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European Journal of Histochemistry
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ANTIXODANTS PROTECT SPERM AGAINST DAMAGE AND IMPROVE THEIR ABILITY TO SUPPORT EMBRYO DEVELOPMENT

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Reactive oxygen species (ROS) are generated during mitochondrial respiration and are involved in several signaling mechanisms. Under pathological conditions, the concentration of ROS may exceed the antioxidant scavenging systems and subsequently lead to cell damage. High ROS levels are detrimental to gametes and compromise their function through lipid peroxidation, protein damage, and DNA strand breakage.[1] Although, antioxidant therapy has been demonstrated to enhance semen quality in subfertile men and has been suggested to improve pregnancy rates, it is still a matter of debate if it can positively influence fertilization outcome and embryo developmental competence. DNA damage in human spermatozoa has been correlated with increased miscarriage rates and morbidity in the offspring.[1] We recently demonstrated that the antioxidants zinc and coenzyme Q10 (CoQ10), and the micronutrient D-aspartate (D-Asp), contained in the dietary supplement Genadis (Merck Serono), have protective effects on human sperm motility, DNA fragmentation, and lipid peroxidation.[2] However, it is still unknown whether sperm exposure to Genadis *in vitro* can affect fertilization outcome and embryo developmental competence. Studies in animal models could provide insights into these fundamental questions. In the present study we evaluated: (1) the effects of zinc, D-Asp, and CoQ10 on bull sperm motility and DNA fragmentation; and their competence in fertilization and in the development of healthy embryos. Finally, to understand the extent of antioxidant protection during sperm handling in assisted reproductive technologies (ART), the xanthine-xanthine oxidase system was used to generate sperm exogenous oxidative stress.

Our data indicate that antioxidants prevent the loss of sperm motility and the rise in sperm DNA fragmentation over time. Moreover, blastocyst rate was found to be significantly higher in oocytes fertilized by treated spermatozoa, and these blastocysts harbored a significantly lower percentage of apoptotic cells.

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ROLE FOR PPAR TRANSCRIPTION FACTORS IN THE ENERGETIC METABOLIC SWITCH OCCURRING DURING NEUROGENESIS

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Neurogenesis takes place throughout life in two main areas of the adult mammalian brain: the subventricular zone (SVZ) of the lateral ventricles (LVs) and the subgranular zone (SGZ) of the hippocampal formation. Although different, these two areas share an extremely organized and specialized microenvironment, where Neural Stem Cells (NSCs) can interact with their progeny, blood vessels, ependymal cells, cerebrospinal fluid and other surrounding cells. This fine architecture defines the so-called "neurogenic niche" that provides several signals regulating proliferation, migration and differentiation of NSCs and their progeny. Among these signals Peroxisome Proliferator Activated Receptors (PPARs) play an essential role in regulating NSCs proliferation and differentiation. Since they are known for being mainly involved in the regulation of energetic metabolism, we tried to correlate the modulation of PPARs with the energetic metabolic switch occurring during neurogenesis both *in vivo* and *in vitro*. The preliminary data obtained seem to indicate an involvement of PPARγ in the storage of glycogen and lipids in undifferentiated cells and that its inactivation is paralleled by the utilization of the energetic storages during the neuronal differentiation. In fact, during neurogenesis, both *in vivo* and *in vitro,* PPARγ inactivation parallels glycogen and lipid droplets consumption, while PPARβ/δ is increased and probably involved in the neuronal maturation, as we have already demonstrated; PPARα is also increased, suggesting a possible function in the acquisition of the cholinergic phenotype.

DEVELOPMENTAL PATHWAY OF THE CALRETININ(+)-S100(+) SUBFRACTION OF CRYPT CELLS POPULATION IN THE OLFACTORY ORGAN OF *POECILIA RETICULATA* FROM BIRTH TO SEXUAL MATURITY

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Crypt cells are the most recently described morphotype of fish olfactory neurons, not found in tetrapods. These cells are less abundant than ciliated and microvillar ones in the sensory epithelium. They are suspected to be involved in reproductive behaviours, even if electrophysiological investigations demonstrated some sensitivity to amino acids but failed to identify response to pheromones. In adult *Poecilia reticulata* the number of crypt cells is different in the two sexes. Using immunohistochemical markers, $G\alpha_{olf}$, calretinin and S100 to identify ciliated, microvillar and crypt cells, we compared the size of their populations in juvenile guppies at various steps from birth to 90 days, when their gonads reach maturity. We observed that the number of crypt cells is sex specific, with independent developmental dynamics between males and females, while ciliated and microvillar cell populations have similar pathways. In the guppy calretinin appeared to be expressed also in a subfraction of S100-positive crypt cells (calretinin(+)-S100(-) crypt cells were not observed). We examined the changes in the amount of calretinin(+)-crypt neurons during growth: cell density reflects the variations observed in the whole population of S100(+) crypt cells in both sexes in the first 45 days. However it decreases reaching 3 months of age, in contrast with the increment registered for calretinin(-)-S100(+) cells. We estimated the total number of calretinin(+) crypt neurons and we observed that it remains very close to the value calculated for all crypt cells until 21 days, but it only slightly rises in the next 2 months, while calretinin(-) crypt neurons sensibly increment their number. We hypothesize that this new identified phenotype, percentually more represented in the first weeks of life than later, could be involved in early non-reproductive function. The known sensitivity for amino acids might indicate a role in the perception of food-related stimuli. On the contrary, calretinin(-) crypt cells become predominant reaching sexual maturity, in line with their supposed involvement in reproductive activity.

IMPAIRED NEURONAL DIFFERENTIATION OF En2 -/- NEURAL STEM CELLS

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Autism spectrum disorders (ASD) are characterized by impaired relationships, impaired verbal and non-verbal communication, restricted and repetitive behaviours. Their identification and classification are difficult as patients show different typical behaviors and phenotypes. Post-mortem studies of both patients and animal models reveal neuroanatomical abnormalities in different brain regions. Defects have also been shown at a cellular level, such as an important reduction of cortical GABAergic interneurons and of cerebellar Purkinje cells, proposed as causes of the pathology.

Two single-nucleotide polymorphisms (SNPs) in the human Engrailed-2 (EN2) gene are associated with (ASD), and one of these was shown to markedly affect EN2 promoter activity. Accordingly, mice lacking the homeobox domain of En2 (En2hd/hd mice; referred to as En2–/–) have been proposed as models for ASD, due to their complex neurodevelopmental, neuroanatomical and behavioral phenotype. En2–/– mice display cerebellar hypoplasia, including a reduced number of Purkinje cells, and a reduced number of GABAergic neurons in the hippocampus and cerebral cortex. Cortical GABAergic interneurons originate from the basal ganglia region between E14 and E18, subsequently migrating to the cortex by precise tangential migration routes. Finally, from behaviour analyses, these mice display reduced social interactions, locomotor impairment, defects in spatial learning and memory.

The molecular mechanisms linking the loss of En2 with the reduction in GABAergic interneurons is still not known. For this reason we have set up an *in vitro* model in which we investigated the role of En2 in neuronal differentiation, and more specifically in GABAergic differentiation. We derived neural stem cells (NSCs) from the basal ganglia of E14 wild-type and En2-/- embryos and assessed their proliferative and differentiation potential. Preliminary results show an increased proliferation and a reduced capability of neuronal differentiation of the En2-/- derived lines with respect to the wild type ones. This is the first molecular evidence linking the transcription factor En2 to the differentiation of GABAergic interneurons.

RELATIONSHIP BETWEEN DNA FRAGMENTATION INDEX AND pAKT IN CUMULUS CELLS: NEW MARKERS OF OOCYTE COMPETENCE

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The specific LH and FSH polymorphisms could influence the growth of follicles and oocytes.Some studies have shown that certain single nucleotide polymorphisms of FSHR are associated with changes in the ovarian activity, having functional implications in human reproduction. Carriers of polymorphic variant of betaLH show sub-optimal ovarian response to the standard long GnRH-agonist down-regulation protocol, when stimulated with recombinant FSH.[2] No studies have been designed relating the polymorphic variants of FSHR and LHB with the oocyte competence.

In previous studies, we demonstrated the correlation between the apoptosis rate and the expression level of some survival pathways molecules, as pAKT, in cumulus cells, as potential markers of oocyte competence.[3]

The aim of this study is to investigate the apoptosis levels of cumulus cells pool collected from the cumulus-oocyte complex of individual patients with specific FSHR and LHB polymorphisms.

Cumulus cells, obtained from 36 selected patients, were used for *in situ* immunocytochemistry by cleaved caspase-3, pAKT and by TUNEL assays. Genomic DNA was extracted from whole blood samples. SNPs of FSHR and LHB gene was amplified by PCR using different primers. We found the following phenotypes:

- for FSHR: A/T–S/N n=18; A/A–S/S n=6; T/T–N/N n=12
- for LHB: W/W–I/I n=23; W/R– I/T n=13

Cumulus cells of patients with phenotype A/T–S/N associated with W/R–I/T (double heterozygous) showed an higher level of apoptosis in terms of DNA Fragmentation Index (DFI) and percentage of active protein caspase-3 $P<0.05$ *vs* all other combinations, and an inverse proportion of the pAKT levels. However no statistical difference was found in clinical data.

In conclusion, these patients with double heterozygous will produce oocytes with a limited competence after ovarian stimulation with rFSH. So, it will be possible to personalize the ovarian stimulation according to polymorphic condition of the individual patient.

