mRNA results. Butyrylcholinesterase (BuChE) activity did not show alterations when compared with control. nAChR4 and 7 mRNA levels significantly decreased in cholinergic interneurons and motor neurons. The analysis of the transcript levels for different cholinergic markers in brain and spinal cord of EAE mice has been also quantified by qRT-PCR. The results suggest that in this phase of the disease, the cholinergic system seems to trigger ACh synthesis. The increase of cholinergic activity might play a relevant role both in the regulation of nervous system activity that in decreasing the neuroinflammation.

6 mT STATIC MAGNETIC FIELD INDUCES U937 CELLS DIFFERENTIATION *IN VITRO*

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The effect of 6 mT homogeneous static magnetic field (SMF)-exposure on the differentiation of U937 monocytes was tested *in vitro*. The 50 ng/mL 12-O-tetradecanoyl-13-phorbol acetate (TPA) was used to induce differentiation and morphological and biochemical parameters have been evaluated. The morphology of U937 cells, plasma membrane exposure and distribution of specific set of molecules (macrophagic markers CD11c and CD14, phosphatidylserine, and sugar residues), cytoskeleton actin filaments reorganization (F-actin component) and distribution and activity of lysosomes, mitochondria, and Smooth Endoplasmic Reticulum (SER) were investigated. Finally, intracellular Ca^{2+} levels were also evaluated by fluorimetric analysis. The SMF exposure during TPA-induced differentiation does not affect cell viability. A time-dependent decrement of differentiation degree in SMF-exposed U937 cells was observed. U937 cells showed cell shape and F-actin modification, inhibition of cell attachment, plasma membrane roughness, blebs appareance on cell surface and alteration in cell surface expression of molecules upon SMF exposure. The intracellular redistribution and polarization at the cell periphery of the SER, mitochondria and lysosomes and alteration of function were observed. An important increase in intracellular Ca^{2+} concentration was observed that probably mediates the above-described changes. In summary, the exposure of U937 cells to 6 mT SMF during TPA-stimulated differentiation causes morphofunctional modifications. Thus, deeper studies are need to clarify the potential harmful consequences for human health of SMF exposure.

EMBRYONIC DEVELOPMENT OF THE ANNUAL FISH *NOTHOBRANCHIUS FURZERI*, THE SHORTEST-LIVED VERTEBRATE

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Annual killifishes inhabit temporary ponds in regions subject to the monsoon and their embryos survive the dry season encased in the mud by entering diapause at the mid-somite stage, a process that arrests embryonic development during hostile conditions. *Nothobranchius furzeri* is an annual killifish adapted to the harshest condition and its natural lifespan is in the order of a couple of months. Here we investigated early cleavage and the transition to diapause in *Nothobranchius furzeri*. Early cleavage of teleost embryos is characterized by a very fast cell cycle (15-30 min) and lack of G_1 and G_2 phases. We used time lapse brightfield microscopy to investigate cell division kinetics during the first developmental stages of several annual- and non-annual species of killifish. All annual killifishes showed cleavage times significantly longer when compared to their non-annual sister taxa (average 35 min *vs.* average 75 min). Epiboly of annual fish is characterized by a dispersed phase when blastomeres migrate randomly for days before aggregating to form the embryonic axis. To visualize cell-cycle dynamics we used fluorescent ubiquitination-based cell cycle indicator (FUCCI) system both after microinjection of mRNAs or in stable transgenic lines. We demonstrate that the first 5 division are synchronous and do not show a G1 phase. Cell cycle synchronization is lost after the 5th cleavage division. We further document the dispersed phase, aggregation of blastomeres entry and exit from diapause. Finally, we studied the effects of temperature on diapause entry by incubating eggs at 28, 24 or 22°C. At 28°C all eggs skipped diapause and developed without stopping. At 24°C about 50% of the eggs entered diapause and at 22°C all eggs stopped during the dispersed phase and did not reach the diapause stage.

D-ASPARTATE PROMOTES THE PROLIFERATIVE PATHWAYS IN GC-1 SPG CELLS

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D-aspartate (D-Asp) is an endogenous amino acid present in vertebrate tissues, with particularly high levels in the testis[1]. *In vivo* studies indicate that D-Asp induces spermatogenesis by activation of hypothalamic-pituitary-gonadal axis inducing the release of GnRH, which in turn elicited LH secretion from the pituitary gland[2]. Further *in vitro* studies demonstrated that D-Asp up-regulates testosterone production in Leydig cells by stimulating gene and protein expression of steroidogenic acute regulatory, which is a key regulatory factor of cholesterol translocation to the inner mitochondrial membrane[3]. To investigate a direct role of D-Asp in spermatogenesis, in this study, a cell line derived from immortalized type B spermatogonia that retains markers of mitotic germ (GC-1 spg) was incubated in a medium containing D-Asp (200 μM) for 0-4 hours. The activities of ERK and Akt, as pathways of cell proliferation, as well as the protein expressions of proliferation cell nuclear antigen (PCNA) and Aurora B, mitotic activity markers, in D-Asp treated cell lysates were compared with controls. Further, we investigated 17-estradiol (E_2) levels as well as P450-aromatase (P450-aro) and estrogen receptor (ER) protein expressions. The results show that D-Asp induces the phosphorylation of ERK and Akt proteins as well as an increase of PCNA and Aurora B expressions. Further, the E_2 increased levels and an enhancement of both P450-aro and ER protein expressions in D-Asp treated cells were observed. In conclusion, present results for the first time demonstrate a direct effect of D-Asp on spermatogonial proliferation. Particularly, the amino acid could induce spermatogonial mitotic activity through ER-mediated ERK and Akt pathways. Since in a previous study[4] we demonstrated that spermatogonia express NR1 and NR2 subunits of NMDA receptor, we hypothesize that D-Asp could be directly involved in spermatogenesis through NMDA receptor activation. Being D-Asp endogenously present also in spermatocytes, spermatides and spermatozoa, it would be interesting to investigate the role of this amino acid in each step of germ cell maturation.

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LINKING ENDOPLASMIC RETICULUM STRESS TO NEURODEVELOPMENTAL DISORDERS

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Autism spectrum disorders are a group of neurodevelopmental disorders with a strong genetic background. One of the most characterized autism-linked mutations is the R451C substitution in the synaptic protein Neuroligin3 (NLGN3). The mutation induces a local misfolding in the extracellular domain causing the retention of NLGN3 in the Endoplasmic Reticulum (ER)[1]. The presence of misfolded protein in the ER can lead to the activation of the Unfolded Protein Response (UPR), implicated in several neurological diseases and in the regulation of neurotransmission and plasticity[2]. Our aim is to ascertain whether the ER retention of the R451C NLGN3 mutant protein activates the UPR. We have generated a new PC12 Tet-On model system with inducible expression of NLGN3, either wild type or R451C proteins, for studying the UPR signaling in time-course experiments. PC12 clones were characterized for NLGN3 expression, by western blots and immunofluorescence. Wild type NLGN3 protein is correctly trafficked to the cell surface, with the R451C NLGN3 being retained in the ER, as shown by sensitivity to endoglycosidase H. Our results indicate that PC12 clones expressing the R451C mutant NLGN3, activate all UPR signaling pathways downstream of the ATF6, IRE1 and PERK stress sensors. Synthesis of R451C NLGN3 induces the up-regulation of UPR target genes, such as BiP and CHOP, before and after differentiating the cells to a neuronal phenotype. In order to understand the potential role of UPR in neurodevelopmental disorders, we are currently investigating its activation in the Knock In mouse model of autism, carrying the R451C mutation in the NLGN3 endogenous gene. Our data represent the first evidence on the effects of the R451C NLGN3 in activating the UPR and represent a solid link between UPR and neurodevelopmental disorders characterized by the retention of misfolded proteins in the ER.

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OCTYLPHENOL AND ESTRADIOL EFFECTS ON HUMAN PROSTATE CELLS

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In the present work we have studied the effects of Octylphenol (OP), Estrogen (E2) and their mixture (OP+E2) on prostate adenocarcinoma epithelial cells (LNCaP) in order to highlight estrogen and xenoestrogen influence on human prostate. Although androgens are the most important hormones in the normal development of the male reproductive system, more recently, it has been suggested a central role for estrogen in male reproductive system. In this view, current epidemiological reports led to the "estrogen thesis" hypothesizing that high level of estrogens may disturb the endocrine control of the male reproductive capability. OP is the estrogen mimicking chemical belonging to alkylphenols. It is an important industrial agent that represents potentially harmful effects to both the ecosystem and human health. This chemical is used in detergents, paints and pesticides and as a result of usage detected in rivers, sewage treatment plants and drinking water. OP belongs to endocrine disrupting chemicals (EDCs), an heterogeneous group of ubiquitous synthetic or natural substances that function like endogenous hormones and disturb the normal biological functions of the endocrine system. We found that both OP and E2 and their mixture were able to increase cell proliferation meas-

ured through MTT assay. Specifically, E2 highly increased LNCaP proliferation at concentration of 10^{-9} M, OP increased LNCaP proliferation reaching the most notable effect at concentration of 10^{-10} M. In order to study estrogen receptor involvement, we have performed a western blot analysis demonstrating that E2 and OP+E2 act inducing an increase of ER receptor whereas OP induced an increase of ER expression suggesting a double activation manner of estrogen pathway. Moreover, we have investigated gene expression of several molecular targets of estrogen pathway through Real Time PCR. We have observed that OP induced an upregulation of Ki-67 whereas E2 induced an upregolation of cyclin D1, genes involved in cell proliferation. On the contrary, OP, E2 and OP+E2 downregulated p53 in accordance with the pro-apoptotic role of this gene. Finally, we have observed an upregulation of proinflammatory cytochines IL-1 after OP treatment. These results confirm the estrogenic activity of OP and suggest that xenoestrogenic EDCs may interfere with molecular pathways of prostate physiology.

