

**PROCEEDINGS OF THE 63<sup>rd</sup> CONGRESS  
OF THE ITALIAN EMBRYOLOGICAL GROUP  
(GEI)**

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# *European Journal of Histochemistry*

## *a journal of functional cytology*

The *European Journal of Histochemistry* was founded in 1954 by Maffo Vialli and published until 1979 under the title of *Rivista di Istochimica Normale e Patologica*, from 1980 to 1990 as *Basic and Applied Histochemistry* and in 1991 as *European Journal of Basic and Applied Histochemistry*. It is published under the auspices of the University of Pavia, Italy.

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## LECTURES

### FROM AN INTEGRIN BINDING PROTEIN TO AN EVOLUTIONARILY CONSERVED TRANSLATION FACTOR NECESSARY FOR THE CONTROL OF METABOLISM

S. Biffo

Laboratory of Molecular Histology and Cell Growth, INGM, and Department of Biosciences, University of Milan, Italy  
E-mail: stefano.biffo@unimi.it

eIF6 (alias p27BBP, Beta4 Binding Protein) was cloned for its capability to bind Beta4 integrin. Rapidly, we learned that it is a factor necessary for ribosome biogenesis and translation,<sup>1</sup> highly expressed in embryonic and cancer cells. eIF6 is rate-limiting for translation under growth factors and oncogenic signaling.<sup>2</sup> eIF6 acts by regulating at the translational level the metabolism of fatty acids.<sup>3</sup> In general, eIF6 reduction increases animal fitness, resistance to tumors and to high fat diet. Why are then high levels of eIF6 maintained *in vivo*? We find that high levels of eIF6 are essential in the immune system. In conclusion, we speculate that translational control acts as a form of "metabolic learning".

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### THEORIA GENERATIONIS: THE ANCIENT ROOTS OF THE MODERN DEVELOPMENTAL BIOLOGY

E. Capanna

Department of Biology and Biotechnologies "C. Darwin", Sapienza University of Rome, Italy  
E-mail: ernesto.capanna@uniroma1.it

Metaphysical concepts are present in Greeks pre-Socratic Philosophy that will form, until present days, the Ariadne thread of the analysis of developmental processes. The debate between *to be* and *to become*, that opposed Parmenides vs Heraclitus, along the two subsequent millenniums, will turn to the dilemma between preformation vs epigenesis, immanence vs transcendence. Aristoteles was the first to transfer the question from Metaphysics to Physics enunciating the *Theoria generationis*. In the Hellenistic period, and during the Renaissance, the autopsy and experimental methods became key to the interpretation of biological processes. The crisis of the Aristotelism was already in place following the studies of Italian anatomists, but the final trespass was due to William Harvey in his *De motu cordis*. Harvey was author of a second work: in his *Exercitatione de generatione animalium* he introduced, with his aphorism *omne vivum ex ovo*, the concept of *ovism*. In the same years, the description of the spermatozoon (*animalculum*) was formed, and Harvey's *ovism* and *animalculism* became counterparts. Both theories were to be read according to preformation and epigenetic approach. With the Enlightenment the dispute over the development process was placed in the Cartesian rationalism, and subjected to rigorous testing. Excels among others the figure of Lazzaro Spallanzani. The positivism of Comte moved to search the material *prime causes* of the development, according the laws of Physics and Chemistry. During this period come the experiences on *prelocalization* of embryonic areas of Carl Vogt,

and the *mosaic egg* of Roux with clear immanent evidence. A finalistic interpretation reemerged from experiences of Driesch concerning the *embryonic regulation*, and from those of Spemann on *embryonic induction*: the morphogenesis was conceived as a dialectical process between inductive power of the organizer and the specificity of the morphogenetic fields. In the middle of last century two notes on Nature by Crick and Watson were published: the millenary fight between preformation and epigenesis was finally solved: the development program is performed in the genome, but varied in epigenetic interactions between parts of the genome itself, and with the cell environment in which the genome operates.

### SEA URCHIN RESEARCH: MILESTONES, MEMORIES, AND FUTURE CHALLENGES

V. Cavalieri

Department of Biological, Chemical and Pharmaceutical Sciences and Technologies, University of Palermo, Italy  
E-mail: vincenzo.cavalieri@unipa.it

The sea urchin eggs and embryos have been used for nearly two centuries as experimental models for classical and modern developmental biology. In the late 1870s, the ground-breaking observations independently obtained by Hertwig and Fol highlighted for the first time that a single sperm enters the oocyte and the male and female pronuclei fuse at fertilization.<sup>1</sup> From that point on, the seminal studies of Boveri, Driesch, and Herbst allowed conceptualization of basic biological themes, such as the chromosome theory of heredity.<sup>2</sup> In the first half of the twentieth century, the embryo manipulation experiments performed by Hörstadius and Runnström further advanced the field, introducing the concept of morphogens double gradient.<sup>3</sup> Later on, with the flowering of molecular biology and the advent of new technologies, scientists of the caliber of Hultin, Monroy, and Davidson emphasized that this echinoderm also represents an excellent model for studying the molecular basis of embryogenesis.<sup>2</sup> In the post-genomic era, the sea urchin embryo continued to be an unsurpassed model for determining the molecular mechanisms responsible for creating a multicellular organism, mainly because of its relative inexpensiveness, optical transparency, rapid synchronous development, and amenability to perform a powerful arsenal of experimental procedures.<sup>4</sup> Although nowadays the carrying capacity is much lower than in years past, the sea urchin embryo is still a convenient model to study gene regulatory networks,<sup>5</sup> response to environmental stressors,<sup>6</sup> biomineralization,<sup>7</sup> stem cell properties,<sup>8</sup> and cancer.<sup>9</sup> Undoubtedly, the breath of all this research makes it clear that the sea urchin embryo could help further generations of investigators to reveal the unsolved mysteries of life.

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## EVO-DEVO: THE UNCERTAIN ARRIVAL OF THE FITTEST

A. Minelli

Department of Biology, University of Padua, Italy

E-mail: [alessandro.minelli@unipd.it](mailto:alessandro.minelli@unipd.it)

Natural selection does not explain why not even a single adult centipede has an even number of leg-pairs, neither why in the giraffe's lineage selection for a longer neck did not result in an increase in the number of cervical vertebrae. With evolutionary developmental biology (evo-devo), research focus shifts at last from the *survival of the fittest* so central to the Darwinian interpretation of biological evolution, to the long overlooked *arrival of the fittest*. The precise relationships between evo-devo and its parent disciplines – evolutionary biology and developmental biology – are still matter of debate, but we can safely take for granted that evo-devo is *at least* a trading zone between them.<sup>1</sup> But this trading is only profitable if both disciplines agree on a number of basic ontological and epistemological issues concerning change in biological systems. In this respect, a leading role must be arguably acknowledged to evolutionary biology, due to its deeper exploration of foundational and philosophical issues. Trading with evolutionary biology, developmental biology would thus take advantage of accepting a conceptual shift towards a distinctly processual and non-teleonomic perspective. This implies, among other things, (1) relaxing the current focus on organogenesis, (2) addressing developmental processes irrespective of the adaptive value of the resulting phenotypes, and (3) abandoning adultocentrism. From this refreshed perspective, the arrival of the fittest will appear less predictable than a strictly Darwinian perspective would suggest. In both developmental and evolutionary perspective, *nirgends ist Neubildung, sondern nur Umbildung* (nowhere are things really new, everything is nothing but transformation).<sup>2</sup> In this framework, the logical equivalent to the common descent with modification of Darwinian evolution will be the inertial behaviour<sup>3</sup> of developing systems, poised between the ramparts of their intrinsic robustness and the diverging paths through the morphospace constrained by their evolvability.