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COBALT OXIDE NANOPARTICLES CAN ENTER INSIDE THE CELLS BY CROSSING PLASMA MEMBRANES

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The ability of nanoparticles (NPs) to be promptly uptaken by the cells by different endocytotic mechanisms makes them both dangerous and useful to human health. Dangerous, as NPs might exert their toxicity, once inside the cell, very close to target organelles as nuclei and mitochondria, a phenomenon which is referred to as Trojan horse effect. Useful, as they can be directed to exert their toxicity toward cancer cells, used for drug delivery, injected as a contrast agent for diagnostic and even for theranostic purposes, and assumed for food supplementation.

It was recently postulated that some NPs might cross the plasma membrane also by a non-endocytotic pathway gaining a direct access to the cytoplasm. To study this possibility, we have resorted to full grown *Xenopus* oocytes that we have filled with Calcein, whose fluorescence is strongly quenched by divalent metal ions. To test if the Calcein was able to detect cobalt uptake, we transfected *Xenopus* oocytes with the Divalent Metal ion Transporter 1 from rat (rDMT1). Since the entry of the divalent metal ions into the transfected cell caused the quenching of Calcein, we decided that Calcein can be used to monitor divalent metal ion concentration changes in the cytoplasm of *Xenopus* oocytes.

After this check, we have used Calcein filled *Xenopus* oocytes to reveal the possible permeation of cobalt (metallic (Co^0) and oxide (Co_3O_4)) NPs inside the cell. Both NP forms undergo dissolution releasing cobalt ions that can be detected by Calcein quenching. Co_3O_4 NPs consistently induced a quenching of

Calcein fluorescence suggesting that they succeed in crossing the oocyte plasma membrane. Co NPs, instead, did not cause a reduction of fluorescence suggesting that were unable to pass through the plasma membrane. This different behavior of cobalt and cobalt oxide NPs could be ascribed to different chemical and physical characteristics of their surfaces.
We are obtaining similar results with iron oxide and this might be of use for food and feed supplementation.

ANKRD1 OVEREXPRESSION IN THE DEVELOPING MYOCARDIUM CAUSES ANOMALOUS VENOUS RETURN AND MORPHOGENETIC DEFECTS BY IMPARING CARDIAC REMODELING

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The acquisition of cardiac anatomical organization is the result of tightly regulated developmental step and even minor mistakes in this chain of events results in congenital heart disease (CHD).[1] Total anomalous pulmonary venous return (TAPVR) is a severe CHD characterized by failure of the pulmonary veins to connect exclusively to the left atrium.[2] We previously identified increased levels of the mechanosensory gene *ANKRD1* in TAPVR patients;[3] however, the link between its increased expression and TAPVR pathogenesis remains unexplored. Here we show that *Ankrd1* defines novel morphogenetic subdomains in the developing myocardium, where it modulates cardiomyocyte structural properties. *Ankrd1* is expressed in discrete subcompartments in the developing mouse heart and its myocardial overexpression in mice strongly impairs cardiac remodeling, including alignment of the developing venous system with the myocardium. Mid-fetal transgenic hearts present complex morphogenetic defects and abnormal pulmonary venous connections accompanied by strong cellular disorganization. Our results define *ANKRD1* as a crucial modulator of heart development, whose regionalized expression is required to refine shape and relative position of cardiac compartments. These findings uncover novel levels of complexity in genetic regulation of cardiac development. We propose that increased *ANKRD1* levels leads to TAPVR as a consequence of impaired remodeling of early venous pole myocardium.

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FIRST DESCRIPTION OF A HISTAMINE RECEPTOR OF CLASS 2 (HRH2) IN A PROTOCHORDATE: EXPRESSION DURING BLASTOGENESIS AND ROLE IN REGULATION OF CILIARY BEAT FREQUENCY

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Histaminergic receptors belong to the family of seven-transmembrane α-helix domain receptors classified in mammals into four distinct classes. Despite being widely studied in vertebrates, few data are available on the invertebrate receptors, with only predicted H1 and H2 sequences for non-chordate deuterostomes. We report the first transcript evidence of an H2 receptor for histamine in the colonial ascidian *Botryllus schlosseri* showing a high degree of conservation with HRH2 mammalian and other vertebrate orthologous proteins. The transcript and protein localisation during blastogenic development through *in situ* hybridisation and immunohistochemistry has been described. The mRNA expression appears first in the ciliary tissues of the alimentary system in filter-feeding adults and the buds, with a particular intensity in the pharynx. Transcription is activated very early, beginning from the inner layer of the disc of the secondary bud. From one generation to the next, the transcript signals become more and more intense at the level of the emergence of primordia of the branchial and peribranchial chambers and, finally, in the cells bordering the stigmata, dorsal lamina, and non-glandular ciliated zones of the endostyle. The translated H2 receptor appears as soon as the primordia of branchial and peribranchial chambers form in the secondary bud, and, in the primary buds, is found mainly in the protostigmata before the two layers of branchial and peribranchial epithelial tissue perforate to form the stigmata. In the adult zooid, the H2 receptor is expressed by ciliated mucous cells involved in food progression throughout the whole length of the alimentary canal. The observation of the effects of histamine and histamine-receptor antagonist (ranitidine) and agonist (dimaprit) drugs on explanted branchial tissue has provided confirmation concerning the receptor class and its role in regulating the ciliary beat frequency. The involvement in the local regulation of ciliary activity is of particular concern for evolutionary considerations because HRH2 seems to have been conserved in the pharynx and its developmental derivatives (*e.g.*, upper respiratory tract and middle ear of mammals) during the evolution of chordates.

A GLYPHOSATE MICRO-EMULSION FORMULATION WIDELY USED WORLDWIDE DISPLAYS TERATOGENICITY IN *XENOPUS LAEVIS*

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Nanopesticides represent an emerging technology that offers a better solubility of the active ingredients (AI) and their release in a slow/targeted manner. However, several issues have been raised about environmental fate and health hazard of these new materials. In this contest, we started to investigate the embryotoxicity of a formulation of glyphosate, which could be a good candidate for the future development of nanoformulations.
Glyphosate (G), is the AI in broad-spectrum herbicide formulations used in agriculture, domestic area and in aquatic weed control. Its market continues to grow with the increase in the cultivation of G-tolerant transgenic crops. The toxic action of G-based herbicides against non-target organisms is still a debated question even if different effects including oxidative stress, mutagenesis, reproductive toxicity and teratogenicity were evidenced.
In this study we tested the effects of Roundup power 2.0®, a next-generation formulation of G on the embryonic development of *X. laevis* using Frog Embryo Teratogenesis Assay-*Xenopus*, a suitable indicator for human developmental hazards and aquatic toxicology risk assessment.
Our results evidenced that Roundup causes lethality only at high concentrations, while sub-lethal doses (96-h TC50 7.3mg/L) are sufficient to induce concentration-dependent anomalies in embryos, such as microphthalmia, oval shaped eyes, craniofacial malformations and abnormal gut coiling. Since the commercial formulation is a complex mixture in which adjuvants improve penetration through the plant surface to the site of action, our study will be extended to the AI alone. Furthermore, to better characterize the observed malformations, histological and ultrastructural analysis will be performed together with the analysis of the expression of selected genes.
Our results allow to increase the toxicity data induced by G-based herbicides and will be useful in producing smart formulations, also in the view of developing nanopesticides where the AI can be encapsulated or nanoemulsified in order to obtained products able to harmonize the biocidal and safety properties.

CROSS TALK BETWEEN M2 RECEPTOR AND Notch1/EGFR PATHWAY IN GLIOBLASTOMA CANCER STEM CELLS: IMPLICATION IN CELL PROLIFERATION

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EGFR (Epidermal Growth Factor Receptor) and Notch signaling pathways are involved in the regulation of cell proliferation, migration and survival of different cell types. They are also important modulators of cell proliferation and maintenance of stemness properties in neural precursors. Their altered expression and function are directly correlated to tumor formation and progression. In fact EGFR and Notch-1 are frequently overexpressed in human gliomas, contributing to its aggressiveness and malignancy. Glioblastoma (GBM) is the most common human brain tumor characterizing by an intense proliferation and widespread invasion of poorly differentiated cells. In GBM, the undifferentiated cells with stemness properties (GCS) have been identified. Similarly to neural precursor cells, Delta/Notch and EGF/EGRFR pathways are expressed in these cells and their activity regulate cell proliferation, survival and self-renewal property, contributing to sustain the GBM growth, progression and invasion. Recently we have investigated the effects produced by the selective activation of M2 muscarinic receptors on GCS proliferation using two GCS cell lines isolated from human biopsy (GB7 and GB8 cells). In these cells, the treatment with the M2 agonist APE, decreases cell proliferation and causes an arrest of cell cycle progression; in particular in GB7 cells the decrease of cell proliferation is time and dose dependent. Pharmacological competition experiments using selective muscarinic antagonists and the M2 silencing by siRNA technique, have indicated that the APE-effects are dependent on selective activation of M2 receptors. The western blot analysis of EGFR and Notch-1 expression has clearly indicated that, in GB7 cells, APE causes a decreased expression of Notch-1 and EGFR. These results suggest that M2 receptor inhibits GCS proliferation probably interfering with Notch-1/EGFR pathway. Considering that GCS present a high chemo-resistance to the conventional therapies, the identification of new drugs able to counteract the GCS proliferation and survival, appears of the strategic relevance in the glioblastoma therapy.