MUSCLE DERIVED PERICYTES FOR ARTIFICIAL SKELETAL MUSCLE HUMAN-LIKE SIZE

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Stem cells and regenerative medicine have greatly increased the expectations of the scientific community and the public for recovering or replacing ablated, injured, aged and diseased tissues. Nevertheless their clinical application for tissue and organs regeneration is currently hindered by problems related to cell survival, inflammatory response, tissue engraftment, vascularization and efficient differentiation. Tissue engineering exploiting biomaterial and stem cells aims to mimic organogenesis in promoting improvement of stem cell engraftment and differentiation. Skeletal muscle tissue engineering is up-and-coming biotechnology that could offer great potential in the near future for muscle repair, but it remains still unsatisfying. Reconstructing the skeletal muscle architecture and function is still a challenge requiring parallel alignment of myofibrils arranged into organized sarcomeres; moreover the new muscle must be vascularized, innervated and it must integrate with host tissue. We show that an anatomical bioreactor-like, represented by the surface of the tibialis anterior muscle (TA), influences maturation and alignment of fibers derived from adult muscle stem/progenitor cells embedded into a poly-ethylene-glycol-fibrinogen (PF) gel, leading to the generation of an artificial normal new muscle[1]. Furthermore by the same approach we succeeded in replacing a complete mouse TA after a massive muscle ablation, recovering morphology and function of the substituting artificial TA[1]. Starting from these observations, we are developing a novel approach for regeneration and/or reconstruction of skeletal muscle tissue segments human-like size in order to translate this technique to clinical application. For this purpose human derived muscle pericytes have been isolated from muscle biopsies and have been investigated for their myogenic potential. Moreover by exploiting the PF property, we demonstrated the noteworthy potential of this cell population for human skeletal muscle tissue engineering.

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SINGLE CELL ANALYSIS OF POPULATION HETEROGENEITY IN RHABDOMYOSARCOMA

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Rhabdomyosarcoma (RMS) is the most common type of soft tissue sarcoma in children and adolescents, under 20 years of age, with an incidence of 4.5 cases per million children/adolescents per year. Rhabdomyosarcoma tumors are classified based on their histology as embryonal, alveolar or other subtypes. The two major subtypes of RMS, embryonal (eRMS) and alveolar (aRMS), differ markedly in their outcome and also in the molecular pathogenesis. Despite these differences, the origin of aRMS and eRMS seems to be, in most cases, the same. The precise cell type that initiates RMS is unclear. Some evidence point to skeletal muscle precursors failing to undergo appropriate terminal differentiation as likely candidates. Other theories support mesenchymal cells as a tumor initiating cell. Thus, unlike other tumors, there is still some uncertainty, about the cell type that initiates rhabdomyosarcoma. Given the inter/intra tumor heterogeneity the identification of the cell type initiating the tumor is a difficult task. The goals of our project are twofold. On one hand we want to identify the cell population(s) that initiate the tumor. On the other hand we want to describe the phenotypic diversity of the cell populations in healthy muscles and compare it with that of a tumor derived from the same tissue, the rhabdomyosarcoma. Since a single-cell approach is critical for studying the diversity of cancer cell populations, we have selected to use mass cytometry, which offers the ability to simultaneously determine up to 40 biomarkers in single cells, thus allowing to characterize extremely rare cell types in mixed populations. For our project we adopt the KrasG12D/+Trp53Fl/Fl conditional mouse model in which the undifferentiated pleomorphic myosarcomas are induced in a spatio-temporal controlled manner by using an adenovirus vector expressing the CRE recombinase.

THE OBESE ZUCKER RATS: AN *IN VIVO* MODEL FOR STUDYING THE LIVER CARBONIC ANHYDRASE CAVII BIOLOGICAL ROLE

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CAVII is a physiologically relevant isoform of the enzyme carbonic anhydrase with high CO_2 hydration activity. In humans and rodents, CAVII is a cytosolic isoenzyme mainly localized in brain tissues where it contributes in generating neuronal excitation. Recently, S-glutathionylation of two cysteine residues from the hCAVII was reported and the effect of native and tetramutated hCAVII in a cell culture was analyzed by stressing cells with an oxidant agent. Results suggested that hCAVII could function as an oxygen radical scavenger for protecting cells from oxidative damage. Here, we aimed to investigate the CAVII protective role toward oxidative insult *in vivo*, using obese Zucker rats (OZRs) as an animal model. The genetically obese (fa/fa) Zucker rats, due to a recessive mutation of the leptin

receptor gene, exhibit hyperphagia and develop hallmark features of metabolic syndrome. The study was performed on the liver of OZRs and their lean counterparts (LZRs) at different ages (12, 16, and 20 weeks from birth; 6 animals each group). Serum values of glucose, insulin, total cholesterol and triglycerides were measured as parameters of dismetabolism. Thiobarbituric acid reactive substances (TBARS) were evaluated in serum and liver samples as a marker of oxidative stress. A specific polyclonal anti-CAVII serum (from S. Parkilla, Finland) was applied for either Western blot analysis and immunohistochemical investigation, this latter performed on 5 μm thick sections from paraffin embedded liver samples. The TBARS levels in OZRs where higher than in the LZRs, confirming the oxidative stress in the obese rats. At all stages examined, CAVII proved to maintain its expression in the liver of obese rats. Indeed, in the immunoblots a band with a MW consistent with that known for CAVII could be identified in either OZR and LZR lysates. An additional higher MW band, detected in all samples, requires to be further characterized. Differences in the immunohistochemical patterns, however, suggested a distinct CAVII distribution in the liver of OZRs, compared with LZRs. Unlike a weak, diffuse staining found in LZR liver parenchyma, a marked cytoplasmic staining was localized in some OZR hepatocytes zonally distributed in the hepatic lobules, in close association with cells showing morphological features of steatosis. Studies are in progress to clarify a possible relation of such preliminary findings to the proposed role of CAVII in oxidative stress.

A NEURONAL MODEL OF THE NEURODEGENERATIVE DEMENTIA FENIB

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The serpinopathies are pathologies caused by mutations that promote polymerisation and intracellular deposition of proteins of the serpin superfamily, leading to cell toxicity and death[1]. The dementia FENIB (familial encephalopathy with neuroserpin inclusion bodies) is caused by mutations in the neuronal serpin neuroserpin (NS) that lead to its polymerisation within the endoplasmic reticulum (ER) of neurons[2,3]. Our aim is to understand how NS polymers accumulate within the ER and what is the mechanism of their cellular toxicity. To create a neuronal model of FENIB, we have generated stably transfected neural progenitor cell lines from mouse brain cortex, expressing the control protein GFP (green fluorescent protein) or human NS in three different versions: wild type, the polymerogenic mutant variant G392E that causes severe FENIB, and deltaNS, a truncated version of NS used here as a misfolding, non-polymerogenic control. We have characterised these cells in the proliferative state and after differentiation to neurons using RT-PCR, SDS and non-denaturing PAGE and western blot, ELISA and immunofluorescence. Our results show that wild type NS is secreted as a monomeric protein into the culture medium, while G392E NS forms polymers that are mostly retained within the ER of neural cells. DeltaNS is absent at steady state due to its rapid degradation, but it is easily detected upon proteosomal blocking. Regarding intracellular distribution, wild type NS is found in partial co-localisation with ER and Golgi markers, while G392E NS fully co-localises with an ER marker. We have used our cellular model of FENIB to perform a transcriptomic comparison of cells expressing GFP and G392E NS by RNAseq and found almost 2000 genes with altered expression at significant levels, which upon bioinformatics analysis fall in different pathways potentially relevant to the pathological mechanism of the dementia FENIB. We have created a neuronal model system that recapitulates the main features of FENIB, which we have used to detect the alterations in gene expression caused by intracellular accumulation of NS polymers, and we are now characterising the most promising candidate genes to discover novel cellular responses to the presence of NS polymers within the ER of neurons.

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AIMING TO UNDERSTAND THE MECHANISMS UNDERLYING THE DIFFERENT RESPONSES TO BERBERINE AMONG DIFFERENT CELL LINES

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Berberine, a bioactive natural isoquinoline alkaloid, is known to generate a variety of pharmacological effects in different cell types. Because of its ability to arrest the cell cycle and cause apoptosis in several malignant cell lines, berberine has received attention as a potential anticancer agent. To investigate the mechanisms underlying the different responses to berberine, we started to analyze its dose-dependent and time-dependent intracellular localization in two human tumor cell lines: MIA PaCa-2 (from pancreatic carcinoma), U343 (from glioblastoma). Human dermal fibroblasts (HDF) were used as a non-tumor control. Berberine presents natural green fluorescence, which allows identification of the intracellular site of accumulation in living cells. We found that the alkaloid may accumulate in different cell compartments, with a dynamic dose-dependent and time-dependent pattern of localization. The results revealed different localization of berberine in cytoplasm and mitochondria and/or nuclei in cancer cells with respect to non-tumor cells. Moreover, berberine treatments reduced cell viability in a cell line-specific manner. To further investigate the effects of berberine, the expression profile of genes involved at different levels in fundamental biological processes, was analyzed. As tumor suppressor genes are often methylated in the process of carcinogenesis, we evaluated DNMT1 and DNMT3B coding for maintenance and *de novo* methyltransferases, respectively. Additionally, MGMT, a geneencoding O6-methylguanine-DNA methyltransferase, and recognized to play a crucial role in the defense against chemotherapy alkylating agents, was analyzed. As literature data demonstrate that berberine may induce apoptosis in cancer cells but not in normal cells and this observation is valuable for development of new anti-cancer therapies, we also compared the variation in the gene expression level of cysteine-aspartic acid protease 3 (CASP3) and the activity of this enzyme in MIA PaCa-2, U343 and HDF cell lines. On the whole

the results indicate that berberine differentially affects the behaviour of MIA PaCa-2, U343, and non-tumor HDF cells.