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## ADULT NEUROGENESIS AND ITS ROLE IN THE CONTROL OF OPPOSITE SEX-ATTRACTION IN MALE MICE

P. Peretto,<sup>1,2</sup> R. Schellino,<sup>1,2,3</sup> S. Trova,<sup>1,2</sup> A. Farinetti,<sup>2,3</sup> P. Giacobini,<sup>4</sup> S. De Marchis<sup>1,2</sup>

<sup>1</sup>Department of Life Sciences and Systems Biology, University of Turin, Italy; <sup>2</sup>NICO – Neuroscience Institute Cavalieri Ottolenghi;

<sup>3</sup>Department of Neuroscience, University of Turin, Italy; <sup>4</sup>Inserm, UMR-S 1172, Laboratory of Development and Plasticity of the Neuroendocrine Brain, Lille, France

E-mail: [paolo.peretto@unito.it](mailto:paolo.peretto@unito.it)

Several studies have shown a link between adult olfactory bulb neurogenesis and reproduction. Male pheromones trigger an increase in newborn neurons in the accessory olfactory bulb (AOB) of female mice, and ablation of neurogenesis negatively impacts some intersex social behaviours. In addition, adenohipophyseal and gonadal hormones are modulators of adult neurogenesis in both sexes. These data support that interplay between adult neurogenesis and hormonal factors may serve to optimize reproductive responses elicited by olfactory-social stimuli. By using Semaphorin

7A knockout mice (Sema7A ko), which show reduced numbers of GnRH-secreting neurons, smaller testicles and subfertility, we found that ko males have low blood testosterone levels, decreased androgen receptor expression in the olfactory bulb and an altered male preference for female scents/individuals. Analysis of adult neurogenesis in the AOB revealed that male pheromones in ko males elicit feminized responses in terms of the integration, selection and activation of newborn neurons. Altered neuronal activation was also found in nuclei of the vomeronasal system (VNS) controlling reproductive behaviour. Remarkably, in the Sema7A ko males, chronic testosterone treatment restored i) the preference for female scents/individuals, ii) the survival and activation of newborn cells under physiological conditions, and iii) the male pattern of neuronal activity in VN circuits. Castration of adult wild-type males phenocopied both the behavioural and cellular responses identified in the Sema7A ko mice and the testosterone treatment restored the preference for opposite sex-stimuli, along with a male pattern of AOB neurogenesis and neuronal activity in the VNS. These data indicate that opposite-sex attraction in male mice is due to fine-tuned testosterone-dependent regulation of adult neurogenesis, supporting cooperation between adult neurogenesis and the endocrine system is required to sustain sexual behaviours elicited by salient chemosensory stimuli.

## THE p53 FAMILY IN CANCER BIOLOGY

G. Melino<sup>1,2</sup>, I. Amelio<sup>1</sup>, F. Bernassola<sup>2</sup>, T. W. Mak<sup>3</sup>

<sup>1</sup>MRC Toxicology Unit, Leicester, United Kingdom;

<sup>2</sup>University of Rome Tor Vergata, Italy; <sup>3</sup>The Campbell Family Cancer Research Institute, Toronto, Ontario, Canada

The p53 family members p73 and p63 are involved in female infertility maternal reproduction (Nature Rev MCB 2011;12,4:259) and as well as in cancer formation (TiBS 2014;39,4:191). We identified their activation during DNA damage, several transcriptional targets, the mechanisms of regulation of cell death, and the protein degradation pathway. To understand the p53 structure-function relationship, we performed a molecular dynamics study, showing an induced-fit interaction of the C-terminal domain with the DNA-binding domain. Direct intra- and intermonomeric long-range communications between the tetramerization and DNA-binding domains are noted, providing a biophysical rationale for the reported functional regulation of the p53 C-terminal region. We also detect 'dynamic' deformations switched on and off by particular p53 tetrameric conformations and measured by the roll and twist parameters in the same base pairs. These different conformations can indeed modulate the electrostatic potential isosurfaces of the whole p53-DNA complex (Oncogene, in press PMID: 26477317). While TAp73<sup>-/-</sup> mice show high tumor incidence with hippocampal dysgenesis, they show an elevated cancer incidence. Accordingly, TAp73 opposes HIF-1 activity, affecting tumour angiogenesis. TAp73 interacts with HIF-1, promoting HIF-polyubiquitination and consequent proteasomal degradation. These findings demonstrate a novel mechanism for HIF-1 regulation and provide an additional explanation for the molecular basis of the growth, progression, and invasiveness of human cancers. (PNAS-USA 2015;112,1:226) (TiBS 2015;40,8:425). P63 is a determinant of skin development. Using a MMTV-ErbB2 murine model, we found that Np63 regulates mammary Cancer Stem Cells self-renewal and breast tumorigenesis via the direct transactivation of Sonic Hedgehog (Shh), GLI family zinc finger 2 (Gli2), and Patched1 (Ptch1) genes. (PNAS-USA 2015. 112,11: 3499-504. PMID: 25739959). At least in part, this seems to be exerted by regulation of the metabolism via Hexokinase II (PNAS-USA 2015. 112,37: 11577-82. PMID: 26324887).

## ABSTRACTS

### **POMACEA CANALICULATA: A NEW MODEL SYSTEM FOR STUDYING DEVELOPMENT AND REGENERATION OF COMPLEX EYES**

A. Accorsi,<sup>1</sup> E. Ross,<sup>1</sup> E. Ottaviani,<sup>2</sup> A. Sánchez Alvarado<sup>1</sup>

<sup>1</sup>Howard Hughes Medical Institute, Stowers Institute for Medical Research, Kansas City, MO, USA; <sup>2</sup>Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy

E-mail: aai@stowers.org

Camera-type eyes with varying morphological attributes are readily found among the vertebrates. All share key components such as a cornea, lens, ciliary margin, retina and an optic nerve. Complex, camera-type eyes are also described in some invertebrate species belonging to annelids, mollusks and crustaceans. While the organogenesis and embryonic development of this sensory organ have been studied in great detail in well-established model systems, the same cannot be said of its regeneration as most if not all of these model systems display little to no regenerative capacity of their eyes. Our research is performed on the freshwater snail *Pomacea canaliculata*, a gastropod with complex, camera-type eyes. Through light and electron microscopy, antibody staining and *in situ* hybridization methods, we describe the structure of the eyes. The lens is round as already observed in other aquatic animals, and the retina appears to be composed of multiple cell types with photoreceptors that have cilia similar to those observed in vertebrate ciliary photoreceptors. Not only do *P. canaliculata* eyes present several anatomical similarities with vertebrate eyes, but they can also regenerate after the amputation. RNAseq data collected from regenerating eyes, allowed us to generate a database of gene expression that we aim to use to molecularly dissect both snail eye embryogenesis and regeneration. The ability to regenerate the eyes, as well as other sensory components like the cephalic tentacles, is not the only important feature that characterize *P. canaliculata*. The ease of culture and reproduction in captivity with abundant and year-round offspring production, direct development and short generation time, are only make this organism a promising model system to advance our understanding of development and regeneration.