ASSESSMENT OF VITRIFICATION-INDUCED STRUCTURAL DAMAGES IN MATURED BOVINE OOCYTES BY RAMAN MICROSPECTROSCOPY

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Vitrification induces ultrastructural and structural damages in different oocyte structures such as membrane, zona pellucida (ZP) and cytoplasm.[1] Recently Raman Microspectroscopy (RMS) has been used to assess the changes caused by vitrification/warming of *in vitro* matured ovine oocytes.[2] RMS is a non-invasive technique for studying the molecular composition of cells, based on the inelastic scattering of laser photons by vibrating molecules. Aim of the present study was to investigate the structural modifications of ZP and cytoplasm of vitrified/warmed *in vitro* matured bovine oocytes by RMS. Abattoir-derived *in vitro* matured oocytes (n=171) were denuded and divided in three experimental groups: control untreated oocytes (CTR), oocytes only exposed to vitrification/warming solutions (CPA) and vitrified/warmed oocytes (VITRI). Oocytes were exposed/vitrified in 20% EG + 20% of DMSO and 0.5 M sucrose and warmed into decreasing concentrations of sucrose (1.25 M-0.3 M). RMS analysis was carried out on CTR matured oocytes (after 22h) and on CPA and VITRI oocytes at different incubation times (0,1,2,3 and 4h) after exposure/warming. The analysis of the large spectral date set, acquired by RMS were performed by Principal Component Analysis (PCA). Our experimental outcomes suggest that vitrification induce a transformation of the protein secondary struc-

ture from the α-helices to the β-sheet form in the ZP, while lipids tend to assume a more-ordered configuration. Both effects induce a mechanical stiffening of ZP, which could explain the reduced fertility of vitrified oocytes with respect to the untreated ones. Intriguingly, these transformations present a certain degree of reversibility, which renders vitrified oocytes more similar to untreated cells after 2h of warming at room temperature.

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INSULIN-LIKE 3 (INSL3) EXPRESSION IN *DANIO RERIO:* MOLECULAR AND BIOINFORMATICS STUDIES TO EXPLORE EVOLUTION OF ITS FUNCTION IN VERTEBRATES

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Insulin-like 3 (INSL3), also known as Leydig cell-specific insulin-like peptide (LEY I-L), is a member of the insulin-IGF-relaxin peptide superfamily. It is a small peptide hormone that is synthesized as pre-prohormone and is secreted mainly in gonadal tissues in males and females[1,2] of all mammal so far analyzed. The fold comprises two polypeptide chains (A and B) linked by two disulphide bonds: all share a conserved arrangement of four cysteines in their A chain, the first of which is linked by a disulphide bond to the third, while the second and fourth are linked by interchain disulphide bonds to cysteines in the B chain.

The organization of *insl3* gene is similar to that of insulin and relaxin consisting of two exons and one intron. Bioinformatics analyses that we performed on almost all known *insl3* genes and cDNA sequences from DataBase reveals that its structure is conserved among fish and several non mammal vertebrate species but intron length is different.

Using RT-PCR analysis we found that *D. rerio* embryos express *insl3* gene as both major and minor splice variants, the former encoding the normal protein, the latter a truncated peptide comprising a C-terminally extended B-domain. Both transcripts are produced in constant relative amounts during embryogenesis. In addition, we report here results of *in situ* hybridization experiments performed in order to localize the alternative transcripts in male and female adult *D. rerio*. As expected, taking into account the analysis reported by Donizetti *et al.*,[3] *insl3* expressing cells were found in brain and oral epithelium in agreement to the spatial organization analyses of receptor rxfp2-like, one of the three receptor rxfp2 homologue genes.

In silico analyses performed on proximal promoter provide molecular evidence on a possible functional role gain of a single copy *insl3* gene in mammalian testis development.

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EFFECTS OF DIBUTYLPHTHALATE ON HUMAN PROSTATE CELLS

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Phthalates are a group of compounds belonging to Endocrine Disrupting Chemicals (EDCs), used to impact plastic flexibility, durability, transparency and longevity. They are present in many common products and human exposure occurs daily. Dibutylphthalate (DBP) is abundantly present in the environment and its effects on reproductive system were demonstrated both *in vitro* and *in vivo*. In this work we studied the effects of DBP and Estrogen (E2) on human 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, it has also been suggested a central role for estrogen in this system.It has been hypothesized that high level of estrogens may disturb the endocrine control of the male reproductive capability. First of all we examined the effects of DBP and E2 on the proliferation of the LNCaP through MTT assay. After 24 h of treatment DBP induces a decrease of cell proliferation at 10^{-8} M, instead E2 at 10^{-9} M stimulates prostate cell proliferation and viability. Subsequently, we evaluated the distribution of cells in the various phases of the cell cycle through FACS analysis showing that DBP induced a S1 arrest. Moreover, in order to study estrogen (ER) and androgen (AR) receptor involvement, we evaluated the cell localization and expression of ERs and AR with immunofluorescence and western blot techniques. Immunofluorescence analyses revealed that both DBP and E2 induced cytoplasm-nucleus translocation of ERα even if E2 was able to induce an early nuclear translocation respect to DBP. DBP and E2 did nott interfere with ERβ localization. After 4 h of treatment only E2 but not DBP interfere with AR nuclear translocation. Our results confirm that DBP may be involved in the deregulation of prostate cell cycle and that it may interfere with both estrogen and androgen hormonal receptor pathways.

3D BIO-PRINTING AND MUSCLE DERIVED PERICYTES FOR ARTIFICIAL SKELETAL MUSCLE HUMAN-LIKE SIZE

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The skeletal muscle tissue exhibits good regenerative capabilities, which are however limited by injury size. As a matter of fact, large muscle lesions are characterized by poor recovery accompanied by scar formation and functional detriment, condition common to people suffering from volumetric muscle loss and needing reconstructive therapeutic approaches. Even if surgical autologous transplantation is a standardized procedure, the outcomes are often unsatisfactory. Hence, the pressing need to develop engineered artificial tissues to replace wasted muscle. Tissue engineering (TE), exploiting stem cells embedded in biomimetic scaffolds, aims to mimic organogenesis by building artificial tissues to replace the damaged ones. Skeletal muscle TE is an up-and-coming biotechnology with great potential for muscle repair, but no conclusive strategy has been demonstrated yet. Reconstructing the skeletal muscle architecture and function is still a challenge requiring the parallel alignment of myofibrils arranged into organized sarcomeres. Recently we demonstrated the great potential of a hybrid biomimetic matrix, namely PEG-Fibrinogen, for enhancing the engraftment of myogenic cell progenitors by providing a suitable 3D environment for mouse muscle reconstruction. Starting from these observations, we developed a novel approach for the regeneration and/or reconstruction of skeletal muscle tissue segments of human-like size by exploiting a population of adult myogenic stem cells, namely pericytes, in combination with 3D bio-printing technology to guarantee a functional architecture. *In vitro* characterization of cell-laden constructs showed enhanced myogenesis and positive myostructure alignment. Thanks to the enhanced control over cell deposition and alignment, the presented technology has the potential to support skeletal muscle repair and regeneration.

THE DEVELOPMENTAL BIOLOGY OF THE COLONIAL ASCIDIAN *BOTRYLLUS SCHLOSSERI.* FROM ARMANDO SABBADIN'S BREAKTHROUGH STUDIES TO NEW SCENARIOS FOR STILL UNSOLVED QUESTIONS

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In over 60 years of studies, the colonial ascidian *Botryllus schlosseri* has become a model for the study of sexual and asexual reproduction.[1] The species was introduced as developmental biology model by Prof. Armando Sabbadin, in the University of Padua (Italy), and is now reared in different laboratories around the world. Its blastogenetic cycle is characterized by three, co-existing blastogenetic generations (filtering adults, buds, and budlets) and by recurrent generation changes. Cyclically, the adult generation undergoes regression and is absorbed by the colony for bud growth. In the meantime, buds become adults, and budlets produce a new generation. This process is highly coordinated and regulated in the colony. Here, we review the main studies on reproduction and colony growth of *B. schlosseri*, with reference to the asymmetry in sexual and asexual reproduction potential, sexual reproduction in the field and the laboratory, and self- and cross-fertilization. These studies were started by A. Sabbadin and then developed over the time by different research groups. Coupling the classical experiments of fusion/separation/controlled-crosses of colonies, to the recent tools of transcriptomic[2] and genomic,[3] the species is used now to deeply elucidate a variety of questions related to developmental processes, such as: stem cell homing and recycling among blastogenetic generations;[4] the somatic and germ line parasitism in chimeric colonies;[5] an *evo-devo* approach to study the cooption of mechanisms and gene-networks in development either among taxa,[6] or between the two reproductive processes (embryogenesis and asexual reproduction);[7] the *eco-devo* analysis of environmental factor.[8]

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INTERNEURON DEFECTS DURING POSTNATAL DEVELOPMENT OF SOMATOSENSORY CORTEX OF ENGRAILED 2 KNOCKOUT MICE, A MODEL OF AUTISM

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The homeobox-containing transcription factor Engrailed-2 (En2) is a candidate gene for autism spectrum disorder (ASD), and En2-/- mice display anatomical and behavioural "autistic-like" features. Previous studies from our laboratory showed that adult En2-/- mice have a reduced number of GABAergic interneurons in the hippocampus and sensory neocortical areas (somatosensory and visual). These defects are accompanied by increased seizure susceptibility spatial learning deficits and a delayed maturation of visual function. Since reduced GABAergic inhibition has been proposed as a possible pathogenic mechanism of ASD, in this study we further investigated the postnatal maturation of inhibitory circuits in the En2-/- somatosensory (S1) cortex. By using quantitative RT-PCR and immuhistochemistry, we showed that the expression of GABAergic interneuron markers parvalbumin (PV) and somatostatin (SST) is significantly reduced in the En2-/- S1 cortex at postnatal day (P) 10, as compared to wild-type (WT) controls. This indicates that the interneuron defects observed in the adult En2-/- neocortex have a developmental origin, and studies are ongoing to further characterize the anatomy of inhibitory circuits in the En2-/- S1 cortex during postnatal development. Finally, to investigate the functional consequences of GABAergic interneuron defects in the En2-/- S1 neocortex, we set up a battery of behavioural tests for the somatosensory function in adult mice. Our study will aim to establish a link between En2, anatomical deficits of GABAergic forebrain neurons and the pathogenesis of ASD.