PROGESTERONE RECEPTOR MEMBRANE COMPONENT 1 (PGRMC1) ACTION DURING BOVINE OOCYTE MEIOSIS: A FUNCTIONAL STUDY

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Progesterone Receptor Membrane Component 1 (PGRMC1) localizes to the centromeres at metaphase-I and II stages of bovine oocyte meiotic division, while it concentrates between the separating chromosomes at ana/telophase-I stages. This localization suggests an essential role for PGRMC1 during meiosis. The aim of the present study is to expand these morphological observations by using small-interfering RNA (RNAi) mediated gene silencing. Cumulus-Oocytes Complexes were collected from ovarian follicles and microinjected to deliver PGRMC1 or CTRL-RNAi into the oocytes cytoplasm, kept in meiotic arrest for 18 h then *in vitro*-matured (IVM) for 24 h. After IVM, efficacy in depleting PGRMC1 expression was assessed by quantitative RT-PCR and western blotting. These analysis revealed a significant 30% reduction of both mRNA and protein expression. After treatment, the oocyte capability to extrude the first polar body (PBI) and the morphology of the MII plates were considered as biological end-points. Our data indicated that PGRMC1's down regulation determines a significant impairment of the oocyte's capability to complete meiotic division. In fact, we observed a 22% reduction of the oocytes that extruded the PBI ($P<0.05$), which was accompanied by altered chromosomes segregation and increase in chromosomal scattering. These results are consistent with PGRMC1 localization and with a putative role during both chromosome segregation and cytokinesis. We hypothesize that altering PGRMC1 function impairs the process of chromosome separation and/or PBI formation that can result in genetic instability.

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IMMUNOHISTOCHEMICAL DETECTION OF AGE-RELATED CHANGES IN HUMAN PERIOSTEAL DERIVED STEM CELLS

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There is a lot of interest in the understanding of possible age-related changes in Mesenchymal Stem Cells (MSCs) in view of their use for regenerative medicine applications also in the elderly. In particular, considering the key role of periosteum in bone biology[1,2] and to acquire data for a cell-based therapy promoting graft osseointegration, we tried to identify specific aging markers and pattern of expression in human periosteal precursor cells (PDPCs). To this aim periosteal tissue was obtained from 8 differently aged healthy subjects, gender matching: two subjects had a mean age of 16 years, two of 28, two of 63 and two of 92 years. PDPCs were isolated from the minced explants of periosteum positioned with their cambium side placed against the dishes; cells were then allowed to adhere in standard cell culture conditions as previously described[3]. Immunohistochemical detection of Ki67 and p53, Nitric Oxide (NO) production and qRT- PCR of a selected gene panel for osteoblastic differentiation (bmp2 and runx2) and bone remodeling (IL-6, RANKL and OPG) were evaluated. Our data evidenced a low percentage of proliferating PDPCs isolated from aged patients (63 and 92 years) compared to the younger ones (16 and 28 years). In contrast to Ki67, we found that p53 expression was statistically higher in PDPCs from patients aged 92 years compared to PDPCs from subjects aged 28 and 16 years. As far as NO release is concerned, our data showed higher levels in PDPCs isolated from the elderly and a good correlation with the immunohistochemical analysis. At last qRT-PCR data indicate that periosteal cell populations modify their potential with age, with a trend toward bone resorption. In conclusion we found that both Ki67 and p53 represent striking markers of cell-cycle arrest in these cells and that their expression correlates with NO production. In addition, age affects genes involved in bone remodeling, with a significant increase in IL-6 mRNA expression as well as RANKL/OPG ratio. We believe that this study, taking into account on age-related changes in human PDPCs, paves the way toward new regenerative medicine strategies in bone aging and/or bone metabolic diseases.

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ANALYSIS OF MICRORNAS IN MEN1 PARATHYROID ADENOMAS IDENTIFIED AN EMBRYONIC STEM CELL-LIKE EXPRESSION SIGNATURE ORGANIZED AS GENE REGULATORY NETWORKS

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The hallmark traits of stem cells - self-renewal and differentiation capacity - are mirrored by the high proliferative capacity and phenotypic plasticity of tumor cells. Moreover, tumor cells often lack the terminal differentiation traits possessed by their normal counterparts. These parallels have given rise to the hypothesis that tumors often arise from undifferentiated stem/progenitor cells, or alternatively, that cancer cells can undergo progressive de-differentiation during their development. It has been hypothesized that the similarities shared by stem cells and cancer cells might relate to shared patterns of gene expression regulation, which might be associated with embryonic state. In order to further investigate the molecular mechanisms involved in MEN1 parathyroid tumorigenesis we performed microarray analysis of global miRNA expression profile in eight MEN1 parathyroid adenomas (four with LOH at

11q13 locus and four still retaining one copy of wild type MEN1 gene) and in a pool of two non-MEN1 sporadic parathyroid adenomas and two non-MEN1 normal parathyroid glands as controls. MiRNA expression has also been investigated in LOH MEN1 parathyroid adenomas with respect to non-LOH MEN1 parathyroid adenomas. Our study identified new miRNAs involved in the MEN1 parathyroid neoplasia, organized as GRNs with genes associated to the development of different inheritable forms of parathyroid tumors. Interestingly, microRNAs identified in parathyroid tumors showed correlation with embryonic transcription factors. These data reinforced also the embryonic signature hypothesis for parathyroid tumor tumorigenesis.

HMGA AND *PATZ* GENES ARE INVOLVED IN XENOPUS NEURAL CREST DEVELOPMENT

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HMGA and PATZ are chromatin remodeling factors that are involved in several forms of human cancer. In particular, HMGA1 and HMGA2 play an active role in the epithelial-mesenchymal transition (EMT) during tumor invasion; PATZ1 may likely play a similar role. We found that in the Xenopus genome only hmga2, but not hmga1, is present. During embryogenesis hmga2 is expressed mainly in the developing central nervous system (CNS) and in the neural crest cells (NCCs). Although hmga1 is absent, we found three divergent AT-hook sequences (Hmg-AT-hook1, 2, 3) within the Xenopus genome. We showed that their mRNAs are expressed similarly to hmga2, though at a lower level. PATZ1, a POZ, AT-hook and zinc-finger containing protein, is a described interactor of HMGA1 and HMGA2. Also patz1 is expressed in Xenopus NCCs. NCCs are a special cell population that undergoes EMT by employing very similar molecular mechanisms to those involved in tumor progression; they represent an *in vivo* embryonic model where to address genetic pathways that underlie cell invasiveness in tumor progression. We have studied the role of hmga2 within the genetic network driving NCC specification, EMT and migration, by gain and loss-of function approaches. We have also studied the role of Hmg-at-hook 1,2,3 in NCCs. Our results show that hmga2 plays a crucial role in NCCs specification and subsequent EMT and migration. Knock-down of hmga2 results in phenotypes where the pharyngeal skeleton is strongly disrupted, and this is anticipated by strong downregulation of NCC relevant genes. Knock-down of hmg-at-hook genes results in much milder phenotypes. We are currently studying patz1 function in a similar approach.

ROLE OF VASOPRESSIN IN THE GROWTH OF BILIARY EPITHELIUM IN EXPERIMENTAL CHOLESTASIS AND IN POLYCYSTIC LIVER DISEASE

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The neurohypophysial hormone arginine vasopressin (AVP) acts by three distinct receptor subtypes: V1a, V1b, and V2. In liver, AVP is involved in ureogenesis, glycogenolysis and neoglucogenesis, and regenerative processes. Cholangiocytes are the target cells in a number of animal models of cholestasis including bile duct ligation (BDL) and in several human pathologies such as polycystic liver disease (PLD) characterized by the presence of numerous cysts within the liver that bud from biliary epithelium. No data exist about the presence of AVP and receptors in biliary epithelium. For that reason, our aim has been to evaluate the role of AVP in experimental model of cholestasis and in course of PLD. *In vivo*, normal and BDL liver fragments from mice and rats plus normal and PLD from human patients were collected to evaluate: (i) intrahepatic bile duct mass (IBDM) by immunohistochemistry for citokeratin-19 (CK-19); and (ii) expression of V1a, V1b and V2 by immunohistochemistry, immunofluorescence and real time PCR. *In vitro*, small and large mouse cholangiocytes, H69 (non-malignant human cholangiocytes) and LCDE (human cholangiocytes from cystic epithelium) were stimulated with AVP in the absence/presence of antagonists such as OPC-31260 and Tolvaptan, before assessing cellular growth by MTT proliferation assay, cAMP levels by a RIA kit. Cholangiocytes express V2 receptor that was upregulated following BDL and in course of polycystic disease. Administration of AVP to cell cultures increased proliferation, cAMP levels of small cholangiocytes and LCDE cells. We found no significant effect in proliferation of large mouse cholangiocytes and H69 cells. Increases that were blocked by pre-incubation with the AVP antagonists. These results showed that AVP play an important role in growth of the biliary epithelium during cholestasis and in cystic epithelium in course of PLD acting on the cAMP signalling pathway and it may be considered an important target factor in the management of biliary growth in course of cholangiopaties.

SILENCING MICAL2 REPRESSES HUMAN CANCER CELL GROWTH AND INVASION INDUCING MESENCHYMAL TO EPITHELIAL TRANSITION

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MICAL (Molecules Interacting with CasL)2 belongs to an evolutionarily conserved family of proteins that catalyze actin oxidation-reduction reactions destabilizing F-actin in cytoskeletal dynamics. Here we show for the first time that MICAL2 mRNA is significantly over-expressed in aggressive, poorly differentiated/undifferentiated, primary gastric and renal human epithelial cancers. Immunohistochemistry showed MICAL2-positive cells on the cancer invasive front and in metastasizing cancer cells inside emboli, but not at sites of metastasis, sug-

gesting MICAL2 expression was "on" in a subpopulation of primary cancer cells seemingly detaching from the tissue of origin, enter emboli and travel to distant sites, to be turned "off" once homing at the metastatic site occurred. *In vitro*, MICAL2 knock-down was clearly associated with induction of mesenchymal to epithelial transition, causing reduction of viability and loss of motility and invasion properties of human cancer cells. Moreover, expression of MICAL2 cDNA in MICAL2-depleted cells induced epithelial to mesenchymal transition. All together our data indicate MICAL2 over-expression is associated with cancer progression and metastatic disease. MICAL2 might be an important regulator of epithelial to mesenchymal transition and therefore a promising target for anti-metastatic therapy.