### **INVOLVEMENT OF A PROKINETICIN DOMAIN-CONTAINING PROTEIN IN THE IMMUNE RESPONSE OF THE EMERGING MODEL *POMACEA CANALICULATA***

A. Accorsi,<sup>1</sup> E. Ross,<sup>1</sup> A. Sánchez Alvarado,<sup>1</sup> D. Malagoli<sup>2</sup>

<sup>1</sup>Howard Hughes Medical Institute, Stowers Institute for Medical Research, Kansas City, MO, USA; <sup>2</sup>Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy

E-mail: davide.malagoli@unimore.it

The golden apple snail *Pomacea canaliculata* is a freshwater gastropod native of South America, and now found in North America, Asia and Europe. *P. canaliculata* is listed as one of the world's worst invasive species and in both EU and USA, its management is already strictly regulated. *P. canaliculata* presents several interesting features also for parasitologists, biochemists and developmental biologists, making it a promising, emerging biological model. The innate immune system of this snail may represent a good target for strategies aimed to control its spread. Recently, we have described the circulating hemocytes

and uncovered evidence indicating likely hematopoietic activity within the pericardial chamber of adult *P. canaliculata*. With the final aim to molecularly describe the maturation process of a molluscan immune system, we screened organ-specific transcriptomes of *P. canaliculata* for sequences that may have a role in immune response function and/or hemocyte maturation. Among several candidates, we identified a 121 aa prokineticin-like protein, *Pc-PLP*. In control snails, the expression of *Pc-plp* is readily detectable in the hematopoietic tissue, ampulla (an organ with a potential role in hemocyte maturation) and central nervous system. qPCR experiments have demonstrated that the *Pc-plp* expression profile significantly change after immune stimulations, such as LPS injection or repeated hemolymph withdrawals. Notably, the modifications intervening in *Pc-plp* expression were organ- and challenge-specific, such that after LPS injection, *Pc-plp* mRNA levels changed in immune-related organs, such as gills and anterior kidney. After repeated, non-lethal hemolymph withdrawals that significantly modify hemocyte population, the most responsive components were found to be the hematopoietic tissue and the circulating hemocytes. Altogether our data present the first prokineticin domain-containing molecule found in gastropods and link it to immune response and hematopoiesis in the golden apple snail, similarly to mammals where prokineticins intervene in several processes, including hematopoiesis, and the immune response.

### **PPAR $\beta/\delta$ ANTAGONISM RESCUES DOPAMINERGIC NEURONS IN AN *IN VITRO* PARKINSON'S DISEASE MODEL**

A. Antonosante,<sup>1</sup> M. D'Angelo,<sup>1</sup> E. Benedetti,<sup>1</sup> V. Castelli,<sup>1</sup> L. Cristiano,<sup>1</sup> M. Catanesi,<sup>1</sup> A. Cimini,<sup>1,2,3</sup>

<sup>1</sup>Department of Life, Health and Environmental Sciences, University of L'Aquila, Italy; <sup>2</sup>Sbarro Institute for Cancer Research and Molecular Medicine and Center for Biotechnology, Temple University, Philadelphia, USA; <sup>3</sup>National Institute for Nuclear Physics (INFN), Gran Sasso National Laboratory (LNGS), Assergi, Italy

E-mail: andrea.antonosante@gmail.com

Parkinson's disease is one of the most common neurologic disorder, affecting about 1-4% of persons older than 60 years. Among the proposed mechanisms of PD generation, free radical damage is believed to play a pivotal role in the development and/or progression of the disease. Recently, PPARs, a class of transcription factors involved in several pathways both in physiological and pathological conditions, have been linked by us and others to neurodegeneration. Particularly, PPAR $\gamma$  and its ligands have been indicated as potential therapeutic targets for the treatment of several pathological conditions associated with neuroinflammation within the CNS. The anti-inflammatory function of PPAR $\gamma$  has attracted attention since agonists exert a broad spectrum of protective effects in several animal models of neurological diseases, including psychiatric diseases. On the other hand, a detrimental role for PPAR $\beta/\delta$  has been proposed in Alzheimer, being closely related to the decrease of BDNF and Trkfl. On these bases, in this work we used a 6-OHDA *in vitro* model treated with a PPAR $\beta/\delta$  specific antagonist. The 6-OHDA treatment determined a significant increase of neuronal death, the presence of the antagonist rescued cell viability, thus indicating that blocking PPAR $\beta/\delta$  activates neuronal survival pathways.

## DEVELOPMENTAL CONSEQUENCES OF PARENTAL AGING IN *NOTHOBRANCHIUS FURZERI* OFFSPRING

M. Api,<sup>1</sup> P. Biondi,<sup>1</sup> E. Terzibasi Tozzini,<sup>2</sup> I. Olivotto,<sup>1</sup> A. Cellerino,<sup>2</sup> O. Carnevali<sup>1</sup>

<sup>1</sup>Dipartimento Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Ancona; <sup>2</sup>Scuola Normale Superiore, Pisa, Italy

Aging is a complex phenomenon likely to be influenced by many genes and controlling elements.<sup>1</sup> Although studies on parental aging are very attractive, it is poorly understood whether parental age affects development of offspring and whether these effects are transferred to adult traits. In this study, we used the killifish *Nothobranchius furzeri*, an interesting aging vertebrate model with the shortest lifespan.<sup>2,3</sup> The embryos from *N. furzeri* are able to either entering into or skipping diapause giving rise to different developmental pathways.<sup>4</sup> Thus, this embryo plasticity allows this model to be used to study different factors that could affect their embryonic development including parental age. The first goal of the present study was to highlight if parental aging could affect the embryonic diapause. To do this, we collected hundreds of F1 embryos from two different groups of breeders (old parents and young parents). We detected the duration of embryonic development and analyzed genes involved in embryo dorsalisation process at 4, 5, 6 dpf. Both molecular and phenotype analyses showed that embryos from young breeders developed faster than embryos from old parents. The second goal was to investigate if embryos developmental plasticity could be modulated by epigenetic process. At this regard, the expression of DNMTs genes was examined. Our data supported the hypothesis that diapause, mostly occurring in embryos from old parents, is related with the methylation process, suggesting an epigenetic control. Finally, we analyzed whether the parental aging could affect metabolism and growth during adult life. Morphometric results and qPCR analysis of genes from IGF system showed a slower growth in adults from old breeders. Moreover, a gender-specificity growth emerged. In conclusion, these results may contribute to better understand the complex mechanism of organism aging.

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## PARACRINE ROLES OF EXTRACELLULAR VESICLES RELEASED BY MOUSE MESOANGIOBLASTS

M.M. Barreca,<sup>1</sup> G. Sconzo,<sup>1</sup> F. Geraci<sup>1,2</sup>

<sup>1</sup>STEBICEF Department, University of Palermo; <sup>2</sup>IEMEST, Palermo, Italy

E-mail: fabiana.geraci@unipa.it

Extracellular vesicles (EV) represent an important mediator of cell-to-cell communication and are involved in both autocrine and paracrine signaling, with a critical role in a number of physiological and pathological conditions.<sup>1</sup> The bioactive molecules contained within EV simultaneously activate several different pathways resulting in the synergistic stimulation of target cells. The discovery and characterization of EV have added a novel understanding to regenerative medicine, namely the finding that stem cells are an abundant source of EV.<sup>1-2</sup> A6 mouse mesoangioblasts, vessel-associated multipotent progenitor stem cells that are capable of differentiating into different mesodermal cell types, are able to release in the extracellular environment membrane vesicles, which contain structural proteins, FGF-2

and the two gelatinases MMP2 and MMP9.<sup>3</sup> Moreover, we have already demonstrated that EV released by these cells contain Hsp70 as a transmembrane protein, which is involved in an autocrine signaling responsible for increased cell migration.<sup>4</sup> In this study we have investigated the possible paracrine effects of A6 derived EV with other cell types, and the effects of these interactions. Firstly, we have focused our attention on their interplay capacity with human endothelial cells, which are induced to form capillary-like structures *in vitro* and to increase their motility. Furthermore, we have analyzed EV immunomodulatory effect on Jurkat lymphocytes, demonstrating that they are able to inhibit both their activation and proliferation. Finally, we have investigated the role of sugar residues on the membrane of A6 derived EV in their interaction with other cell types, by enzymatic removing of N-linked glycans on their membrane. In particular, PNGase F that is responsible for the cleavage between asparagine and GlcNAc in all types of glycan chains induces a substantial reduction in EV-target cell interaction. On the contrary, the use of EndoH, which is responsible for the cleavage between two residues of GlcNAc, increase target cell-EV interaction.