EXPOSURE OF SEA URCHIN *ARBACIA LIXULA* EMBRYOS TO CuO NPs AFFECTS LARVAL MORPHOGENESIS, NEUROTRANSMISSION AND SKELETOGENESIS

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In the last decades, a great deal of concern has been raised over the extensive use of nanoparticles (NPs) in a variety of fields and their potential toxicity to the environment and human health. In this context, the embryotoxicity of copper oxide (CuO) NPs was assessed in the black sea urchin *Arbacia lixula*, an intertidal species commonly present in the Mediterranean. Fertilized eggs were exposed to five doses of CuO NPs ranging from 0.009 to 2.9 µM, until the pluteus larva stage. Developmental delay and morphological abnormalities were observed, as well as interference with the normal neurotransmission pathways. In detail, evidence of serotoninergic and cholinergic systems affection were revealed by a reduction in serotonin (5-HT) and inhibition of AChE enzymatic activity, as further supported by dose-dependent decreased levels of *N*-acetyl serotonin and choline, respectively, as detected by a NMR-based metabolomics approach. Moreover, because of the evident alterations of the skeletal spicules induced by CuO NPs, the expression of skeletogenic genes, i.e. msp130 and sm30, was investigated and found to not differ among groups, allowing to hypothesize altered PMC migration. Evidence of interference in the skeletogenesis of sea urchin embryos was also provided by a significant CuO NP-dependent increase of anhydroglucose and glycine, which are components of matrix proteins and/or proteins involved in the biomineralization process, respectively. Noteworthy, other unknown metabolites were detected from the NMR spectrum of sea urchin embryos, and their concentrations found to be reflective of the CuO NP exposure levels. Overall, these findings demonstrate the toxic potential of CuO NPs to interfere with larval morphogenesis, neurotransmission and skeletogenesis of sea urchin embryos. Moreover, the embryotoxicity tests, conducted with sea urchins as model organism, represent a highly sensitive and effective tool for assessing the effects of NPs on the health of aquatic biota.

WHEN A DEVELOPMENTAL BIOLOGY MODEL SPECIES CHANGES NAME: THE CASE OF *CIONA INTESTINALIS*

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The ascidian *Ciona intestinalis* is the main tunicate species for developmental biology studies. Its larva joins simplicity to a chordate body plans, rendering it suitable for evo-devo researches. The species was described by Linnaeus[1] in 1767. In 2007, molecular analyses on specimens sampled in various localities evidenced that different taxa were hidden[2] under the name *C. intestinalis* (L.). In particular, two cryptic taxa, named *C. intestinalis* types A and B, showed disjoint geographical distribution and were molecularly distinguishable. In 2015, two papers[3,4] evidenced that *C. intestinalis* type A is *Ciona robusta*,[5] whereas *C. intestinalis* type B is *Ciona intestinalis* (L.). *C. intestinalis* larvae have a longer pre-oral lobe, longer and narrower total body length, and a shorter ocellus-tail distance than *C. robusta* larvae. Only *C. robusta* adult individuals show tubercular tunic prominences. The nomenclature change caused concerns among developmental biologists: since *C. robusta* has a wider distribution than *C. intestinalis*, most researchers realized that they are actually working with *C. robusta*. Moreover, the genome published in 2002 as that of *C. intestinalis*, *de facto* belongs to *C. robusta*. This name revision represents a challenge for the tunicate community and the first case concerning a genome-released model organism. Firstly, an initially recognized single taxon now consists of two distinct species. Second, the name *C. intestinalis* will continue to be used for one of the two species (this could create confusion in citing literature). Third, a large body of data (genomic, transcriptomic, proteomic, *etc.*) currently used and stocked in public databases should be re-assigned. The entire tunicate community is actively involved in the development and implementation of solutions of this challenging issue.

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ROLE OF THE CHROMATIN REGULATOR SETD5 DURING ZEBRAFISH BRAIN DEVELOPMENT

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Loss-of-function (LoF) mutations in one SETD5 allele in humans have been recently identified as relatively frequent mendelian causes of both intellectual disability (ID) and autistic spectrum disorders, the most common neurodevelopmental disorders (3-5% of the population). Interestingly, SETD5 falls within the critical interval deleted in the 3p25.3 microdeletion syndrome which is characterized by ID, microcephaly and congenital heart defects. These findings suggest that SETD5 haploinsufficiency is sufficient to cause the disease. SETD5 encodes for a putative histone H3 methyltransferase highly expressed in the brain, whose activity has not been clearly demonstrated yet. The aim of this study is to generate and characterize zebrafish models in which *setd5* has been knocked down or knocked out. As a first approach, we have targeted zebrafish *setd5* with a specific antisense morpholino oligonucleotide. External inspection and *in situ* hybridization analysis with district-specific markers indicate that *setd5* knockdown embryos display microcephaly, cardiac edema and reduced locomotor activity response. Compared to embryos injected with a control morpholino, *setd5* LoF brains, despite their remarkable reduced size, show a statistically significant increase of mitotic cells as assessed by immunostaining with anti-phospho-H3 antibodies, while immunoreactivity to the neuronal differentiation marker HuC is not changed. We are currently evaluating whether *setd5* knockdown results in a possible mitotic arrest of developing brain cells followed by apoptosis. Furthermore, exploiting the Crispr/Cas9 genome editing strategy, we are planning to establish stable setd5 knockout zebrafish lines. These animal models will be extremely useful to identify the molecular mechanisms underlying SETD5 LoF phenotype and to screen for drugs and chemical compounds able to rescue or alleviate the developmental defects.

TOXICITY INDUCED BY GADOLINIUM IONS ON DEVELOPING SEA URCHIN EMBRYOS

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Pharmaceuticals are a class of emerging environmental contaminants. Gadolinium (Gd) 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. We investigated the effects of exposure to sublethal Gd concentrations on the development of four phylogenetically and geographically distant species: two Mediterranean species, *Paracentrotus lividus* and *Arbacia lixula*, and two species living in the East coast of Australia, *Heliocidaris tuberculata* and *Centrostephanus rodgersii*. Measures of the Gd and Ca content inside embryos by ICP-MS showed a time- and dose-dependent increase in Gd, in parallel with a reduction in Ca, suggesting that Gd competes with Ca for binding to the Ca channels. In all the four species, we observed a general delay of embryo development at 24h post-fertilization, and a strong inhibition of skeleton growth at 48 h, with species-specific threshold levels of sensitivity.
Further experiments were carried out on *P. lividus* embryos. Removal of Gd after 24 h caused partial recovery of embryo development 48 h post fertilization. We detected an increase of the LC3 autophagic marker at 24 and 48 h, while confocal microscopy studies confirmed the increased number of autophagosomes and autophagolysosomes. In contrast, immunofluorescence studies showed no apoptotic induction.

PRE-BIRTH SENSE OF SMELL IN WILD BOAR (*SUS SCROFA*), THE ONTOGENY OF OLFACTORY MUCOSA

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Artificial selection began to override natural selection in domesticated wild boar and other species about 10,000 years ago. The intentional selection of a desired phenotypic trait is a complex process, and comes along with unexpected or even unwanted changes in other traits, because of epistatic gene effects, and ontogenetic constraints.[1] The loss of brain mass in domestic ungulates is related to selection for reduced reaction to external stimuli. Wild boar, more than pig, must be beforehand ready for the chemical universe that will house it. As a matter of fact, it is showed a more intense neuronal activity of olfactory mucosa in wild boars, compared to pigs.[2] With this in mind here we have identify the morpho-functional development of olfactory mucosa. Here we face implications of pre-birth adaptation, using mRNA expression analysis of Olfactory Marker Protein and Neuropeptide Y involved in olfactory sensory neurons (OSNs) functionality. We show the early activation of OSNs in wild boar and remarkable differences compared to pig at neonatal stage.

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AUTOSOMAL RECESSIVE RETINITIS PIGMENTOSA: TARGETING IMPG2

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The InterPhotoreceptor Matrix (IPM) consists of the extracellular space between photoreceptors and retinal pigmented epitelium (RPE) and is involved in their interconnection and functions. In the IPM of the eye have been found proteins (opsin, the alpha-subunit of transducin, interphotoreceptor retinoid-binding protein and others), but also carbohydrates and proteoglycans. This composition suggests an involvement of IPM in intercellular communication, cell survival, membrane turnover, photoreceptor function and retinoid transport. Data suggests that alterations in IPM could be correlated to different retinal diseases, such as Retinitis Pigmentosa (RP). Recently, a new form of autosomal recessive RP has been found to be correlated to IMPG2, a proteoglycan specific to the vertebrate IPM. In human, six different nonsense point mutations seem to affect human IMPG2 functions and five of them produce a truncated IMPG2. Affected patients present an altered rod-cone pattern and a reduced or deleted ERG response demonstrating loss of vision. Until now, no animal models are available to study IMPG2-related retinopathies. Moreover, what is the possible role of IMPG2 in vertebrate retinal development and how a truncated IMPG2 could lead to RP degeneration is not yet known. Zebrafish transgenic lines represent a powerful tool to study human neurodegenerative diseases and to identify candidates for therapeutic approaches. We propose to study IMPG2 proteoglycan during zebrafish retinal development and to generate zebrafish transgenic lines by a CRISPR/Cas9 genome editing. We plan to introduce in the zebrafish IMPG2 the human mutations, in order to truncate IMPG2 mimicking the human alteration. This will create a strong animal model that will allow us to study the cell and molecular biology of IMPG2-related retinopathies. With this animal model, it will also be possible to test on a large scale the therapeutic use of new compounds.