HISTONE DEMETHYLATION BY JHDM1D REGULATES PHOTORECEPTOR GENERATION IN XENOPUS RETINA

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The histone demethylase JHDM1D, also known as KDM7 and KIAA1718, catalyzes demethylation of both mono- or dimethylated H3K9 and H3K27, epigenetic marks associated with transcription repression. Although this chromatin modifier has been shown to control neural induction and differentiation, its role in retinal development remains unexplored. In this study, we address the retinal function of JHDM1D taking advantage of specific features of the *Xenopus laevis* model system. JHDM1D is expressed in the eye field and in retinal progenitors of optic vesicles and cups. JHDM1D overexpression in the early eye field does not significantly affect the retinal expression of markers of cell proliferation and differentiation or the expression of retinal progenitors markers. However, when JHDM1D is injected in a 16-cell stage blastomere fated to give rise partially to the retina, the generated retinal clones display an increase of photoreceptors and a decrease of bipolar cells, compared to control GFP injected embryos. Late overexpression, obtained by lipofecting retinal precursors of optic vesicles with JHDM1D cDNA, yields the same results. Furthermore, immunostaining with a rod-specific antibody shows that JHDM1D overexpression leads to a significant increase in rod-to-cone ratio. Intriguingly, JHDM1D knockdown also leads to an increase of photoreceptors, although without changing the rod-to-cone ratio. These results suggest that the balance between methylated and demethylated H3K9 and H3K27 controlled by JHDM1D is a crucial component of a histone code leading to photoreceptor specification. We are currently assaying the functional interactions between JHDM1D and JMJD3, a histone demethylase that removes a methyl group from trimethylated H3K27, thus providing further substrates for JHDM1D.

DEVELOPMENTAL DEFECTS INDUCED BY GADOLINIUM IONS IN SEA URCHIN EMBRYOS OF PHYLOGENETICALLY DISTANT SPECIES

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Gadolinium (Gd) is nowadays an emergent environmental pollutant: it is a metal of the lanthanide series of the elements whose chelates are commonly employed as contrast agents for magnetic resonance imaging and subsequently released into the aquatic environment. Sea urchin embryos are highly sensitive to several kinds of stressors and able to activate different defense strategies. The aim of this study was to analyze the consequences of embryo exposure to sublethal Gd concentrations. We compared the effects of Gd on the development of four phylogenetically distant sea urchin species: two Mediterranean species, *Paracentrotus lividus* and *Arbacia lixula*, and two species living in the East coast of Australia, *Heliocidaris tuberculata* and *Centrostephanus rodgersii*. In all these species, we observed a general delay of embryo development at 24 h post-fertilization (gastrula), and a strong inhibition of skeleton growth at 48 h (pluteus), frequently displayed by an asymmetrical pattern. Since it was demonstrated that the autophagic and apoptotic processes are important defense mechanisms against stress in *P. lividus* embryos[1-3], we further investigated the induction of these two processes. Autophagy in *P. lividus* embryos was inspected by acridine orange (AO) vital staining, Western Blot analysis of total lysates and LC3 (an autophagic marker) immunofluorescence on whole-mount embryos. Overall, the results showed an increase of the LC3 protein at 24 and 48 h and confocal microscopy confirmed the increased number of autophagosomes and autophagolysosomes. In contrast, the study of apoptosis performed by immunofluorescence (IF) staining using an anti-cleaved-caspase-3 antibody on whole-mount embryos after 24h and 48h exposure showed no apoptotic induction. In conclusion, the results showed that Gd highly perturbs skeletogenesis in the four species analyzed, even if with species-specific threshold levels of sensitivity, and that autophagy is a molecular process activated in *P. lividus* embryos, probably acting as a cell survival strategy to defend the developmental program. Taken together, the results pose serious questions on the hazard of Gd in the marine environment.

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UNUSUAL METHYLATION ON NASCENT RNA FIBRILS AND STORED RNA GRANULES

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DNA methylation is an epigenetic modification occurring at CpG islands in the regulatory region of genes. It induces reversible transcriptional silencing and is involved in several biological processes. Its alteration often occurs during neoplastic transformation. While methylation is widely investigated in DNA, it is a newly described process in mRNA and ncRNA and its funtion is still unclear[1]. So far, no data are available at electron microscopy on the detection of DNA methylation levels. In order to investigate the subcellular localization of methylated bases, we have identified immunocytochemically 5-methylcytosine (5mC) at transmission electron microscopy and also at fluorescence microscopy level. Specific staining methods have been used to discriminate between DNA and RNA. DNA methylation was observed on heterochromatin and this result was verified performing DNA-staining with osmium ammine. 5mC-immunolabelling was evident on metaphase chromosomes too, thus confirming that the ultrastructural localization of this DNA modification obtained with this approach was correct. Interestingly, a not negligible signal was also detectable in perichromatin granules and at the border of condensed chromatin areas, where terbium staining revealed fibrils of nascent RNA[2]. After fluorouridine incorporation, we used anti-5mC and anti-fluorouridine antibodies to ascertain whether newly synthesized RNA was methylated. Moreover, the presence of labelled RNA was proved by a double labelling for hnRNPs and 5mC. Latter data indicate that hnRNA and probably mRNA can display this epigenetic marker. Given the localization of 5mC both on nascent (perichromatin fibrils) and on stored (perichromatn granules) RNA, it seems reasonable that methylation might be a sort of signal, although we do not know to which process it is linked or if any pathological pathway is involved.

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ROLE OF MIR-7 IN NEURAL DEVELOPMENT OF THE ASCIDIAN *CIONA INTESTINALIS*

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MicroRNAs are small, non-coding RNAs of 21-23 nucleotides that regulate gene expression at post-transcriptional level[1]. miR-7 mature sequence is evolutionary conserved across Bilateria[2]. During embryo development miR-7 neural expression was reported in mouse and teleosts as well as in animals distantly related as annelids. The majority of studies involving this microRNA were focused on its role in cancer, where miR-7 seemed to be context-specific as evidence exists for both oncogenic and tumor suppressor roles[3]. The first *in vivo* studies were performed on fly and demonstrated that miR-7 mutant flies develop defects in sensory structures only under stressful conditions[4]. In human neural stem cells miR-7 overexpression induced an increase of synapsin gene expression in the derived neurons playing an important role in synaptic formation[5]. A miR-7 sequence, identical to human one, is also present in the ascidian *Ciona intestinalis* genome. We investigated miR-7 expression during *C. intestinalis* development by *in situ* hybridization with LNA probes. Mature transcripts were diffuse in early embryos from gastrula to neurula stage. In late tailbud stage expression was restricted into a population of mesenchyme cells of the trunk while in late larva miR-7 was present in tail peripheral neurons. miR-7 function was studied by injecting anti-miR-7 Peptide Nucleic Acids (PNAs) in unfertilized eggs. PNAs are synthetic pseudopeptides bearing the four nucleobases mimicking the antisense oligonucleotide base-matching properties. The hatching ratio of control and injected samples was not affected by anti miR-7 PNA injections but morphological alterations were observed. Knockdown embryos displayed rounded trunk region, smaller than controls. In situ hybridization experiments revealed that although the nervous system was differentiated properly the expression of Ci-syn was drastically reduced in injected embryos. Synapsins are neuronal proteins involved in neurotransmitter release, neurite elongation and synapse formation[6]. Our first results are therefore consistent with what reported in human rising the hypothesis that neural role of miR-7 is conserved between vertebrates and ascidians.

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NOGGIN AND TGF ORCHESTRATE FOREBRAIN REGIONALIZATION

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The initial phases of neural plate specification and organization require a complex series of signaling events, which are highly conserved in vertebrates. During regional differentiation of the neural plate, the roof plate and floor plate become local signaling centers and orchestrate subsequent regionalization and differentiation of the surrounding brain areas.[1] The roof plate, under the influence of the overlying epidermal tissue, produces BMPs and Wnts, driving the formation of dorsal neural structures.[2] Midline floor plate cells, instructed by the underlying axial mesendoderm, secrete TGF and SHH, conferring a ventral identity to the adjacent neural cells.[2] Following these stimuli, the anterior part of the neural plate gives rise to the forebrain that will subsequently divide to form telencephalon and diencephalon.[1] The aim of the work is to clarify the involvement of Noggin and TGF in the regionalization of forebrain. We performed microinjection of different doses of Noggin1 in *Xenopus laevis* embryos (two-cells stage). Microinjected embryos were used either for ACES cells explants at stage 8.5 or collected at neurula stage (stage 15) for whole mount in situ hybridization and real-time quantitative polymerase chain reaction (RT-

qPCR) experiments. Whole embryos and microinjected ACES cells was treated with TGF agonists (Activin and Nodal) or antagonist (SB431542) respectively to activate and inhibit TGF signaling. Here, we report an important role for Noggin1, a BMP inhibitor, to establish a forebrain identity during *X. laevis* development and to convert pluripotent Xenopus cells in telencephalic or diencephalic/retinal precursors in a dose dependent manner. Moreover, we report a role of different TGF in the regionalization of forebrain. In fact, Activin and Nodal respectively contribute to the acquisition of telencephalic or diencephalic/retinal identity. Finally, we suggest a new role for Nodal signaling in recruiting the SHH pathway, necessary for Noggin1-mediated retinal induction.