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## PROPOSAL OF A PREDICTIVE IN SILICO MODEL FOR MIXTURES OF TERATOGENIC AZOLE FUNGICIDES PROBABLY ACTING ON THE SAME MOLECULAR INITIATING EVENT

M. Battistoni,<sup>1</sup> F. Di Renzo,<sup>2</sup> L. Palazzolo,<sup>3</sup> U. Guerrini,<sup>3</sup> I. Eberini,<sup>3</sup> A. Moretto,<sup>4,5</sup> F. Metruccio,<sup>5</sup> E. Menegola<sup>1</sup>

<sup>1</sup>Department of Environmental Science and Policy, University of Milan; <sup>2</sup>Department of Biosciences, University of Milan; <sup>3</sup>Department of Pharmacological and Biomolecular Sciences, University of Milan; <sup>4</sup>Department of Biomedical and Clinical Sciences University of Milan; <sup>5</sup>ICPS, ASST Fatebenefratelli Sacco, Milan, Italy

E-mail: elena.menegola@unimi.it

Single azole fungicides, including triadimefon (FON) and flusilazole (FLUSI), affect early cranio-facial morphogenesis in different animal models, including postimplantation rat whole embryo culture (WEC). In the context of the skeletal craniofacial adverse outcome pathway, the proposed molecular initiating event (MIE) for azole teratogenicity is the inhibition of embryonic CYP26 isozymes involved in retinoic acid (RA) catabolism with the consequent local increase in endogenous RA levels, leading to branchial defects in embryos. The aim of the present work is the development of an *in silico* tool, combining pathway modelling and molecular docking in order to simulate the formation of physiological RA levels in the rat embryo hindbrain and predict their perturbation after exposure to single azole fungicides and to their binary mixtures. *In vitro* obtained data on RA, FON and FLUSI teratogenic effects have been used in order to set parameters. Data obtained by the exposure to the FON-FLUSI mixture were used to verify the accuracy of the predictions. Results 1- demonstrated that the developed model is able to adequately predict the outcome of *in vitro* exposure of embryos to azoles mixtures, and 2- suggest the the two tested azoles should share the same MIE.



## CELL LINES (FIBROBLASTS, IPSCs AND NEURONAL) FROM JUVENILE HUNTINGTON DISEASE WITH LARGE CAG REPEAT EXPANSIONS

E. Bidollari,<sup>1,2</sup> G. Rotundo,<sup>1</sup> L. De Filippis,<sup>3</sup> M. Marano,<sup>3</sup> F. Squitieri,<sup>1</sup> A. Vescovi,<sup>1,3</sup> J. Rosati<sup>1</sup>

<sup>1</sup>Cellular Reprogramming Unit, IRCCS Casa Sollievo della Sofferenza - Mendel Institute, Rome; <sup>2</sup>IRCCS Casa Sollievo della Sofferenza, San Giovanni Rotondo, FG; <sup>3</sup>Department of Biochemical Sciences "A.R. Fanelli" Sapienza University of Rome, Italy

Juvenile Huntington Disease (JHD) subjects are excluded from therapeutic trials due to missing biomarkers, validated evaluation scales and their atypical, yet unpredictable, presentation. We started to collect data and tissues from young children with HD mutations, some carrying large size expansions, in the attempt to seek potential markers of JHD. We have so far collected skin biopsies from 20 mutation positive subjects (from pre- to advanced HD stages), including two JHD subjects with large mutations (i.e. 85 and 64 CAG repeats), and controls. Patients' skin fibroblasts are currently reprogrammed into iPSCs introducing four pluripotency episomal factors (SOX2, KLF4, c-MYC AND OCT4) by virus-free protocol. All iPSC clones that show a uniform flat morphology are characterized for their stemness and pluripotency, both *in vitro* through embryoid bodies formation and *in vivo* through teratoma formation assay. A new protocol is optimized for differentiation of iPSCs derived embryoid bodies expressing all the three germ layers (ectoderm, mesoderm and endoderm) in neurospheres of Neural Stem Cells (NSCs). We have obtained a neural population of astrocytes, oligodendrocytes and neuron cells by spontaneous differentiation of neurospheres from a young child with 85 CAG repeat mutation, showing typical HD abnormalities including mitochondrial alterations, increased caspase activation and altered viability. The careful JHD clinical and genetic stratification and the investigation of subjects with very large mutations and infantile onset may open clues on new mechanisms and markers of JHD.

## MICRO RNAs ARE INVOLVED IN ACTIVATION OF EPICARDIUM DURING THE REGENERATION TRIAL OF ZEBRAFISH HEART

D. Bonvissuto,<sup>1</sup> C. Carlantoni,<sup>2,3</sup> M.A. Missinato,<sup>4</sup> M. Ceci,<sup>3</sup> B. Di Giacomo,<sup>3</sup> R. Contu,<sup>5</sup> G.L. Condorelli,<sup>5</sup> M. Tzang,<sup>4</sup> N. Romano<sup>3</sup>

<sup>1</sup>Faculty of Medicine and Surgery, Catholic University of Sacred Heart, Milan, Italy; <sup>2</sup>MPI-Heart and Lung Research, Department of Developmental Genetics, Bad Nauheim, Germany; <sup>3</sup>Department of Ecological and Biological Sciences, University of Tuscia, Viterbo, Italy; <sup>4</sup>Department of Developmental Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA; <sup>5</sup>Humanitas, University of Milan, Italy

E-mail: nromano@unitus.it

After the resection of the 20% of ventricular apex, the adult zebrafish is able to regenerate efficiently the heart. This rescue process requires the concert activation of the endocardium and epicardium as well as some preexisting cardiomyocytes capable together to replace the lost tissue. The molecular mechanisms involved in this activation process are not completely understood. In this work, the research has focused on the miRs133 (a-b) and the miR1 expressions during the regeneration process with the aim to understand if the down-regulation of this miRs are eventually linked with the other processes observed, such as the activation of epicardium. Experiments of regeneration detected from 24

hours(hrs) onwards by using qPCR from the whole heart and, by using the laser micro-dissection, from epicardium cells and from the regenerative clot has indicated that already at 24hrs occur the down-regulation of miR133a and miR1 in epicardium, whereas the myocardium showed only the down regulation of miR1. All the miRs significantly continuously decreased up to 7 days post-surgery. With the aim to study the heart's component activation in combination with miR, it has also developed the immunohistochemistry of the antibodies directed against the Wilms Tumor 1 (WT1) a marker of epicardium in mammals; HSP70, a chaperon activated during the regeneration processes and the cardiac Troponin T (cTnT) a marker of differentiated cardiomyocytes. WT1 and HSP70 strongly marked the regeneration site just at three days when miRs are start to be significantly down-regulated. In coherence, cTnT intensively marked on the regeneration portion from 7 days onward. The miRs-1 and -133(a,b) have been strongly involved in the activation of the epicardium during the regeneration process in zebrafish.