HIGH FAT DIET CONTRIBUTES TO TESTICULAR TOXICITY INDUCED BY DDE IN RATS

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The 1,1dichloro2,2bis(p-chlorophenyl)ethylene (DDE), the major metabolite of DDT, is an organic pollutant and a male reproductive toxicant. Parallel, obesity induced by hypercaloric diet is a pervasive problem for the human health; the simultaneous intake of fats and lipophilic xenobiotics, such as DDE, increases assimilation of pollutants and cell stress.
In this study, independent and combinative toxicities of high fat diet and DDE were tested for testicular toxicity. Male rats were exposed to DDE (10 mg/kg bw) and fat (245 g/Kg food, 45% J/J) daily, individually or in combination for 30 days, then the four groups of rats (1:control, *N* group; 2:high fat, *HF* group; 3:DDE-treated, *DDE* group; 4:*DDE+HF* group) were sacrificed and testes were removed and immediately processed for morphological and molecular analyses.
Results demonstrated marked histopathological lesions in both *HF* and *DDE* groups. Exposure to DDE or HF diet resulted in

the reduction of the size of seminiferous tubules and in the damaging of spermatogenic epithelium. In addition, *DDE* rats showed hypertrophy of Leydig cells and disruption of the arrangement of spermatogenic layers. The seminiferous tubules contained few spermatogenic cells; spermatogonia showed cytoplasmic vacuolization with pyknotic nuclei and sperms were scattered in the lumen of the tubules. Spermatids retention, degenerating germ cells and occlusions of the efferent ductules were also evident. These alterations were more significant in testis of *DDE+HF* rats.

Physiomolecular approaches demonstrated that mitochondrial respiratory rates decreased in *DDE* rats, suggesting a DDE-induced damage of mitochondrial functions. Real-Time PCR analysis showed no significant differences in the expression of the cytoprotective protein metallothionein (MT between *N* and *HF* group and between *HF* and *DDE+HF* group; surprisingly, an appreciable decrease in MT mRNAs was found in *DDE* group. Data were confirmed by MT immunolocalization.

In conclusion, the exposure to DDE or HF diet adversely affected the male reproductive organ, and a higher testicular toxicity was detected upon exposure to combinations of DDE and HF. Further studies will be needed to clarify if this toxicity impairs the reproductive functions.

THE CELL DEATH PHENOMENON DURING TUBER ECTOMYCORRHIZA MORPHOGENESIS

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Cell death phenomenon was investigated during the formation and establishment of Tuber ectomycorrhiza (ECM) with host trees, both *in vitro* and in pot culture using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) reaction, Transmission electron microscopy (TEM) and Enzyme-linked immunosorbent assay (ELISA). *Tuber borchii* mycelium and plantlets of *Tilia plathyphyllos* were used for *in vitro* ECM synthesis, whereas *T. melanosporum*, *T. aestivum* and *T. borchii* spores and seedlings of *Corylus avellana*, *Quercus pubescens* and *T. plathyphyllos* were employed in pot cultures. Non-mycorrhizal roots showed TUNEL-positive nuclei at the level of cap cells and tracheary elements as a result of physiological root morphogenesis. In contrast, during the presymbiotic phase and the following ECM developmental stages, progressive accumulation of tannin/polyphenol deposits developed in epidermal and cortical cells, leading to the cell death but without TUNEL positivity. After this necrosis, a further unexpected autophagic cell death was observed in apparently healthy mycorrhizae, first affecting mycoclena and then the Hartig net hyphae. This series of cell death phenomena involving both root cells and fungal ectomycorrhizal hyphae points to the existence of a genetic orchestration between the two symbiotic partners during ECM morphogenesis and deserves further investigation to elucidate the underlying molecular mechanisms and signaling pathways.

ENVIRONMENTAL COCAINE CONCENTRATION EFFECTS ON *DANIO RERIO* DEVELOPMENT

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The illicit drugs can be considered the latest group of emerging pollutants, widespread in the aquatic environment, as a consequence of the enormous increase in the global consumption of these drugs.[1] Although their strong pharmacological activities[2] let foresee toxic effects to the aquatic organisms, only few studies have been performed to evaluate the real risks to the aquatic species.[3] In particular, previous data showed that when the European eels (*Anguilla anguilla*) are chronically exposed to environmental cocaine concentrations, they bioaccumulate cocaine in their tissues, especially muscles[4] and undergo endocrine[5] and histological[6] alterations. Therefore, the aim of the present study was to evaluate if environmental cocaine concentrations could affect the development of the zebrafish, *Danio rerio*, a well-known bioindicator of environmental pollution. Toxicity tests at environmental cocaine concentration (20 ng/l) were performed on zebrafish embryos and larvae. No mortality and phenotypic alterations were revealed on both developmental stages at 24, 48 and 72 h of cocaine exposure. However sections of larvae fixed in Bouin's solution, embedded in paraffin for light microscopy and stained with ematoxylin and eosin (HE) showed alterations of the muscle tissue, that appeared reduced in thickness and disorganized. An increase of apoptosis was also observed in the tail cells of larvae by acridine orange stain in whole mount. These results agree with unpublished data, showing similar changes in the muscles of *A. anguilla*, chronically exposed to cocaine. Therefore, the present results indicate that cocaine, at environmental concentrations, could impair the skeletal muscle development in zebrafish larvae.

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ADVERSE EFFECTS OF CADMIUM AND ALUMINUM ON ZEBRAFISH DEVELOPMENT

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Exposure to several metals during pregnancy has been shown to be harmful to the developing fetus.[1,2] The use of zebrafish embryos and larvae is always more widespread and accepted in the scientific community for the screening of dangerous effects exercised by pollutant.[3] Zebrafish embryos at shield stage[4] were separately exposed to different concentrations of aluminum chloride ($AlCl_3$) and cadmium chloride ($CdCl_2$) for 48 h with the purpose to assess the toxic actions of these metals on their development. After 24 and 48 h we carried out phenotypic and lethality analysis. We observed a greater toxic action of $CdCl_2$ on these stages of development. In fact at 24 and 48 h of exposure the total lethality (LC_{100}) was observed at 1 and 0.5 mM for cadmium respectively and at 40 and 25 mM for aluminum. The phenotypic analysis doesn't show alterations induced by $AlCl_3$ while the embryos exposed to $CdCl_2$ showed pericardial oedema, hypopigmentation, alterations in the development of the tail and

of the body axis. Considering the minor effects of $AlCl_3$ we carried out toxicity tests on larval stage, a system commonly recognized as more susceptible to pollutants toxicity.[5] Larvae at protruding mouth stage[4] were exposed to different concentration of $AlCl_3$ for 48 h and in these experiments we observed a LC_{100} of 0.25 and 0.20 mM respectively after 24 and 48 h of exposure. These values were comparable to those obtained with $CdCl_2$. We observed the ability of $AlCl_3$ to induce heart alterations with pericardial oedema and a reduction in heart rate after exposure to 50 and 100 μM. Cytotoxic effects of these metals were also observed on the nervous system in embryos and in larvae. We find an increase of apoptotic index in embryos exposed to 9 μM of $CdCl_2$ for 24 h while in larvae exposed for 48 h to 100 μM of $AlCl_3$ there was a reduction in number of GFAP-positive cells. These data show that $CdCl_2$ is more toxic than $AlCl_3$ on zebrafish development, both are neurotoxic, but $AlCl_3$ has also cardiotoxic ability.

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POTENTIAL TOXICITY OF IMPROPERLY DISCARDED EXHAUSTED PHOTOVOLTAIC CELLS

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Low tech photovoltaic panels (PVPs) installed in the early '80s are now coming to the end of their life cycle and this raises the problem of their proper disposal. As panels contain potentially toxic elements, unconventional, complex and costly procedures are required to avoid environmental health risks and in countries where environmental awareness and economic resources are limited this may be especially problematic. This work was designed to investigate potential risks from improper disposal of these panels. An exhausted panel was broken into pieces, placed in water for 30 days and the resulting solutions were analyzed to determine chemical release and the potential toxicity of the solutions in established test protocols. These tests included end point seed germination (on *Cucumis sativus* and *Lens culinaris*) and effects on early development in three larval models: two crustaceans, *Daphnia magna* and *Artemia salina,* and the sea urchin *Paracentrotus lividus*. Our results show that the panels release small amounts of electrolytes (Na, Ca and Mg) into solution along with antimony and manganese and nickel at potentially toxic concentrations. Developmental defects are seen in the plant and animal test organisms after experimental exposure to the solutions leached from the broken panel.

IN VITRO MODULATION OF CADMIUM GENOTOXICITY BY TIO2 NANOPARTICLES IN *DICENTRARCHUS LABRAX* EMBRYONIC CELLS (DLEC)

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Titanium dioxide nanoparticles (n-TiO_2) continuously released into waters may cause harmful effects to aquatic organisms and their potential interaction with conventional toxic contaminants represents a growing concern for biota. Interaction between n-TiO_2 and cadmium ($CdCl_2$) has been investigated in different freshwater models leading to conflicting results. The aim of this study was to assess *in vitro* the genotoxicity n-TiO_2 (1 μg/L) and to investigate the genomic effects generated by the co-exposure with of $CdCl_2$(0,1 μg/mL). The study was performed using sea bass continuous embryonic cell line (DLEC) as a model for 3, 24 and 48 h of exposure. The genotoxic potential was assessed by DNA strand breaks, degree of apoptosis and molecular alterations at the genomic level. Three methods were used: Comet Assay, Diffusion Assay and RAPD-PCR technique, that was utilized to calculate the Genomic Stability of the Template (GTS%). Data were analyzed for statistical significance using unpaired Student's *t*-test ($P \leq 0.05$). The results showed that n-TiO_2 generated genotoxic effects for all exposure time, in fact induced a statistically significant loss of DNA integrity and of genomic stability and an increase of apoptotic process. In the same way, the $CdCl_2$ exposure induced an increase of DNA damage accompanied by a decrease in genome template stability at 3, 24 and 48 h of exposition. The co-exposure (n-TiO_2+ $CdCl_2$) did not induce a statistically significant genotoxic effects for all exposure times. These results suggested an antagonistic role of n-TiO_2 in abating $CdCl_2$ genomic damage. It can thus be hypothesized that n-TiO_2 were able to form aggregates with cadmium, reducing so its genotoxicity. The DNA damage significantly decreased after co-exposure to n-TiO_2+$CdCl_2$, with respect to that found by the single exposure, since their interaction form a 'sandwich structure' in which cadmium, placed at the centre, is fully incorporated by n-TiO_2, which instead displayed on the outer surfaces of the structure. Further research are necessary to elucidate genotoxic mechanisms induced by mixtures of NPs and toxic contaminants.