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SEROTONIN 2B RECEPTOR SIGNALING IS REQUIRED FOR EYE MORPHOGENESIS IN XENOPUS

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Serotonin (5-HT) is a neuromodulator that plays many roles in adult and embryonic life. Among the 5-HT receptors, 5-HT2B is one of the key mediators of 5-HT functions during development. We demonstrated that 5-HT2B modulates postmigratory skeletogenic cranial neural crest cell (NCC) behavior. 5-HT2B overexpression induced the formation of an ectopic visceral skeletal element and altered the dorsoventral patterning of the branchial arches. Loss-of-function experiments revealed that 5-HT2B signaling is necessary for jaw joint formation[1,2]. Moreover, 5-HT2B morphants have defective eyes with a loss of the ventral optic fissure closure (coloboma). Interestingly, the 5-HT2B gene is expressed both in the retina and in periocular mesenchyme (POM), a key signaling center required for eye morphogenesis. POM is composed by cranial NCC and cranial paraxial mesoderm derived cells. In 5-HT2B morphants the expression of key genes involved in POM development, such as Pitx2, FoxC1 and Raldh3, is affected. NCC remain gathered in the ventral part of the eye failing to conclude their migration within the optic fissure. POM also participates to the morphogenesis of the optic nerve and periocular muscles that result affected in 5-HT2B morphants. Embryos pharmacological treatments, with a selective 5-HT2B antagonist, reproduce all the morphants phenotypes. By using NCCs transplantation assay and time laps *in vivo* analysis, we propose a dual role of 5-HT2B signaling in the retina and in the POM possibly cooperating to modulate different events of eye morphogenesis and optic fissure closure. These results unveil a new role for 5-HT2B signaling during development and propose it as a new possible target for congenital eye disease such as coloboma.

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THE EXTRA-CELLULAR PROTEIN TSUKUSHI REGULATES NOTCH AND RECEPTOR TYROSINE KINASE SIGNALLING PATHWAYS IN MOUSE NEURAL PROGENITORS

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Tsukushi (TSK) is an extra-cellular protein belonging to the small leucine-rich proteoglycan family[1]. TSK binds to ligands and/or receptors of the Transforming Growth Factor beta, Wnt, Notch and Receptor Tyrosine Kinase (RTK) signalling pathways and coordinates their activities during gastrulation and neurulation in frog and chick embryos[1,2]. Knock-out mutant mice lacking TSK function display abnormal brain morphogenesis, as shown by a patently enlarged telencephalic lateral ventricle and a strong reduction of the anterior commissure[3,4]. The molecular mechanisms of TSK function during brain development, however, remain unclear. Using *in vitro* systems of neural progenitor cells (NPCs) derived from the mouse embryonic cerebral cortex, we have generated transgenic NPCs with stable expression of a membrane-tethered form of TSK (TSK-TM), obtained by in frame fusion of mouse TSK open reading frame with the transmembrane domain of the Platelet Growth Factor Receptor. This approach results in extra-cellular exposure of the membrane-tethered TSK-TM protein, which retains comparable biological activity to that of the soluble protein[2,3]. We have also produced control NPC lines stably expressing Green Fluorescent Protein (GFP). Real-time PCR, Western blot and immunofluorescence analyses confirmed robust expression of TSK-TM or GFP mRNA and protein in transgenic NPCs. To gain insight into the molecular pathways regulated by TSK in NPCs, we compared the transcriptome of TSK-TM and GFP transgenic NPCs by microarray analysis and we found 125 genes that appeared to be differentially regulated in TSK-TM *versus* GFP NPCs. A subset of these genes were selected for further validation by real-time PCR. This approach led to the identification of 15 genes consistently misregulated in TSK-TM NPCs in comparison with GFP NPCs. These include genes encoding for components of the Notch (Dll3, Mfng, Hes5) and RTK (Fgf8, Fgfr3, Erbb4) signalling pathways, along with genes implicated in cell adhesion, motility or survival. These results indicate that TSK activity can induce a specific transcriptional response in NPCs and suggest possible molecular mechanisms underlying the brain defects of TSK mutant mice.

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NEURONAL DIFFERENTIATION OF MESENCHYMAL STEM CELLS DERIVED FROM AMNIOTIC FLUID

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More than 600 disorders afflict the nervous system (NS); among them, neurodegenerative diseases are characterized by progressive NS dysfunction, with degeneration and/or death of neurons. Neurodegenerative diseases, whose frequency is increasing because of the rising elderly population, are incurable and debilitating conditions. In the search for new strategies for their treatment, cell therapy with mesenchymal stem cells (MSCs) is one of the most promising developments. MSCs are able not only to replace the degenerate elements but also to rebuild the interrupted connections, thus restoring normal brain function. Among the others[1-3], MSCs derived from Amniotic Fluid (AF-MSCs) are an emerging alternative source, since they have clonogenic, self-renewal and differentiative abilities. In addition, AF-MSCs are characterized by low immunogenicity and do not give rise to tumors. Here, MSCs were isolated from amniotic fluids, cultured and characterized by testing the immunophenotype, the expression of selected genes and the differentiative potential[4-6]. After the assessment of their stemness, AF-MSCs were induced towards neuronal differentiation by co-culture with human glial cells 1321N1, trying to recreate a physiological microenvironment in which the paracrine effect surely plays a key role. After co-culture, the proliferation rate, the morphology and the expression of more than 80 genes related to stemness and neurogenesis (by PCR array) and of specific neuronal proteins (by immunohistochemistry) were analyzed. AF-MSCs before co-cultures were used as control. Data after co-cultures indicate that the cell morphology changed (cells showed neurite-like formation), the proliferation rate and the expression of genes related to stemness decreased; in contrast, genes and proteins involved in neurogenesis were upregulated. Taken together, these results suggest that the paracrine effect exerted by glial cells enhances the differentiation of AF-MSCs towards neuronal cells, but further experiments are necessary to better define the neuronal progeny and their membrane excitability by electrophysiological experiments.

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NEURONAL DIFFERENTIATION OF MESENCHYMAL STEM CELLS DERIVED FROM AMNIOTIC FLUID: A COMPARATIVE STUDY BETWEEN TWO DIFFERENT APPROACHES

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Mesenchymal Stem Cells (MSCs) are characterized by self-renewal and differentiative potential towards osteogenic, chondrogenic and adipogenic lineages. Nevertheless, it is now well accepted that these cells are also able to transdifferentiate in cells of other germ layers, such as neurons. This ability makes MSCs a good candidate for use in regenerative medicine, in particular for the treatment of nervous system pathologies that lack effective therapies, with the aim of replacing damaged or lost cells. MSCs derived from different sources show variable skill to transdifferentiate; among the others[1-3], MSCs isolated from amniotic fluid (AF-MSCs) seem to have high differentiative potential and low immunogenicity, two fundamental aspects for their use in medicine. A common accepted protocol for the neuronal differentiation of MSCs is still lacking; different approaches have been tested and some of them completely forsaken. Actually, two methods are mainly considered: co-culture with glial cells (trying to mimic the physiological microenvironment with the paracrine effects exerted by glial cells) and the use of culture medium supplemented with specific compounds. In this work, after the isolation and the characterization of AF-MSCs [4-6], cells were induced to neuronal differentiation by co-culture and by specific culture medium. After differentiation, the proliferation rate, the expression of genes and protein related to stemness and neurogenesis were analyzed to detect the most performing approach. Our data indicate that both protocols induce neuronal differentiation of AF-MSCs, with significant decrease of genes/proteins related to stemness and increase of markers of neurogenesis. To clearly identify the most suitable protocol, additional experiments will be performed firstly to investigate the real ability of differentiated cells to respond to electrical stimuli.

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RISC-MEDIATED CONTROL OF SELECTED CHROMATIN REMODELERS FEATURES THE GROUND STATE PLURIPOTENCY OF MOUSE CELLS

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Embryonic stem (ES) cells are intrinsically unstable as they spontaneously differentiate if not shielded from external stimuli. Although the nature of such instability is still controversial,

growing evidence suggests that protein translation control may play a crucial role. We performed an integrated analysis of RNA and nuclear proteins at the transition between naïve ES cells and cells primed to differentiation. During this transition, protein translation of a set of genes coding for chromatin remodelers was specifically released from the inhibition mediated by the RNA-Induced Silencing Complex (RISC). Interfering with the chromatin remodeling activity of these genes impeded priming to differentiation. We propose that RISC-mediated inhibition of specific sets of chromatin remodelers is a primary mechanism of preserving ES cell pluripotency while maintaining cells in a metastable state.

POSTNATAL NEUROGENESIS IN DOLPHINS

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Adult neurogenesis is a sort of `protracted` developmental neurogenesis linked to homeostatic functions in specific neural systems: hippocampus and olfactory bulb[1]. Since aquatic mammals, such as dolphins, are devoid of olfaction, they can be used to clarify if (or how) adult neurogenesis originating in the subventricular zone (SVZ) and destined for the olfactory bulb does exist independently from a specific physiological role in olfaction. By using a stock of fixed material stored in a Marine Mammal Tissue Bank (Padua University), we analyzed the periventricular region of *Tursiops truncatus* and *Stenella coeruloalba* brains, at neonatal and postnatal stages. Firstly, we realized an atlas of the neonatal/early postnatal dolphin forebrain in order to allow identification of the SVZ which appeared extremely small (same size than in mice, in a brain which is 42-fold that of mice). By using histology and immunocytochemistry for neurogenic markers (doublecortin -DCX- and Ki67 antigen) we reconstructed the SVZ area. In the neonate, it consists of a compact mass of neuroblasts lining the lateral ventricle from which many small chains of neuroblasts ramify radially within the white matter, directed towards cortical areas of the whole brain hemisphere. Surprisingly, very rare proliferating cells were detectable within the DCX^+ cell masses. Such scarce neurogenesis was almost exhausted at early postnatal stages (ten times reduction of the SVZ area in 3-6 months old dolphins). Current experiments aimed at assessing the occurence of SVZ neurogenesis in adult dolphins indicate that small SVZ-like structures can still be present in some individuals, with no cell proliferation but occurrence of mature neurons within the cell masses. These data suggest that SVZ neurogenesis in dolphins dramatically drops around birth, only vestigial remnants then persisting in adulthood. Comparative studies showed that adult neurogenesis can be very low or absent in the hippocampus of cetaceans[2]. Our results confirm the same trend, which in the dolphin SVZ also correlates with the highly advanced development of the brain and the lack of olfaction. Yet, taking into account the average lifespan of 30-50 years in these aquatic mammals, the persistence of a vestigial SVZ (not absence) in the adult dolphin brain also raises intriguing evolutionary hypothesis.