## NUCLEAR AND MITOCHONDRIAL ALTERATIONS IN LONG-TERM CULTURES OF INDUCED PLURIPOTENT STEM CELLS

R. Borghi,<sup>1,2</sup> S. Petrini,<sup>3</sup> F. Colasuonno,<sup>1</sup> V. D'Oria,<sup>3</sup> A. Niceforo,<sup>1,2</sup> E. Bertini,<sup>2</sup> C. Compagnucci,<sup>2</sup> S. Moreno<sup>1</sup>

<sup>1</sup>Department of Science-LIME, University of Rome "Roma Tre"; <sup>2</sup>Department of Neuroscience, Unit of Neuromuscular and Neurodegenerative Diseases, Bambino Gesù Children's Hospital, IRCCS, Rome; <sup>3</sup>Confocal Microscopy Core Facility, Research Laboratories, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy  
E-mail: sandra.moreno@uniroma3.it

Induced pluripotent stem cells (iPSCs) share important features with embryonic stem cells, as they are derived from somatic cells through reprogramming into cells able to self-renew and to differentiate into any cell type. iPSCs may be used to model rare disorders *in vitro* and to screen disease- and patient-specific drug treatments.<sup>1</sup> The concept that iPSCs can be maintained and propagated indefinitely in culture, preserving their properties, has been recently challenged by the finding that long-term cultured iPSCs ("aged" iPSCs, a-iPSCs) display altered mitochondrial status and inability to undergo neurogenesis, when compared to "young" iPSCs (y-iPSCs).<sup>2</sup> To address the issue of possible aging processes occurring in iPSCs, we investigated cell senescence hallmarks affecting nuclear and cytoplasmic compartments, in short-to-long term cultures. Specifically, the architecture of the nuclear envelope (NE) was analysed by confocal microscopy following immunofluorescence for lamin A/C-prelamin isoforms, lamin B1, emerin, nesprin-2, demonstrating nucleoskeletal components imbalance in a-iPSCs. Accordingly, ultrastructural analysis by FIB/SEM demonstrates nuclear dysmorphism, with abnormal shapes and occasional blebs. Electron microscopic investigation also highlights changes in a-iPSCs mitochondria, which appear more numerous, dispersed in the cytoplasm, along with showing elongated morphology, and higher number of *cristae*, compared to y-iPSCs. These results are further supported by molecular data concerning the expression of *SIRT7*, a modulator of mitochondrial biogenesis, which is reduced in a-iPSCs. We conclude that a-iPSCs display NE features reminiscent of normal cell senescence and premature-aging syndromes.<sup>3</sup> These findings validate the use of long-term cultured iPSCs as a model for studying normal and pathological aging processes.

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## HUMAN SPERM CELL AS EARLY BIOINDICATOR OF MALE HEALTH AND REPRODUCTION

L. Bosco,<sup>1</sup> G. Ruvolo,<sup>2</sup> C. Martino,<sup>1</sup> R. Chiappetta,<sup>3</sup> D. Carone,<sup>3</sup> G. Lo Bosco,<sup>4</sup> L. Carrillo,<sup>2</sup> A. Ferrigno,<sup>1</sup> M.C. Roccheri<sup>1</sup>

<sup>1</sup>Dipartimento di Scienze e Tecnologie Biologiche, Chimiche e Farmaceutiche (STEBICEF), Università degli Studi di Palermo; <sup>2</sup>Centro di Biologia della Riproduzione, Palermo; <sup>3</sup>Centro di Riproduzione e Andrologia, Taranto; <sup>4</sup>Dipartimento di Matematica e Informatica, Università degli Studi di Palermo, Italy

Environmental factors could have a key role in the remarkable and continuous decline of sperm quality observed in the last fifty years. This study addressed the gap of knowledge on the effect of air pollutants on sperm DNA fragmentation, comparing the seminal parameters<sup>1</sup> from men living in locations with different levels of air pollution. The detrimental effects of environmental pollution in the Taranto area are alarming: the high level of pollutants released from the steel plants in the atmosphere can cause health and fertility issues. Our study analyzed sperm samples from three groups of patients: i) workers of Taranto steel plants; ii) Taranto residents; iii) Palermo residents, assumed as controls. Results demonstrate that patients from the “steel plants workers” group, constantly exposed to environmental pollutants for professional reasons, show a mean percentage of sperm DNA fragmentation above 30%. In contrast, patients from the “Taranto residents” group and controls show mean percentages ranging between 16.8% and 25%, respectively. We suggest that sperm DNA evaluation<sup>2</sup> can either be an indicator of individual reproductive health, and a suitable tool to connect the surrounding environment with its effects. On the other end, preliminary data of the Eco Food Fertility project<sup>3</sup> indicated an impairment of several semen quality parameters, including increased sperm DNA damage, in clinically healthy male volunteers living in areas with high environmental impact. Since the methods to study pollutants effects have still to be validated, we suggest that studying sperm DNA fragmentation could serve as a valuable biomarker of the presence and effects of pollution, and we candidate the human sperm cell as an early bioindicator of male health and reproduction.

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## TRANSLATION INITIATION FACTOR eIF6 REGULATES APOPTOSIS IN *D. MELANOGASTER* EYE DEVELOPMENT

P. Calamita,<sup>1,3</sup> A. Russo,<sup>1,2</sup> G. Gatti,<sup>1,3</sup> R. Alfieri,<sup>1</sup> E. Pesce,<sup>1</sup> C. Cheroni,<sup>1</sup> K. Soanes,<sup>4</sup> S. Biffo<sup>1,3</sup>

<sup>1</sup>INGM “Romeo ed Enrica Invernizzi”, Milan, Italy; <sup>2</sup>DiSIT UPO, Alessandria, Italy; <sup>3</sup>UniMi, Milan, Italy; <sup>4</sup>IMB National Research Council, Canada

E-mail: calamita@ingm.org

Eukaryotic initiation factor 6 (eIF6) regulates the initiation of translation by binding the 60S subunit<sup>1</sup> and it is upregulated in some cancers.<sup>2</sup> The cause-effect of eIF6 high levels and tumorigenesis is unknown but we demonstrated that eIF6 haploinsufficient mice show a reduction in lymphomagenesis.<sup>3</sup> The *eif6* gene is highly conserved from yeast to humans. The well characterized development of *D. melanogaster* and the easy manipulation of its genetics led us to the fly model to study the effects of eIF6 gene dosage. Ubiquitous overexpression of eIF6 is lethal. We then focused on the eye, which is dispensable for life. Here,

eIF6 overexpression results in a *rough eye*. The fly eye is composed of ~800 units, called ommatidia, forming a crystalline-like structure. The retinal differentiation is a process that is largely characterized.<sup>4</sup> By genetic crosses we found that eIF6 dosage is fundamental for the correct eye development, as both up- and downregulation causes the disorganization of the structure. Using promoter-specific drivers, we also found that two cell types are responsible for the phenotype: altered eIF6 levels in cone or pigment cells are sufficient for a *rough eye*. It is well established that the crosstalk between these two cell types during the pupal stage is responsible for the programmed cell death (PCD) of extra-numerary pigment cells to determine the correct structure of the adult eye.<sup>4</sup> We found that eIF6 is critical at this stage, because its overexpression is associated to a delayed and increased PCD, resulting in an aberrant eye. Moreover, we found an alteration of translation efficiency when eIF6 is overexpressed and RNASeq analysis showed alterations in eye's specific genes and surprisingly, a tremendous decrease in genes related to ecdysone pathway. This is the first evidence of PCD regulation by the translation initiation factor eIF6 via the ecdysone metabolism.