FUNCTIONAL CHARACTERIZATION OF NOVEL GENES INVOLVED IN BRAIN AGING AND DEVELOPMENT

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Adult neurogenesis is the process by which new neural cells are generated from a small population of multipotent stem cells located in specific area of the central nervous system (CNS). The age-related incidence of many CNS diseases coincides with a reduced adult neurogenic potential. The regenerative capability and the amount of adult neural stem cells (aNSC), in fact, decline with age, contributing to the reduced functionality of the aged brain. Despite the great interest in age related diseases, in Italy alone over-65 people will rise to the 18% value of 2010 to more than 30% in 2050, the molecular factors responsible for age-dependent decay of neural stem cell function are almost unknown. We envisage that genes controlling age-dependent processes act in continuity between development, adulthood and

aging. The starting point of our work was a list of brain age-regulated mRNAs that we have previously obtained by RNA-Seq and validated by qPCR and *in situ* hybridization.[1] Among them, we are currently studying the expression profile and the function of *Mex3A* and *Znf367* genes, codifying respectively for a RNA binding protein and a transcription factor, in embryonic neurogenesis. These genes, of unknown function, are expressed in neuroblasts and retinoblasts of Zebrafish and *Xenopus laevis* embryos and in the aNSC of the short-lived fish *Nothobranchius furzeri*. By means of gene gain and loss of function approaches in *Xenopus* and Zebrafish embryos, we started to clarify the specific function of these genes in regulating the maintenance of a stem phenotype in the developing CNS and in regulating survival and differentiation of the primary neurons. The same genes will be tested to verify their function also in neural stem cells of adult fishes. The identification of genetic mechanisms involved in embryonic and adult neurogenesis represents the first step in defining interventions that can increase neurogenesis in the aged brain and that could lead to improved maintenance and even repair of neuronal function.

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NEUROTOXIC EFFECTS OF CHLORPYRIFOS EXPOSURE ON NEONATAL MICE

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Chlorpyrifos (CPF) is one of the most used organophosphorus insecticide (OP) and putative developmental neurotoxicant in different organisms and also in humans.[1,2] Maternal exposure to CPF was found to be dose-related to slower brain growth and associated to several structural abnormalities such as thinning of cerebral cortex.[3] In mice, prenatal CPF exposure has the potential to affect long-term brain cognitive function, but the mechanisms are still unknown.[3] It has been demonstrated that even sub-lethal doses of neurotoxic pesticides affect behaviour by inhibiting acetylcholinesterase (AChE). To elucidate through what mechanism CPF induces alterations in neurogenesis, we used the M*us musculus* as animal model. F2 offspring (3-8 month) of *Mus musculus* females, treated with 3 doses of CPF (0.1-1-10 mg/kg/d) during pregnancy, were used to study the effects of chronic exposure to this compound. The analysis was performed on 84 genes associated to Parkinson disease, using RT^2 Profiler PCR Arrays. There was a statistically significant dose-dependent correlation between increasing CPF concentration and the down-regulation for many genes studied at 3 and 8 month mice. It was interesting to note that, among the genes significantly down-regulated, there were those more directly related to Parkinson's disease, such as the Park2, Sv2b, Gabbr2, Sept5, Atxn2, whereas the expression of the Ubiquitin C, Rgs4 and Chgb showed an up-regulation in 8 month mice. Furthermore, we found alteration of the AChE activity, that was inhibited by the exposure to CPF. The results of our study confirm that CPF can elicit alterations in the gene expression profile during neurogenesis and reinforce the view that the critical window of vulnerability of the developing brain to CPF extends from gestational exposure through postnatal period.[4]

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LARVAL AND ADULT NERVOUS SYSTEMS IN *RHABDOPLEURA MERONUDA*

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Nervous systems enable organisms to sense external and internal stimuli, process and integrate multiple sources of information, and produce from simple to elaborate patterns of responses - from single cell to whole body level - by means of neurosecretory and motor effector systems.[1] The evolution of nervous system among deuterostomes is a still debated question.[2] Deuterostomes include Chordata and Ambulacraria, a taxon encompassing echinoderms and hemichordates. In the recent years, both enteropneust and (less frequently) pterobranch hemichordates have been proposed as emerging model organisms for evolutionary and developmental studies.[3-6] To shed light on the evolution of nervous system in Ambulacraria, this study describes for the first time the neuroanatomies of both the zooid and the crawling 'planula-like' larva of a new pterobranch species, *Rhabdopleura meronuda* from the Mediterranean Sea. By scanning electron microscopy, confocal laser scanning microscopy, immunohistochemistry, and histological sections, we described the nervous system organization of both adult and larval stages and its rearrangement during metamorphosis.

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ANTI-PROLIFERATIVE AND ANTI-MIGRATORY EFFECTS MEDIATED BY M2 MUSCARINIC RECEPTORS IN SCHWANN-LIKE CELLS INDUCED FROM ADIPOSE MESENCHYMAL STEM CELLS: IMPLICATIONS IN NERVE REGENERATION

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The peripheral nervous system has an intrinsic regeneration capability. Nevertheless, the nerve regeneration is often not satisfactory, leaving patients with physical morbidity. Schwann Cells (SCs) play a central role in the response of the axon injury, in the removal of myelin debris, in the modulation of inflammatory response, and in the production of growth factors that enhance nerve regeneration. However, SCs show some drawbacks for tissue engineering, as the difficulty in collection and culture and the slow *in vitro* expansion potential. For this reason, the attention of the researchers has moved toward other cell types presenting best properties for regenerative medicine. The adipose tissue contains mesenchymal stem cells (ASC). Recently, it has demonstrated that ASC can be differentiated *in vitro* in Schwann-like (dASC). dASC expresses SCs markers and promote neurite outgrowth *in vitro.* dASCs, as SCs, express

receptors for different neurotrasmitters (*i.e.*, GABA, acetylcholine) that can modulate physiological processes such as proliferation, migration and myelination. Acetylcholine (ACh) has the property to control Schwann cell proliferation and differentiation via M2 muscarinic receptors. In present work we characterized the effects mediated by M2 receptors in rat dASC. dASC express all muscarinic receptor subtypes and in particular, M2 subtype. In dASC, M2 agonist APE caused a decreased cell proliferation, the inhibition of cell migration without affecting cell survival. After M2 agonist treatment, we have also observed modulated expression of neurotrophic factors and myelin proteins. Considering the ability of dASC to respond to cholinergic stimuli, we are setting up co-cultures of sensory neurons/dASCs in order to study the *in vitro* myelination in presence of cholinergic agonists. The data obtained may contribute to identify new therapeutic strategies for peripheral nerve regeneration based on the combination of cell therapy and pharmacological treatments.

LEGISLATION ON ANIMAL EXPERIMENTATION: MEDIATING BETWEEN ANIMAL RIGHTS ASSOCIATIONS AND THE SCIENTIFIC COMMUNITY

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Over the last 40 years, scientific knowledge and animal rights associations have influenced the way our culture sees animals and the way it values their life. As a result, European laws regarding the delicate subject of animal experimentation have become stricter than before and more oriented towards ensuring the wellbeing of the animals. However, it has gotten to the point where some of the prohibitions and limits included in the laws are almost incomprehensible for the scientific community - for example, it is no longer possible to use certain animals in specific fields of research.[1] Protecting the animals and ensuring their welfare remains crucial for scientists and researchers as well, but unreasonable bans and laws that are too strict can be damaging for research itself.

The EU Directive 63/2010[2] highlights two fundamental aspects of the legislation, that is 'animals are sentient creatures and as such they need to be considered' and 'animals have an intrinsic value and for this they must be respected and protected for the only purpose of themselves'. Nevertheless, animal rights associations are still battling against laboratory animal use by stating that it is absolutely immoral and unethical and most of all useless; whereas the scientific community is trying to speak in favor of this crucial aspect of biomedical research by underlying its necessity and its concrete application to everyday life.

At this point, the question is whether or not it is possible to find a middle ground between the two opposite parties by encouraging an open dialogue based on accurate data and conducted in an honest and reasonable way. This is the hope of the scientific community and the aim of SPERA. SPERA represents those who are morally and scientifically committed to the proper use of laboratory animals in scientific research; it supports their role through a campaign to raise awareness about the aims and instruments of scientific research and the results it has accomplished so far. SPERA intends to promote curiosity with a series of different projects that would involve scientists and students at all levels.

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PACAP/RECEPTORS SYSTEM IN *COTURNIX COTURNIX* TESTIS

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PACAP (Pituitary adenylate cyclase-activating polypeptide) is a neuropeptide of secretin/glucagon superfamily with a widespread distribution, involved in a number of biological processes like growth, cell proliferation and differentiation; increasing facts indicate its direct involvement in spermatogenesis process too, as demonstrated so far in mammal, reptile and fish testis. This neuropeptide exerts its functions through three receptors: PAC_1, $VPAC_1$ and VPAC2.