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CHOLINERGIC CONTROL OF CELL GROWTH AND MIGRATION IN UNDIFFERENTIATED AND SCHWANN CELL-LIKE DIFFERENTIATED ADIPOSE-MESENCHIMAL STEM CELLS

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Peripheral nerve injury is a common disease and represents a major economic burden for society. The development of novel strategies, including cell therapy, to enhance peripheral nerve regeneration is therefore of great social and clinical relevance. The use of synthetic nerve conduits, in combination with different cell types may represent a promising therapy. The great regenerative capacity of peripheral nervous system (PNS) is due to a permissive environment provided by Schwann cells that proliferate, migrate and release growth factors either during development or after nerve lesions. Mesenchymal stem cells (MSCs) are an attractive cell source for nerve tissue regeneration. They are able to self-renew and possess multi potent differentiation properties. In particular adipose MSCs (A-MSCs) appear the most promising source of MSCs. Recent studies have shown that A-MSCs can be differentiated in Schwann-like cells, representing an alternative and reliable source of peripheral glial cells. Acetylcholine (ACh), the main neurotransmitter in central and PNS, has the property to modulate neurite outgrowth and to control Schwann cell proliferation and differentiation. ACh plays important role also in non-neural tissue, but its functions in MSCs has been poorly investigated. In present work we have characterized the muscarinic cholinergic agonist effects in rat A-MSC and in differentiated Schwann-like obtained from A-MSCs. Analysis by RT-PCR has demonstrated that the A-MSCs express all muscarinic receptor subtypes. MTT analysis and wound healing assay have also demonstrated that the selective activation of M2 receptors caused an inhibition of cell growth and migration of MSCs indicating the ACh as possible modulator of MSC proliferation and migration. In Schwann cell-like derived from A-MSC, similarly to that observed in Schwann cells, the M2 muscarinic agonist caused a decrease of cell proliferation without affecting cell survival. Further analysis are addressed to evaluate the capability of these receptors to mediate the differentiative processes in Schwann cell-like, as previously observed in Schwann cells. In conclusion, we hypothesize that a combination of autologous MSC, differentiated in Schwann-like, and selective ACh mimetics may represent a successful strategy to achieve better results in peripheral nerve regeneration.

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SEROTONIN DEPLETION AFFECTS DEVELOPMENT AND MAINTENANCE OF SEROTONERGIC NEURONAL CIRCUITS

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Serotonin is a neurotransmitter implicated in the modulation of several behavioral and physiological processes within the central nervous system (CNS) including mood, control of sleep, appetite, aggressivity and sexual behavior. In neurons serotonin is synthesized in two main steps with tryptophan hydroxylase 2 (Tph2) as the rate-limiting enzyme. Serotonergic neurons are one of the most early born neuronal systems in the mammalian brain and provide a widespread innervation to the whole CNS. The synthesis of serotonin during embryonic development together with a dynamic expression of serotonergic receptors in the CNS has led to the hypothesis that serotonin could act as a growth regulator in specific neurodevelopmental events such as neurogenesis, neuronal migration and circuitry formation. Although recent discoveries from animal models and human genetic studies have highlighted the importance of serotonin homeostasis maintenance during CNS development, the precise role of this molecule in specific morphogenetic activities remains poorly understood. To address the consequences of time-controlled serotonin depletion on CNS development, we have generated a Tph2 conditional (floxed) allele and used it in combination with a Tph2-GFP knockin mouse line allowing the visualization of serotonergic neurons and fibers[1]. We were able to demonstrate that abrogation of serotonin synthesis in adult mice affects the proper serotonergic wiring, thus indicating that the serotonergic system exhibits a previously unexpected plasticity in response to serotonin signaling. Our results together with previous observations suggest that appropriate serotonin homeostasis is crucial not only for proper development of the serotonergic neuronal circuit but also for its maintenance during adulthood.

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TESTICULAR STEROIDOGENESIS IN OBESE RATS

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Obesity is associated with a state of hypoandrogenism in men[1]. Evidence indicates that testosterone deficiency induces adiposity increase and, at the same time, increased adiposity induces hypogonadism; however the specific pathogenetic mechanisms involved in this phenomenon are complex and not completely understood[2]. To assess the effect of obesity on testicular steroidogenesis in rats treated by a high fat diet, we evaluated the testicular morphology and the expression of key enzymes involved in steroid production, as 3-beta-HSD, 17-beta-HSD and aromatase Cyp-19, using immunolocalization and real-time experiments. No significant difference was observed in testis structure and in 3-beta-HSD and 17-beta-HSD localization, while we show in obese rats an increase in aromatase mRNA expression. Using immunohistochemical investigations, we show that along with this increase a widespread enzyme localization in different cytotypes in obese rats compared with control animals occurs.

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HUMAN SPERM CRYOPRESERVATION: COMPARATIVE STUDY OF DIFFERENT PROTOCOLS BY MEANS OF MICROSCOPY, CYTOMETRY AND MOLECULAR APPROACHES

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Sperm cryopreservation represents a key strategy for the preservation of fertility in males with various diseases inducing progressive loss of the semen quality. Nevertheless, the current protocols of freezing are neither optimal nor standardized between different laboratories. The aim of this study was to evaluate the efficiency of different methods of freezing, by comparing some protocols of human spermatozoa cryopreservation, and applying both traditional and improved analysis of sperm quality, in order to define the critical steps of the process and identify possible chance of improvement of the technique. According to the ethical standards of the Hospital, we recruited 21 patients referred to the PMA center of the Fondazione IRCCS Policlinico San Matteo (Pavia, Italy) who were defined normospermic according to WHO parameters 2010. The excess volumes of semen samples used for the procedures of PMA were frozen, according to 5 different protocols: M1 (Method 1): 30 min at 4°C, 10 min on nitrogen vapors; M2: cooling of seminal fluid and cryoprotectant at 4°C, mixing of sample and cryoprotectant at 4°C, 30 min at 4°C, 10 min on nitrogen vapors; M3: 2h at 4°C, 10 min on nitrogen vapors; M4: 30 min at 4°C, 30 min at -20°C, 30 min at -80°C; M5: 30 min at -20°C, 30 min at -80°C. Each phase ended with direct immersion of the sample in liquid nitrogen. Cryopreservation of human spermatozoa has been related to decreased motility associated with impaired velocity and viability of sperm pre-freeze and post-thaw. For all applied methods there was a significant reduction of progressive and total motility (P) as a result of freezing. M2 and M4 are the worst protocols in terms of recovery of viability (p=0.010 and p=0.041, respectively) and M5 was discarded since it appears to be the worst method in terms of recovery of total (p=0.0163) and P (p=0.0278) motility, and of the post-thaw

vitality (p=0.012). There were no significant differences between M1 and M3 in terms of reduction in sperm motility and viability. Other non-routine analysis were performed to determine whether the cooling time to 4°C may affect the procedure negatively. 5 additional cryopreserved samples by M1 and M3 were analyzed with Comet Assay (to assess the degree of sperm DNA fragmentation), flow cytometry (to study light scatters patterns and membrane integrity) and electron microscopy (to investigate ultrastructural cell details). No significant differences were found between M1 and M3 methods.

THE INVOLVEMENT OF VIP IN STEROID HORMONE SECRETION DURING *PODARCIS SICULA* SPERMATOGENESIS

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Spermatogenesis is a complex process that is regulated by both the hypothalamus-pituitary axis through the release of the pituitary gonadotropins, and local factors including the vasoactive intestinal peptide (VIP)[1]. VIP is a pleiotropic neuropeptide belonging to the superfamily PACAP/glucagon/secretin, and is involved in multiple events, including spermatogenesis. Investigations performed on mammals, particulary, have demonstrated that VIP regulates the testosterone synthesis[2]. In non-mammalian vertebrates the investigations are limited to a few species including the cartilaginous fish *Torpedo marmorata*[3] and the lizard *Podarcis sicula*, where Agnese *et al.*[4,5] have show that VIP and its receptors are widely distributed in both germ and somatic cells during all reproductive cycle, thus suggesting a possible role of VIP in the control of *Podarcis sicula* spermatogenesis. Now, we reported the effects of the VIP on steroidogenesis in significant periods of the *Podarcis* reproductive cycle: winter stasis, reproductive period and summer stasis. Using VIP treatments in testis culture in absence or presence of receptor antagonists in different combinations, we demonstrated for the first time that in *Podarcis sicula* VIP is involved not only in thetestosterone synthesis, as in mammals, but in 17-estradiol synthesis too probably through the control of aromatase expression.