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## THYROID HORMONE-REGULATED MOLECULAR MECHANISMS DRIVING MOUSE BLASTOCYST IMPLANTATION

L. Campagnolo,<sup>1</sup> D. Piccirilli,<sup>1</sup> M. Massimiani,<sup>1</sup> E. Baldini,<sup>2</sup> S. Ullisse,<sup>2</sup> C. Moretti<sup>3</sup>

<sup>1</sup>Department of Biomedicine and Prevention, Section of Histology and Embryology, University of Rome “Tor Vergata”; <sup>2</sup>Department of Surgical Sciences, University of Rome “La Sapienza”; Department of System Medicine, Section of Reproductive Endocrinology, University of Rome “Tor Vergata”, Rome, Italy

Email: ampagnolo@med.uniroma2.it

Embryo implantation is one of the earliest and critical events of the mammalian reproduction.<sup>1</sup> A clinical association between implantation failure and thyroid dysfunction has been extensively reported, although the molecular mechanism governing this correlation has not been elucidated yet. TH machinery is expressed in the fetomaternal unit at the time of implantation, suggesting a local action of TH. We have investigated the role of TH in mouse hatching and outgrowth, since dysregulation of these events induces implantation failure, leading to infertility.<sup>2</sup> Mouse blastocysts were cultured on a feeder-layer of primary endometrial cells or on plastic, with or without TH supplementation, and hatching and outgrowth evaluated. TH stimulation was also studied on endometrial cell cultures without blastocysts. By qRT-PCR, the expression of two proteases involved in blastocyst hatching in mice (Isp1 and Isp2<sup>3,4</sup>), and MMPs was studied. TH supplementation significantly increased the number of hatching blastocysts cultured on the endometrial cells, while had limited effect on blastocysts cultured on plastic. Isp1 and Isp2 were significantly up-regulated in both blastocysts and endometrial cells, independently analyzed after an overnight co-culture with TH; a less pronounced effect was observed in both blastocysts and endometrial cells cultured alone. TH also induced a significant up-regulation of several MMPs in endometrial cells, independently from the presence of the blastocysts, although the co-culture induced a much higher increase. TH also significantly increased the expansion of trophectodermal cells in

both culture conditions, however expansion was more pronounced in the presence of the endometrial feeder layer. TH plays a key role in the bidirectional crosstalk between the competent blastocyst and the receptive endometrium at the time of implantation. TH supplementation may improve implantation success.

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### EFFECTS OF CuO NANOPARTICLES ON EARLY LIFE STAGES OF BLACK SEA URCHIN *ARBACIA LIXULA*

T. Cappello, A. Giannetto, S. Oliva, V. Parrino, S. Fasulo, A. Mauceri, M. Maisano

*Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Italy*

*E-mail: tiziana.cappello@unime.it*

Copper oxide nanoparticles (CuO NPs) are among the most used metal nanomaterials, but the increasing interest for environmental health has led in the last years to focus on the risk assessment of these materials and their tolerable levels by biological systems. In this perspective, embryotoxicity tests were applied using fertilized eggs of black sea urchin *Arbacia lixula*, exposed to five CuO NPs concentrations (0.009  $\mu\text{M}$ , 0.09  $\mu\text{M}$ , 0.9  $\mu\text{M}$ , 1.9  $\mu\text{M}$ , 2.9  $\mu\text{M}$ ), until the pluteus larva stage. By dynamic light scattering (DLS) analysis, it was revealed that CuO NPs in seawater form clusters characterized by an average hydrodynamic radius of about 140 nm, and exposed *A. lixula* embryos are able to internalize them. Developmental delay and morphological alterations, including skeletal abnormalities, were observed and given the evident damage to skeletal spicules formation, the expression of skeletogenetic genes (i.e. msp130, SM30) was also investigated by molecular analysis. A NMR-based metabolomics approach confirmed the altered skeletogenesis and also highlighted a significant alteration of the cholinergic and serotonergic neurotransmission systems in sea urchin embryos. Moreover, biomarkers of oxidative stress and metal exposure (i.e. SOD, CAT and MT), investigated by molecular analysis of gene expression, demonstrated that CuO NPs induced oxidative toxicity and transcription of MT in exposed embryos. Findings from this research demonstrate the potential of CuO NPs to affect the early life stages of black sea urchin. Therefore, the embryotoxicity tests here applied and the embryos of *A. lixula* used as bioindicators are useful and suitable to evaluate the biological risk and effects of NPs released in the aquatic environment.

### CADMIUM EFFECTS AT A SUB-LETHAL CONCENTRATION IN EMBRYOS AND ADULTS OF *DANIO RERIO*

T. Capriello, A. Monaco, V. Schiano, M.C. Grimaldi, I. Ferrandino

*Department of Biology, University of Naples Federico II, Italy*

*E-mail: ida.ferrandino@unina.it*

Cadmium is known as a biologically not essential-metal and as toxic agent for many organs, including brain. It is in fact in the list of top 20 hazardous substances. Cadmium can be toxic even at low doses since it accumulates and has a long biological half-life. In the last years, this metal has also been indicated as a possible etiological factor in neurodegenerative diseases<sup>1</sup>. Recently we demonstrated that cadmium accumulates in *Danio rerio* brain in which it induces evident histo-morphological damages

and alterations of glial cells, key components of the protection and function of neurons<sup>2,3</sup>. In this study we have analysed the effects of cadmium on embryos and adult specimens of *D. rerio* exposed to 1mg/L of CdCl<sub>2</sub>. Embryos of 6hpf were exposed to CdCl<sub>2</sub> and observed after 24 and 48 hours; adult were instead treated for 16 days and analysed at 2, 7 and 16 days. Acridine orange staining was used for the study of cell death in whole-mount embryos; the Fluoro-Jade B histochemical stain and the immunohistochemical revelation of amyloid<sub>1-42</sub> peptide were instead applied on sections of adult brains to observe the occurrence of degenerative processes. Western blotting experiments were also performed to verify the antibody specificity and to analyse quantitatively the accumulation levels of amyloid<sub>1-42</sub> peptide in adult brains. Collected data show that at 1mg/L of CdCl<sub>2</sub> cadmium induces neurodegeneration in the adult and an increase of cell death in embryos. Considering cadmium implications in neurodegenerative disorders, interestingly enough is the observation of the increased levels of amyloid<sub>1-42</sub> peptide in treated fish brain. Since this peptide is the principal pathological agent in Alzheimer's disease and other amyloidopathies this study might represent a starting point for discovering possible correlations between cadmium and neurological disorders using *D. rerio* as model organism.