In this work, we investigated the expression and the localization of PACAP and its receptors in the testis of the bird *Coturnix coturnix* during the reproductive period. Using immunohistochemical investigations, we demonstrated that PACAP is localized in Leydig and peritubular cells and in spermatocytes, in a perinuclear area. Furthermore, the peptide is present in endothelial cells of testicular capillaries. About receptors, PAC1 and VPAC2 are localized in Leydig cells and spermatocytes, VPAC1 in Leydig cells only. Using molecular tools, we obtained the partial coding sequence of PACAP and performing an *in situ* hybridization we find that *PACAP* mRNA was synthesised in endothelial cells of testis capillaries; furthermore, BLAST analysis shows high identity of the *Coturnix* PACAP sequence with published PACAP sequence of other birds and of mammals too.

EXPRESSION OF L*IN-28* GENES IN AMPHIOXUS AND ZEBRAFISH: EVIDENCE FOR AN EVOLUTIONARY CONSERVED ROLE IN THE BRAIN DEVELOPMENT

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lin-28 was initially identified as a heterochronic gene in *Caenorhabditis elegans*, necessary as a regulator of developmental timing.[1] More recently, *lin-28* genes emerged as factors that define stemness in several tissue lineages and as regulators of skeletal myogenesis, germ cell development, neurogliogenesis, blood cell differentiation and glucose metabolism. Mammals possess two *lin-28* genes, *lin-28a* and *lin-28b* and both are required for normal neural progenitor cell proliferation and brain development.[2] Recently, it was reported the presence of two *lin-28* related genes in zebrafish: *lin-28a* and *lin-28b*.[3] In the present work, we identified, cloned and studied the expression pattern of *lin-28* genes in both zebrafish and amphioxus (a cephalochordate used as proxy for the last invertebrate ancestor of the chordates). Our results show that *lin-28a1*, *lin-28a2* and *lin-28b* are expressed during development in hematopoietic tissues and different areas of the developing nervous system. All zebrafish *lin-28* genes were not present in adult organs. Thus, we conclude that *lin-28* genes are important in the first stages of embryogenesis and probably are involved in the differentiation programs for brain development. As other invertebrates, amphioxus possesses only one *lin-28* homolog. *Amphilin-28* starts to be expressed at late gastrula stage in the presumptive neuroectoderm. At early neurula stage, *lin-28* is found in the neural plate borders and as development proceeds it is highly expressed in the neural tube. Finally, at larval stage the expression of *lin-28* in nervous system decreases, whereas it is maintained at level of some endodermic structures. These data sug-

gest that as in *C. elegans lin-28* expression in both zebrafish and amphioxus is high in developing tissues and decreases as differentiation proceeds. Moreover, *lin-28* is ancient and has an important developmental role in CNS of animals of diverse phylogeny.

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ROLE OF P450 AROMATASE IN *PODARCIS SICULA* TESTIS

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Spermatogenesis is a complex process under the control of gonadotropins and testicular factors, including estrogens, for long time regarded as typically female hormones.[1,2] The aim of present study was to investigate the localization and the expression of P450 aromatase together with the hormonal profile of sex hormones in the *Podarcis* testis in the different periods of its reproductive cycle: summer stasis, early-middle autumnal resumption, winter stasis, spring resumption, and reproductive period. Using hormonal immunoassay, we showed that in *Podarcis* testis the highest levels of testosterone were recorded in reproductive period, while the highest levels 17β-estradiol were evident during stasis periods. Immunohistochemical investigations demonstrated that P450 aromatase was always present in somatic and germ cells of *Podarcis* testis, particularly in spermatids and spermatozoa, save in early autumnal resumption, when P450 aromatase was evident only within Leydig cells. Real-time PCR and semi-quantitative blot investigations of P450 aromatase showed that both mRNA and protein were expressed in all periods, with two peaks of expression during the stasis periods. The highest levels of P450 aromatase were in line with the increase of 17β-estradiol, liable for *Podarcis* spermatogenesis block. Differently, in autumnal resumption, the level of P450 aromatase dramatically decreased, as well as the 17β-estradiol level, while testosterone titre increased, with subsequent renewal of spermatogenesis even if not followed by spermiation. Finally, in both spring resumption and reproductive periods, we found intermediate P450 aromatase expressions together with low 17β-estradiol titres, and the highest testosterone level with spermatogenesis resumption followed by spermiation. These data strongly suggest an active role of P450 aromatase in the control of both *Podarcis* steroidogenesis, as changes in P450 aromatase expression are followed by changes in sex hormones, and spermatogenesis, as the differentiation of male germ cells is accompanied by a peculiar distribution of P450 aromatase.

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DEVELOPMENTAL AND CELL TOXICITY OF THE POSITIVELY-CHARGED BPEI-COATED SILVER NANOPARTICLES

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Silver Nanoparticles (AgNPs) are among the most exploited nanomaterials (NMs) in consumer products due to their antimicrobial properties. Surface coatings, often used to functionalize or stabilize AgNPs, can modify surface chemistry or charge, influencing NP toxic profile and biocompatibility.
In our lab the biointeractions and toxicity of negatively charged Citrate (Cit) and positively charged Branched Polyethyleneimine (BPEI) coated 10nm AgNPs were investigated on *in vivo* and *in vitro* models. The effects were compared with the exposure to Ag^+. Dynamic Light Scattering and Electron Microscopy (EM) analyses were performed to study AgNP physicochemical properties. Developmental toxicity, investigated previously by FETAX, suggested that Ag^+ was strongly lethal, more than AgNPs. Contrary to Cit-AgNPs, BPEI-AgNPs showed a high teratogenic index pointing out the role of coating in determining lethal and teratogenic effects. Since the histological analysis of BPEI-AgNP treated larvae revealed irregular intestinal diverticula coupled with edematous surrounding connective tissue, we deepened the study investigating the presence of NPs in the intestinal barrier. Using two-photon excitation confocal microscopy, NP aggregates were mapped throughout the intestinal mucosa. In Human Alveolar Epithelial Cells A549, used as a model of respiratory barrier, cytotoxicity of BPEI-AgNPs resulted to be up to 100-times than that of Cit-AgNPs. Confocal microscopy showed the internalization of both AgNPs by A549 cells. EM analyses revealed the adsorption both Cit- and BPEI-coated AgNPs onto the surface of A549 cells, although the Cit-AgNPs were not toxic to A549 cells.
We can conclude that the positive surface charge of BPEI-AgNPs influences their capability to interact and produce toxic effects in cell cultures and developing embryos. Anyway, the modality of NP-tissue and -cell interactions needs deeper and detailed investigations.

TEROIDOGENIC ENZYME AND SEX HORMONE RECEPTOR GENE EXPRESSIONS IN THE BRAIN OF THE FROG *PELOPHYLAX ESCULENTUS*: SEASONAL CHANGES AND SEX DIMORPHISM

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Although it is firmly established that the brain synthesizes steroids *de novo* from cholesterol, the regulatory mechanisms which govern the concentrations of neurosteroids are unclear. Seasonal breeders represent excellent models to understand physiological changes in neurosteroid levels and their regulatory mechanisms. Our study first investigated gene expressions of Steroidogenic acute regulatory (StAR) protein and neurosteroidogenic enzymes in the brain of adult males and females of the frog *Pelophylax esculentus,* in both reproductive and post-

reproductive periods. The mRNA levels of both androgen and estrogen receptors were also examined. We found that the highest mRNA level of StAR, protein required for shuttling cholesterol across the mitochondrial membrane, was detected in the diencephalic-mesencephalic region (D-M) of reproductive males and in the telencephalon (T) of post-reproductive females. The gene expressions of both 3β-Hydroxysteroid dehydrogenase and 17β-Hydroxysteroid dehydrogenase (the enzymes that catalyze the conversion of 3β-hydroxysteroids into 3-ketosteroids and 17-ketosteroids into testosterone, respectively) were higher in the D-M of male and female reproductive frogs than post-reproductive frogs. The mRNA levels of P450 aromatase, an enzyme that catalyzes the conversion of testosterone into 17β-estradiol, were higher in the D-M of females of both periods than those of males. The highest mRNA level of 5α-Reductase, an enzyme that catalyzes the conversion of testosterone into 5α-dihydrotestosterone, was observed in the D-M of females during the breeding period. Interestingly, D-M of both reproductive and post-reproductive females showed the highest expression of both androgen and estrogen receptors. These results indicate that brain steroidogenic enzymes exhibit seasonal changes with the highest expression in the reproductive period. The evident sexually dimorphic expressions of both steroidogenic enzymes and sex hormone receptors may contribute to sex-specific, hormone-behavior relationships.
Our findings will be useful for future research on neurosteroidogenesis in amphibian with the aim clarifying their putative role in reproductive biology.