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A THREE-DIMENSIONAL ULTRASTRUCTURAL ANALYSIS OF ACTIN INVOLVEMENT IN THE APOPTOTIC NUCLEUS

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Apoptosis is an essential biological function required during embryogenesis, tissue homeostasis, organ development, immune system regulation as well as in a variety of pathological conditions[1]. During apoptosis cytoskeleton proteins appeared damaged and enzymatically disassembled, leading to the formation of apoptotic features. Actin, involved in chromatin remodeling in different biological processes, under apoptotic stimuli polymerizes actively inside the nucleus, partially leaving the cytoplasm where it exists in depolymerized form[2]. Field Emission in Lens Scanning Electron Microscope (FEISEM) permits to obtain three-dimensional information regarding protein localization and cellular components and has been employed in this study to better characterize chromatin and cytoskeleton behavior in apoptotic condition[3]. For that, HL-60 cells have been exposed to hyperthermia (1 h, 43°C) followed by 4h post incubation[2], processed for immunogold reaction and FEISEM analysis. The latter showed, in cryosectioned untreated cells, the diffuse chromatin clearly detectable and mainly characterized by 10 nm fibers. Hyperthermia-treated cells evidenced a strict separation between condensed and diffuse chromatin which appeared similar to that observed in control condition. Moreover, apoptotic chromatin rearrangements involved the translocation of actin filaments from the cytoplasm to the nucleus under apoptotic stimuli. In treated samples, gold particles appeared, in apoptotic nuclei, localized in the diffuse chromatin and organized in straight or curvilinear clusters, evidencing the presence of structures longer than 100 nm. This result strongly suggests a role of nuclear actin filaments in achieving the well-known spatial separation between the normal-functional chromatin and that inactivated by the apoptotic process.

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EXPOSURE TO ENVIRONMENTAL DOSES OF BPA AFFECTS EMBRYO DEVELOPMENT AND INHIBITS ZEBRAFISH REPRODUCTION THROUGH DEREGULATION OF EPIGENETIC PATTERN

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Bisphenol A (BPA) is an Endocrine Disruptor Compound which interferes with vertebrates' reproduction by binding to nuclear receptors ER-alpha and ER-beta, ultimately affecting gene expression. BPA is likely able to alter epigenetic mechanisms through methylation of the CpG sites. The matter is still uncertain, but BPA is supposed to influence histone modification, hence the structure of chromatin correlated with activation or repression of transcription. Our project aimed to determine the effects of environment-like BPA doses on female reproductive physiology and to assess whether different transcriptional trends of genes related to the process could be caused by histone modifications. Given the results we obtained, we also tested BPA doses on embryo development. Experiments were carried out on adult Daniorerio females treated for three weeks with three doses of BPA: 5, 10 and 15 μg/L. Oocyte growth and maturation, autophagy and apoptotis, as well as histone modifications and DNA methylation were studied through qPCR, histology and ChIP-qPCR. As for embryo development, Whole-Mount in

situ Hybridization was performed. The lowest dose of BPA caused the most severe effects. It inhibited reproduction and particularly affected oocyte maturation, as to lowered ovarian mRNA levels of esr1, esr2a , lhcgr and pgrmc1. Instead, apoptosis (p53 and caspase3) was up-regulated. Histology reinforced these results, because it evidenced atresia in almost all mature follicle of the BPA-treated group. ChIP-qPCR results agree with transcriptional studies: H3K4me3 and H3K27me3 enrichments in the Transcription Start Site of star and fshr genes decreased in treated groups, while the TSS of lhcgr, whose gene expression was down-regulated by 5 µg/L of BPA, showed a decrease and an increase in H3K4me3 and H3K27me3 marks, respectively. Focus on DNA methyltransferases additionally showed the decrease of dnmt1 and dnmt3, supporting data from ChIP. About embryo development, we found that 5 µg/L of BPA was able to interfere with crestin localization. To conclude, our study demonstrates that the most harmful of the tested BPA doses is the lowest. It interfered with *Danio rerio* embryo development, oocyte growth and maturation, and down-regulated signals involved in the last phase of oogenesis. Likely, the negative effects of BPA on reproduction are due to an upstream capacity of such pollutant to deregulate the epigenetic mechanism.

SUPERMIGRATION CAPABILITY IN A SUB-POPULATION OF MOUSE MESOANGIOBLAST STEM CELLS SELECTED BY OXIDATIVE PRECONDITIONING

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In the development of cell-based therapies many efforts are addressed to improve stem cell survival for transplantation. It has been shown that the release of several types of factors, such as cytokines and growth factors, from damaged tissues causes massive cell death during the first days after transplantation compromising stem cell homing capability toward the target site and consequently the therapeutic efficacy. Mesoangioblasts (mabs) are stem cells derived from dorsal aorta of mouse embryos with the ability to proliferate in culture condition and to differentiate. In a previous study we found that an H_2O_2 oxidative preconditioning of mouse mabs selected a subpopulation, with some features more suitable than the rest of mab population. The isolated sub-population maintained stemness characteristics such as proliferation and differentiation capability and acquired resistance against a second oxidative stress. By using transcriptome microarray analysis, we identified 800 significant ($q<0.05$ with FC+/-2) differentially expressed genes between mabs and the subpopulation. We found that genes involved in the detoxification of oxidised molecules were overexpressed in the subpopulation, including several glutathione-S-transferases and one gene indirectly involved in a cell proliferation mechanism that operates via the inhibition of p38 MAP kinase activity. Now we have highlighted a new noticeable finding by wound healing assays. The sub-population cells have a higher migration capability than that of parental mabs. This result is supported by transcriptome data in which several genes that are inhibitors of migration are down regulated favouring cell migration. Moreover, the p selectin gene and the integrin beta7 gene are up-regulated in the subpopulation making the cells more able for speed rolling (p selectin) and for transmigration/extravasation (integrin beta7). In addition, we found that in normal cell growth condition the subpopulation cells have the Hsp70.1 gene up regulated by two fold change respect to that of the rest of mabs. The link between migration and the amount of Hsp70 had been found by our other experiments in mabs that have demonstrate that mab migration depends on the amount of extracellular Hsp70 that acts as an autocrine signal on 2/4Toll-like receptors. In conclusion, the higher migration ability makes the subpopulation cells together to other features suitable for the regenerative tissue application.

CULTURED CELLS EXPOSED TO LOW OZONE CONCENTRATIONS UNDERGO CYTOPLASMIC AND NUCLEAR REORGANIZATION

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Ozone therapy is a modestly invasive procedure based on the regeneration capabilities of low ozone concentrations and used in medicine as an alternative/adjuvant treatment for several diseases[1,2]. Despite ozone has been applied for therapy since the end of the 18th century, the cellular and molecular mechanisms accounting for the positive effects of ozone treatment are still largely unknown[3]. In the present study we have investigated the effects of low ozone concentrations (1, 10 and 20 µg O_3/mL O_2) on cell dynamics and organelles` structure and function by means of morphological, morphometrical, cytochemical and immunocytochemical techniques at bright field, fluorescence and transmission electron microscopy. In order to analyse under controlled experimental conditions the direct effects of mild ozonization on some cellular mechanisms, we used an *in vitro* cell system, which allows to exclude the confusing influence of the organismic reaction occurring in animal models *in vivo*. HeLa cells were exposed for 10 min to an oxygen-ozone gas mixture with different ozone concentrations[4] and then analysed after 24 h. Our results demonstrate that, under our experimental conditions, cell proliferation and death (necrosis or apoptosis) rate are unaffected by mild ozonization. Moreover, no evidence of a significant increase in reactive oxygen species was found. Conversely, exposure to low ozone concentrations proved to influence cytoskeletal actin organization, mitochondrial structure and function, and nuclear transcription. The most effective treatment proved to be the one with 10 µg O_3/mL O_2, which was able to induce positive long-lasting cellular responses. Our results confirm that the effects of ozone exposure depend on gas concentration, suggesting that ozone treatments should take into account the cytological and cytokinetic features of the different tissues to ensure permanent cellular activation.

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PROTECTIVE ACITIVITY OF THE CHOLINE CONTAINING PHOSPHOLIPID CHOLINE ALPHOSCERATE, ON CEREBROVASCULAR TREE OF SPONTANEOUSLY HYPERTENSIVE RATS

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Since 1980, pre-clinical and clinical evidence, identified the cholinergic system as relevant for learning and memory processes. Based on this hypothesis precursor loading strategies were proposed for countering cognitive impairment occurring in adult-onset dementia disorders. Cholinergic precursors may increase choline availability and acetylcholine synthesis/release in different brain areas. Among compounds of this class, choline alphoscerate (alpha-gliceryl-phosphoryl-choline, GPC) is the most effective in enhancing acetylcholine biosynthesis and release in animal models. This study was designed to assess the effect of long term treatment with GPC on cerebrovascular tree in a model of vascular brain injury represented by spontaneously hypertensive rats (SHR). Male SHR aged 32 weeks and age-matched normotensive Wistar-Kyoto (WKY) rats were treated for 4 weeks with GPC (150 mg/kg/day) or vehicle. Wall and lumen area of pial and intracerebral arteries of different brain areas were analyzed by morphometric techniques. Vascular astrocytes, blood brain barrier (BBB) and endothelial markers of inflammation were assessed by immunohistochemistry associated with quantitative analysis. In SHR, no significant changes in the size of perivascular astrocytes were observed compared to WKY rats. The expression of the BBB markers aquaporin-4 and claudin-5 was increased and decreased respectively, in the brain of SHR. In terms of activity on BBB, GPC treatment countered changes of aquaporin-4. Hypertension affected to a different extent endothelial markers and vascular adhesion molecules expression in pial and intracerebral vessels. In SHR a decrease of vascular endothelial growth factors sensitive to GPC treatment was noticeable. The observation that treatment with GPC countered cerebral microanatomical changes occurring in SHR may represent the basis for investigating the activity of the compound in cerebrovascular patients with cognitive dysfunction.