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### MSC CONDITIONAL MEDIUM RESCUES NEURONS FROM HYPOXIA/REPERFUSION INJURY

V. Castelli,<sup>1</sup> I. Antonucci,<sup>2</sup> D. Iannotta,<sup>1</sup> M. d'Angelo,<sup>1</sup> D. Di Tizio,<sup>2</sup> E. Benedetti,<sup>1</sup> M. Catanesi,<sup>1</sup> A. Antonosante,<sup>1</sup> L. Stuppia,<sup>2</sup> A. Cimini<sup>3,4</sup>

<sup>1</sup>*Dipartimento di Medicina Clinica, Sanità Pubblica, Scienze della Vita e dell'Ambiente, Università dell'Aquila;* <sup>2</sup>*Dipartimento di Scienze Psicologiche, della Salute e del Territorio, Università di Chieti;* <sup>3</sup>*Sbarro Institute for Cancer Research and Molecular Medicine and Center for Biotechnology, Temple University, Philadelphia, PA, USA;* <sup>4</sup>*National Institute for Nuclear Physics (INFN), Gran Sasso National Laboratory (LNGS), Assergi, Italy*  
*E-mail: vanessa.castelli@graduate.univaq.it*

Stroke remains a leading cause of death and disability in the world. The neurological functional disruption caused by stroke is often severe. Stem cell- derived paracrine effects have emerged as promising strategy for the reactivation of endogenous mechanisms of repair and regeneration in several disease models. Emerging evidences have shown that transplanted stem cells can release trophic signals that influence the microenvironment. Recent studies have shown that the beneficial effects observed following stem cell transplantation in several preclinical models of experimental ischemic disease and injury could be mediated by stem cell secretome. In particular, many studies have reported the potential efficacy of secreted vesicles in stimulating neural plasticity following stroke. In this study in vitro model of hypoxia/reperfusion was used as in vitro stroke model. Injured cells were treated with human amniotic fluid derived stem cells (hAFS)- conditioned media obtained from three different pregnant women and cells were assayed for viability and for the expression of proteins responsible for neuronal survival such as BDNF, TrkB, ERK5 and neuronal death such as pro-BDNF, p75, JNK. The results obtained indicated a strong neuroprotective activity of hAFS- conditioned media by activating neuronal survival pathways and decreasing neuronal death. Interestingly a significant difference in counteracting neuronal death was observed among the different pregnant women

## GRAPHENE OXIDE TRIGGERS NEURONAL DIFFERENTIATION BY THE HIPPO PATHWAY

M. Catanesi,<sup>1</sup> E. Benedetti,<sup>1</sup> G. Fioravanti,<sup>2</sup> F. Perrozzì,<sup>2</sup> L. Cristiano,<sup>1</sup> A. Antonosante,<sup>1</sup> M. D'Angelo,<sup>1</sup> V. Castelli,<sup>1</sup> L. Ottaviano,<sup>2</sup> R. Ippoliti,<sup>1</sup> C. Gargioli,<sup>3</sup> S. Cannata,<sup>3</sup> A. Cimini<sup>1,4,5</sup>

<sup>1</sup>Department of Life, Health and Environmental Sciences, University of L'Aquila, Italy; <sup>2</sup>Department of Physical and Chemical Sciences, University of L'Aquila, Italy; <sup>3</sup>Department of Biology, University of Tor Vergata, Rome, Italy; <sup>4</sup>Sbarro Institute for Cancer Research and Molecular Medicine and Center for Biotechnology, Temple University, Philadelphia, PA, USA; <sup>5</sup>National Institute for Nuclear Physics (INFN), Gran Sasso National Laboratory (LNGS), Assergi, Italy

E-mail: mariano.catanesi86@gmail.com

Recently, graphene (as the thinnest two-dimensional nanomaterial in the world) has been the focus of intense research owing to its fascinating physico-chemical properties and the most promising applications especially in bio-nanotechnology. Very recently, biomedical applications of graphene oxide in nervous systems have attracted much attention. In fact, since neural cells and their activities are electrical-dependent, the unique electrical properties of graphene can provide an excellent advantage for neuronal stimulation, therapeutic or other clinical diagnostics and treatments. Moreover, graphene can be easily functionalized by proper molecules to enhance the effective neural differentiation. Moreover, it has been demonstrated the effective influence of graphene oxide on enhanced differentiation of human neural stem cells (hNSCs) into neurons. However, the mechanisms underlying the effects of graphene oxide in inducing neuronal differentiation are still poorly understood. In this work we used graphene oxide (GO) and slight reduced GO (rGO) and strongly reduced GO (rGO<sup>+</sup>) as scaffolds for neuroblasts growth and differentiation. The biofilms of the different graphenes were not harmful for cell viability, while the different graphenes differently affected neuronal differentiation, with the rGO as the better choice for neuronal differentiation. The electrical properties of graphene and its stiffness determined the downregulation of the final effectors of the hippo pathway YAP and TAZ and their translocation to the cytoplasm in the inactive form.

## REWIRING FIBRO-ADIPOGENIC PROGENITORS DIFFERENTIATION TRAJECTORIES TO COUNTERACT MUSCLE MYOPATHIES

A. Cerquone Perpetuini, A. Calderone, M. Marinkovic, E. Micarelli, S. Pirro', A. Reggio, M. Rosina, R. Stefanelli, C. Gargioli, L. Castagnoli, G. Cesareni

Department of Biology, University of Rome "Tor Vergata", Rome, Italy

Several pathological conditions, including Duchenne muscular dystrophy, affect skeletal muscle regeneration potential and ultimately its function. Besides Muscle Satellite cells (MuSC), which are directly responsible for the generation of new myofibers, distinct interstitial stem cell populations are involved in muscle regeneration and the complex interplay between these diverse populations is essential to coordinate the regeneration process. Fibro-Adipogenic Progenitors (FAPs), support muscle regeneration through the release of cytokines, the productions of components of the extracellular matrix and the clearance of necrotic debris. However, within the dystrophic context, they are considered the source of fibrotic and fat tissue infiltrations that impairs muscle function. Here we present a High-Content Screening platform to identify compounds that reshape the dif-

ferentiation trajectories of these multipotent stem cells. We identified several classes of compounds that prevent adipogenic or fibrogenic differentiation of FAPs isolated from a dystrophic mouse model. Among them distinct glucocorticoids, despite sharing a common chemical structure, exert different effects on FAPs differentiation trajectories, suggesting distinct mechanisms behind their modulation of FAPs fibro-adipogenic differentiation.

## A HISTOLOGICAL INVESTIGATION ON THE HEPATOPANCREAS OF *GAMMARUS ELVIRAE* (CRUSTACEA, AMPHIPODA) EXPOSED TO ARSENIC

C. Chimenti,<sup>1</sup> C. Giannelli,<sup>1</sup> E. De Matthaeis,<sup>1</sup> D. Davolos,<sup>1,2</sup>

<sup>1</sup>Department of Biology and Biotechnology "Charles Darwin", Sapienza University, Rome; <sup>2</sup>Department of Technological Innovations and Safety of Plants, Products and Anthropic Settlements, INAIL, Research Area, Rome, Italy

Arsenic (As) is a toxic, genotoxic and carcinogenic metalloid. The main chemical forms of inorganic arsenic in the environment are arsenate (As (V)) and arsenite (As (III)); As (V) is generally found in oxidized sites, while As (III) dominates in geochemically reducing conditions. Arsenic redox status affects its mobility, bioavailability, and toxicity. Arsenic pollution of surface waters and aquifers is generally due to natural processes. However, arsenic contamination occurs also presumably as a result of improper anthropic activities, including mining and agricultural practices, the use of fossil fuels, and the accidental or deliberate discharge of industrial waste. Although being highly relevant to public health, knowledge of the toxic and genotoxic effects of low As doses in freshwater environments (the European regulatory limits in drinking water are of 10 µg L<sup>-1</sup> As) is still limited. In recent years, the crustacean *Gammarus elvirae* has been considered as a model organism for toxicological studies in aquatic environments.<sup>1</sup> Experiments conducted by means of the Comet test allowed us to identify effects at the DNA level in individuals of *G. elvirae* exposed to arsenic (5, 10, and 50 µg L<sup>-1</sup> of As) in the forms of arsenite and arsenate.<sup>2</sup> The aim of this work was to examine the potential effects of arsenic on the hepatopancreas of *G. elvirae* by adopting the histological methods<sup>1</sup> since this gland plays a pivotal role in the response/vulnerability to toxic contaminants. In particular, individuals of *G. elvirae* were exposed to river waters (Capo d'Acqua, Amaseno, Latium, central Italy; As concentration of ca. 1 µg L<sup>-1</sup> As)<sup>1</sup> subsequently supplemented with either As (V) or As (III) at a final concentration of 5, 10, and 50 µg L<sup>-1</sup> of As. River waters from Capo d'Acqua were used as a negative control. In addition, based on our previous *G. elvirae* LC (50-240 h) estimations for As (V) (1,550 µg L<sup>-1</sup>) and As (III) (1,720 µg L<sup>-1</sup>)<sup>2</sup>, we carried out a histological investigation of the hepatopancreas of this species exposed to freshwaters containing mg L<sup>-1</sup> concentrations of As in the forms of either As (III) or As (V). Preliminary results of our histological examination on the two pairs of hepatopancreatic structures of *G. elvirae* are discussed in relation to previously data on toxic effects of arsenic published for other crustaceans.