NEGATIVE IMPACT ON OVARIAN RESERVE DUE TO INTRACELLULAR OXIDATIVE STRESS IN HUMAN GRANULOSA CELLS

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The reactive oxygen species (ROS) act physiological roles during folliculogenesis and oocyte maturation, but excessive ROS production may create an unsuitable environment for reproduction and could be related to pathological conditions. The assisted reproductive techniques (ART) outcome is adversely affected if an imbalance exists between ROS and antioxidant defense in the oocyte microenvironment. The aim of this research was to assess the relationship between intracellular ROS levels in granulosa cells (GCs) and ART outcome parameters in reduced ovarian reserve-ART patients. This prospective study compared oocyte quality, fertilization rate and embryo quality with intracellular ROS in GCs of 15 patients <35 years, expected low responders (defined as having AMH ≤1 ng/L) (A Group) and 15 patient <35 years, expected normal responders (AMH>1 ng/L) (B Group) undergoing ICSI-ET cycles. PCOs, endometriosis, metabolic and endocrine diseases and severe OAT were not included. GCs samples were isolated by centrifugation from follicular fluid that were obtained on the day of oocyte retrieval. The intracellular ROS levels were assessed by H_2DCF-DA (2′,7′-dichlorofluorescein diacetate) fluorescent probe. The data were analyzed using the unpaired Student's t-test and considered significant if P-value≤0.05. Statistically significant differences between the two groups in intracellular ROS levels in GCs have been observed. In particular, ROS percentage was significantly higher in young women with reduced ovarian reserve compared normal responders (P≤0.05). The B group showed a better oocyte quality associated with a higher percentage of fertilization and embryo development compared to A group (P≤ 0.05). The results showed that increased intracellular oxidative stress in GCs is a possible enlightenment for a low ovarian response in young women. Molecular studies are in progress to characterize the influence of expression level gene related to endogenous antioxidant defense on reduced ovarian reserve. These findings could lead to the development of specific antioxidant therapies to support fertility condition in young woman.

TERATOGENIC EFFECTS OF THERMAL STRESS ON LIZARD *PODARCIS SICULA* EMBRYOS

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Geographic distribution of living organisms is greatly controlled by environmental temperature, that is particularly important in determining developmental rates, morphological, behavioural and performance-related traits, and final size of organisms, especially ectotherms. In reptiles, temperatures experienced by developing embryos can determine offspring's sex. It is also known the influence of embryonic incubation temperature on the duration of the incubation, hatchling size, locomotion, and growth in lizards of the genus Podarcis. Nowadays, the current models of climate changes predict the increase of the average global temperature and the frequency and intensity of periods with extreme temperatures, threatening many ectothermic species.
In this study, we examined the effects of continuous (from ovoposition to day 20 post ovoposition, op) or temporary (5 days from day 5 or 15 op) thermal stresses on the development of the wall lizard *Podarcis sicula*. Either cold (10°C and 15°C) or warm (31°C) stresses were applied; the effects on morphogenesis and on the expression of HSP70 were analyzed. HSP70 is a molecular chaperone involved in cellular stress response, widely used as a biomarker of thermal-stress in a broad range of animals, including reptiles. The results showed lethality of constant thermal stress and of temporary stress when given at early stages of development (5 days op). At later stages (15 days op), temporary heat-stress also exerted lethal effects, while temporary cold-stress allowed development but induced teratogenesis. The spatial and temporal localization of HSP70 transcripts was investigated by *in situ* hybridization; results demonstrated significant changes in expression, partly justifying the occurrence of malformations. A preliminary gene expression profiling analysis retrieved further changes in lizard embryos transcriptome in response to cold shock.
These findings demonstrate that *P. sicula* embryos tolerate a very narrow temperature ranges and that thermal stress during embryonic development may be a critical factor for offspring adverse effects. This apparent higher sensitivity of embryos also suggests that many lizard populations will be most affected by global climate change, with severe reductions in hatchling production.

TOWARDS AN ARTIFICIAL TENDON TISSUE

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Skeletal muscle tissue engineering is a considerably growing

research field in last years. We already obtained a fully functional artificial muscle in mice,[1] however this muscle lacked tendons and ligaments; hence to obtain a complete biological substitute it is necessary to build an artificial tendon tissue. Tendons are anatomic structures which have to bear high tensile force and for this reason are subject to injuries such as rupture and laceration.[2] Unfortunately, their hypocellular and hypovascular nature makes the healing process slow and the resulting tissue inferior in structure and function.[3] It was demonstrated that stimulation with growth factors, such as TGF-β, is crucial for the induction of tenogenic differentiation leading to an increment in the expression of important tenogenic genes, such as Scleraxis.[4] Moreover, the mechanical stimulation of cell constructs embedded into biomaterials is also able to promote the tenogenic differentiation.[5] Therefore, in this work we isolated cells with tenogenic potential from different human tissue (adult tendon and periosteum); afterwards we differentiated these and other cell lines (murine C3H10T1/2) in 2D and 3D condition with or without chemical stimulation (TGF-β and Ascorbic Acid). For the realization of 3D constructs, we used a PEG-fibrinogen (PF) based hydrogel. Finally we designed and developed a bioreactor to apply an uniaxial tension to 3D constructs in order to improve the tenogenic differentiation of cells and to ameliorate the alignment of collagen fibers. In our experiments we observed that treatment with TGF-β and ascorbic acid improves the production of collagen both in 2D and 3D cultures for used cellular types. Moreover, in 3D experiments, we observed an increase of extra-cellular matrix and an improvement of collagen fibers organization in mechanically stretched constructs.

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HOXB1 AND *HOXB2* ARE INVOLVED IN THE DETERMINATION OF THE RHOMBOMERE 4-DERIVED VESTIBULAR SISTEM

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The central auditory pathway consists of sensory nuclei that transmit the ascending acoustic information, and efferent motor neurons that modulate primary afferent responses. We have previously shown that rhombomere 4 (r4) contributes to structurally and functionally linked sensory afferent and motor efferent components of the central auditory system, and that in the absence of *Hoxb1* mutant mice have severe hearing problems.[1]We subsequently demonstrated that *Hoxb2* and *Hoxa2* and their genetic interactions are also necessary for the proper development of r4. The vestibular nuclear complex, in part derives from r4 and consists of a collection of sensory nuclei that integrates and relays information for the coordination of eye movements, balance and posture. We still used *Hoxb1* mutant mice to investigate the contribution of r4 in the developmental patterning of vestibular projection neurons, with particular focus on the lateral vestibulo spinal tract (LVST). Retrograde labelling and marker analysis on *Hoxb1* mutant embryos and postnatal pups confirmed specific absence of the LVST and of the vestibular efferent neurons (VEN), in accordance with loss of r4 identity and ectopic production of r3 neurons.[2] However, transmission electron microscopy experiments in adult mice show the presence of both afferent and efferent nervous endings. It is thus plausible that in the adult mouse mutant a compensatory mechanism overtaken by other neuronal tracts is able to compensate for the early absence of the vestibular nuclei, in line with a partial rescue of the vestibulo-spinal reflex.[2] We also analyzed vestibular afferences and efferences of *Hoxb2* and *Hoxa2* mutant mice and demonstrated presence of afferent and efferent endings. To this purpose, we are in the process of using newborn mutant pups to assess whether these projections are already lost at birth and whether new connections gradually appear during the first month of life.

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NEW INSIGHTS ON THE BIOSYNTHETIC INTERFERENCE OF ESTROGEN-LIKE COMPOUNDS ON *PODARCIS SICULA* MALE

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Spermatogenesis is regulated by several hormones and their balance ensures the right progression and ending of the process. In the last years, the hormonal balance is threatened by the interferences of substances with hormone-like action (Endocrine Disruptor Chemicals, EDC) that may harm the reproduction mainly through the food chain. Synthetic EDC, such as the alkylphenol nonylphenol (NP), are found in pesticides widely used in intensive agriculture. On the other hand, EDC derived by metabolites of the steroid hormones could be present also in manure used as fertilizer in the organic farming. In oviparous vertebrates, the most validated biomarker of the exposure to estrogenic substances is the induction in male liver of the vitellogenin (VTG), an estrogen dependent protein expressed only in females during the reproductive season. Lizards are considered good sentinels of the terrestrial habitat since they are characterized by strong site fidelity and are key components of the food chain.

Nowadays, the organic farming has exponentially grown and the manure as soil fertility source is becoming the principal choice. Studies on wildlife exposed to runoff from manure-fertilized cropland are limited; so, we decided to investigate the expression of VTG in the male lizard *Podarcis sicula* caught in areas devoted to organic farming and in males experimentally fed with NP-polluted food. A comparative morphological analysis on testis status was also performed. Results demonstrated that lizards from the two different groups displayed the hepatic biosynthetic alterations typical of an estrogenic contamination: hepatocytes, in fact, contained VTG transcripts and proteins, detected by ISH and ICC investigations, respectively. Surprisingly, under the same conditions, we found the expression and synthesis of VTG also in testes, in all germ cells of the seminiferous epithelium. The failure to detect transcripts for VTG receptors in testis demonstrated that the testicular VTG derived only from local production triggered by estrogen metabolites or NP. However, histological analysis showed for the testis of males collected in the organic farms a normal architecture of seminiferous epithelium, in accordance to the reproductive stage; testis of NP-treated lizards displayed an impaired testicular organization.

CHANGES IN THE MORPHOLOGY AND DIFFERENTIATION OF HUMAN SKIN AFTER IRRADIATION

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The use of charged particles in radiotherapy has several advantages *e.g.* sparing of healthy tissue. The application is increasing constantly during the last years and more and more different types of cancer can be treated succesfully. But in most radiation treatments the skin can not be spared completely and is exposed to low or moderate doses. Therefore, a deeper understanding of early and late side effects occuring in skin is requiered. We measured cellular and molecular changes related to the early inflammatory response of human skin irradiated with carbonions, in particular induction of cell death, as well as changes in differentiation and proliferation of epidermal cells during the first days after exposure. Model systems for human skin of different complexity, *i.e.*, keratinocytes, coculture of skin cells, 3D skin equivalents, and skin explants, were used to investigate the alterations induced by carbonions (spread-out Bragg peak, dose-averaged LET 100 keV/μm) in comparison to X-ray and UV-B exposure.

Whereas in none of the model systems apoptosis or necrosis was observed after ionizing irradiation, changes in proliferation and differentiation were detected. High doses of carbonions were more effective than X-rays in reducing proliferation and inducing abnormal differentiation. In contrast, changes identified following low-dose exposure (≤0.5 Gy), *i.e.*, enhanced proliferation and change in the polarity of basal cells, were induced more effectively after X-ray exposure.