PROGESTERONE RECEPTOR MEMBRANE COMPONENT-1 PLAYS A ROLE IN BOVINE GRANULOSA CELLS MITOSIS

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Progesterone Receptor Membrane Component-1 (PGRMC1) is expressed in bovine granulosa cells (bGC) during all stages of folliculogenesis. The aim of the present studies is to assess PGRMC1`s role in regulating bGC mitosis since studies in rat and human ovarian cell lines and bovine oocyte indicate its direct involvement during chromosome segregation and cytokinesis. First, PGRMC1's sub-cellular localization was evaluated by immunofluorescence in bGCcollected from antral follicles andcultured *in vitro*. These studies revealed that in mitotic cells, PGRMC1 concentrates in the area of the spindle apparatus, localizing in the midzone and the midbody during Ana/Telophase and Cytokinesis, respectively. Then, in order to assess the effect of disrupting PGRMC1 function on bGC cell mitosis, bovine PGRMC1 expression was down-regulated using small interfering RNA (RNAi). Cells were transfected with PGRMC1 or CTRL RNAi and cultured for up to 72h. Transfection efficiency was tested using fluorescent-labeled RNAi and was estimated to be 80%. Quantitative RT-PCR and western blotting analysis confirmed that RNAi treatment reduced PGRMC1 expression, when compared to the CTRL-RNAi treated group (t-test, $p<0.05$). The effect of PGRMC1 depletion on cell proliferation was assessed by cell counting and flow cytometry which, respectively, revealed a lower growth rate by 41.7% at 72 h ($p<0.05$) associated to a higher % of cells arrested at G2/M phase compared to CTRL RNAi treated group at 72h (2.3±0.9% and 9.7±1.2% $p<0.05$). Furthermore, live imaging studies confirmed that PGRMC1 silencing directly affects mitotic division. In fact, we observed an increased percentage of abnormal and/or incomplete mitosis in PGRMC1 RNAi treated cells, when compared to control cells (Fisher exact test, $p<0.05$). Finally, to investigate PGRMC1 putative mechanism of action in mitotic cells we conducted immunofluorescence and *in situ*-Proximity Ligation Assay studies, which is a new immuno-based technique that allows localized and quantifiable detection of protein-protein interactions directly on fixed cells. Specifically, we considered a possible interaction between PGRMC1 and clathrin. In fact, clathrin acts during cell division concentrating at the spindle apparatus, where it stabilizes fibers of the mitotic spindle aiding chromosomes separation. The results show that PGRMC1 colocalizes and directly binds to clathrin during interphase and all stages of the M-phase. Our data confirm a direct involvement of PGRMC1 during granulosa cell mitosis and set the stage for further mechanistic studies.

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CIRCADIAN RHYTHMS DISRUPTION INDUCES AGING-LIKE CHANGES IN ADULT NEURONAL STEM CELLS OF *NOTHOBRANCHIUS FURZERI*

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Nothobranchius furzeri is an annual fish with a captive lifespan of 3-7 months that emerged as a model organism for aging studies. We studied adult neurogenesis in *N. furzeri* to quantify the effects of aging on neuronal progenitors proliferation, and the expression of glial fibrillary acid protein (GFAP) in the radial glia as measure of neuroinflammation; in teleosts, the glial component, including neuronal Stem Cells (nSCs), is totally represented by radial glia, both in the embryos and adult fish. In this previous study we described an age-dependent reduction of the proliferative activity of adult nSCs and neuronal precursors, as well as age-dependent gliosis, into the most prominent neurogenic niches of *N. furzeri* brain. Circadian rhythms alteration is the most common form of environmental pollution in the modern society and may have consequences on human health. So, we

decided to investigate the effects of circadian rhythms disruption on adult nSCs of *N. furzeri*. From week 7 to 20, we applied to an experimental group a "shift work simulation" protocol (SW), by interrupting the normal dark phase with 4h of light exposure. Then, 21 weeks-old animals (treated and age-matched controls) were sacrificed for brain dissection and analysis. Result 1: circadian rhythms disruption reduces neurogenesis and stem cells proliferation potential. We counted the total number of proliferating ($PCNA^+$) cells of the brain with an unbiased stereological method, to quantify neurogenesis in the anterior telencephalon (aTEL) and the posterior optic tectum (pOT): we found in both areas a significant reduction of neurogenesis in the treated group compared to the controls. Moreover, in the aTEL we observed the age-dependent reduction of the number of proliferating ($PCNA^+/S100b^+$) glial cells, suggesting a reduction of the nSCs proliferative potential. Result 2: circadian rhythms disruption induces gliosis. We analysed gliosis using two specific glia markers (S100b and GFAP for normal glia visualization and inflammatory activation, respectively); then we performed a semi-quantification of the GFAP (gliosis marker) fluorescence over threshold, normalized on S100b: SW-treated group showed a significant increase of gliosis compared to controls. Our findings suggest that circadian rhythms disruption in the *N. furzeri* can have negative effects on adult neurogenesis thereby impacting on brain functions.

BUILDING AN ARTIFICIAL TENDON

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Tendons are anatomic structures which connect muscles and bones transmitting the force generated in muscles to the bone, allowing movement. For this reason tendons sustain high tensile force thus being prone to injuries such as rupture and laceration. Unfortunately, their natural healing process is slow and the resulting tissue is nether in structure and function due to their hypocellular and hypovascular nature. For this reason, tissue engineering is looking for new strategies based on stem cells and scaffold biomaterials to replace damaged tendon tissue. Chemical stimulation with growth factors such as TGF-beta and/or mechanical stimulation are demonstrated to be very important to promote the tenogenic differentiation leading to an increment in the expression of Scleraxis (Scx), the tenogenic pivotal gene, and others typical components of tendon ECM (collagen I/III and tenascin C). Therefore in this work we have first isolated mesenchymal stem cells from two different tissues, the adult tendon tissue (TDSCs) and the periosteum (PDCs). Then we have differentiated these cell populations in 2D and 3D culture using a PEG-fibrinogen (PF) based hydrogel treating the cultures with TGF-beta and Ascorbic Acid (A.A.) in order to ameliorate tenogenic differentiation. Moreover we have developed a bioreactor to apply an uniaxial tension to the cell and PF 3D constructs in order to improve the tenogenic differentiation and the proper alignment of the collagen fibers. By our experiments we have observed increasing production of collagen after cell treatment with TGF-beta and A.A. both in 2D and 3D cultures. Moreover the 3D environment of PF alone is able to improve the expression of the tendon extracellular matrix components.

OBESITY-RELATED CEREBROVASCULAR INJURY IN OBESE ZUCKER RATS

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Increased food intake, reduced physical activity and altered metabolic processes are variables affecting energy balance and inducing obesity. Obesity is becoming in the industrialized countries a relevant medical challenge being associated with the development of chronic diseases affecting also nervous system. Metabolic syndrome (MetS), defined by a constellation of an interconnected physiological, biochemical, and metabolic factors is directly correlated to obesity. It increases the risk of atherosclerotic cardiovascular disease, type 2 diabetes mellitus (T2DM) and all cause mortality. The obese Zucker rats (OZRs), with a mutation in leptin receptor, represent a model of obesity exhibiting T2DM with a moderate degree of arterial hypertension. OZRs are characterized by the simultaneous occurrence of hyperglycaemia, hyperinsulinaemia and hyperlipidaemia. The aim of this work was to characterize brain microanatomy of OZRs compared to their non-obese cohort lean Zucker rats (LZRs) to assess possible cerebrovascular injury related to obesity. Male OZRs and LZRs of 12, 16 and 20 weeks of age were used. Body weight, blood pressure and blood parameters were checked in different groups. Neuronal, glial and blood-brain-barrier changes were assessed by immunochemical and immunohistochemical techniques. In frontal cortex and hippocampus a decrease of neuronal specific nuclear protein positive cells in older OZRs group compared to age matched LZRs was noticeable. These changes were accompanied with a reduced neurofilament immunoreaction without changes of the dendritic protein MAP-2. In OZRs an increased expression of glial fibrillary acidic protein immunoreactive astrocytes was observed compared to LZRs. Blood brain barrier of older OZRs revealed an increased expression of aquaporin-4 and glucose transporter-1 compared to LZRs. These data suggest that OZRs developed a specific neuronal, glial and cerebrovascular changes further suggesting that OZRs may represent an useful animal model for assessing the influence of obesity/MetS on the brain. This could contribute to clarify the pathophysiology of nervous system damage reported in obese individuals and/or affected by MetS and to identify possible treatment strategies.

COMPARATIVE EVALUATION OF THE NANOPARTICLES ACTIVITY ON *XENOPUS LAEVIS* EMBRYOS

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Nanotechnology is a rapidly growing industry of global economic importance. The properties of nanoparticles (NPs), that make them useful for industrial applications, have led to concerns regarding their potential impact on human and environmental health. Because of their properties NPs are attractive for basic science, technical or medical applications. Size, surface

charge, geometry, and its modifications contribute to NPs toxicity. The aquatic environment is at risk of exposure to NPs, as it acts as a sink for environmental contaminants[1]. There are insufficient evidence for environmental concentrations and derived harms for almost all NPs. This finding requires more standardised approaches for NPs hazard identification. The possibility to relate the data obtained in *Xenopus laevis* with higher vertebrates, including humans, makes it a convincing study model[2,3]. Our aim is to evaluate the activity of three different NPs: AgNPs, AuNPs and SiO_2NPs, 20nm sized, on the embryogenesis of *X. laevis*, These commercial nanoparticles were used as model to understand the possible consequences of similar NPs utilized in medicine. We purchased NPs from MKnano (Canada). Dinamic Light Scattering was performed at 21°C to measure NPs size and Z-potential. The embryos were reared starting from st 4/8 in FETAX containing 0.01, 1 and 5 mg/L NPs. All embryos were harvested at st 47/48. The mortality, morphology, length, heartbeat and pigment distribution were statistically analyzed[4]. Real Time-PCR for rax1, pax6, sox9, fgf8 and egr2 were carried out from embryos st. 46, see[5]. Our data show that all NPs, that we tested, aggregate in FETAX and do not cause mortality. Embryos treated with AuNPs are longer and bradycardic compared with control, whereas those treated with AgNPs or SiO_2NPs are shorter and tachycardic (AgNPs).The pigment distribution and gene expressions are altered after treatment with SiO_2NPs. In conclusion our studies indicate that the NPs that we tested affect embryonic development impairing the expression of genes involved in the early embryogenesis.

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