### Acknowledgements

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## DEVELOPMENT OF MOUSE HIPPOCAMPAL NEURONS *IN VITRO*: A 6-STAGES MODEL TO STUDY DIFFERENTIATION DEFICITS AND RECOVERY IN RETT SYNDROME

A. Colliva\*, S. Donegà\*, G. Baj, E. Tongiorgi

Laboratory of Cellular and Developmental Neurobiology,  
Department of Life Sciences, University of Trieste, Trieste, Italy

\* These authors contributed equally.

E-mail: tongi@units.it

Neuronal development is a process that can be subdivided into several, partially overlapping phases that depend on complex molecular interactions. Numerous models of neuronal cultures were successfully used to clarify neuronal development mechanisms. The most accurate description of neuronal development available today refers to rat hippocampal neurons and was published in 1988 by Dotti<sup>1</sup> and collaborators who identified 5 stages of development, up to 7 days *in vitro* (DIV). However, this pattern was incomplete and there was no equivalent staging system in the mouse, although there are many available murine models of genetic mutations. By comparative morphometric analysis on rat and mouse hippocampal neurons cultures from 1 to 15 DIV, we constructed a new 6-stage model for both species. Then, we used the new staging system for mouse neurons to identify which phases of the maturation process are altered in Rett syndrome, a genetic disorder due to inactivation of the MeCP2 gene, characterized by a brain development delay with severe mental retardation. Hippocampal cultures from MeCP2 knockout mice exhibited decelerated growth of dendritic branches from Stage 4 (DIV4) onwards. The number of synapses was reduced in MeCP2-ko neurons at stages 5-6 (DIV9-15), suggesting a greater elimination of synapses.<sup>2</sup> These results indicate that there is a deficiency in Rett syndrome both in the initial stages of differentiation and in the late maintenance phases of form and function of neurons. Subsequent to this initial study, using an animal model of Rett syndrome we compared the morphology of hippocampal neurons *in vivo* and in culture at DIV 12, i.e. the time at which we observed the maximal developmental deficits. We found shrinkage of pyramidal neurons somata and reduced diameter of apical dendrites both *in vivo* and *in vitro*. In cultured neurons, we also found that dendrites were shorter and with less branches. In the attempt to recover Rett hippocampal neurons from atrophy, we treated MeCP2-ko mice for 15 days with 50 mg/kg mirtazapine, an antidepressant that increases the release of serotonin and norepinephrine. To mimic this treatment *in vitro*, we incubated hippocampal cultures with 10  $\mu$ M mirtazapine for 9 days (from DIV3 to DIV12) or for 3 days (from DIV 9 to DIV12) and we found recovery of neuronal atrophy both *in vivo* and *in vitro*.<sup>3</sup>

### Acknowledgements

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## BIO-INTERACTIONS AND EFFECTS OF IRON NANOPARTICLES IN *XENOPUS LAEVIS* EMBRYOS: A MODEL TO STUDY THE EFFICACY AND SAFETY OF NANOINGREDIENTS FOR FOOD FORTIFICATION

A. Colombo,<sup>1</sup> P. Bonfanti,<sup>1</sup> M. Saibene,<sup>1</sup> R. Gornati,<sup>2</sup> G. Bernardini,<sup>2</sup> U. Fascio,<sup>1</sup> P. Mantecca<sup>1</sup>

<sup>1</sup>Department Earth and Environmental Sciences, Research Centre POLARIS, University of Milano "Bicocca"; <sup>2</sup>Department of Biotechnology and Life Sciences, University of Insubria, Varese, Italy

E-mail: anita.colombo@unimib.it

Iron deficiency affects 1–2 billion people worldwide, in particular risk groups like pregnant, lactating women and young children. Iron sources approved for food fortification (e.g. Fe<sub>2</sub>SO<sub>4</sub> and Fe<sub>4</sub>O<sub>21</sub>P<sub>6</sub>) have disadvantages such as organoleptic changes and poor absorbability. Decreasing particle size to the nanoscale could be a strategy to improve Fe bioavailability; however, the benefits and risks associated with the use of nanoparticles (NP) in the food industry need to be studied especially during the critical window of development. In this study we used the Frog Embryo Teratogenesis Assay-*Xenopus* to evaluate the safety of FeNP (zerovalent Fe and Fe<sub>3</sub>O<sub>4</sub>) compared to salt (FeCl<sub>3</sub>) (5–100 mg/L) and their bio-interactions with larvae intestinal tract with the view of a possible food fortification application. Unexpectedly FeCl<sub>3</sub> resulted embryotoxic, causing 100% mortality at 50 mg/L, this is probably due to the formation of Fe(OH)<sub>2</sub> not soluble in FETAX solution. No important adverse effects were evidenced at lower concentrations (5 and 25 mg/L). Conversely, FeNP were not toxic even though Fe<sub>3</sub>O<sub>4</sub>NP could cause abnormal gut coiling in a dose-independent mode. It is noteworthy that FeNP at all tested concentrations and FeCl<sub>3</sub> at 5 mg/L evidenced higher embryo length compared to not exposed samples. Prussian blue staining and laser scanning confocal microscopy in reflection mode allowed to observe Fe accumulation in the gut lumen and on the enterocyte brush border, confirming the uptake of FeNP and Fe(OH)<sub>2</sub> precipitates. In particular, FeNP were abundantly mapped in structurally well preserved gut epithelia. ICP-OES and TEM analyses will be performed to better evaluate Fe accumulation in larval tissues and the modality of FeNP uptake by enterocytes. These preliminary results indicate that *Xenopus* embryos could be considered as a valuable tool to evaluate the efficacy and safety of functional NP relevant for ingestion, and that FeNP might be considered for future strategies to prevent iron deficiencies.

## PCDH19 AND THE ESTABLISHMENT OF PLANAR CELL POLARITY IN IPS-DERIVED DEVELOPING NEURONS IN A X-LINKED MODEL OF EPILEPSY

C. Compagnucci,<sup>1</sup> S. Petrini,<sup>2</sup> M. Trivisano,<sup>3</sup> N. Specchio,<sup>3</sup> E. Bertini<sup>1</sup> A. Terracciano<sup>1</sup>

<sup>1</sup>Unit of Neuromuscular and Neurodegenerative Diseases;

<sup>2</sup>Confocal Microscopy Core Facility, Research Laboratories;

<sup>3</sup>Division of Neurology, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy

E-mail: claudia.compagnucci@opbg.net

PCDH19 (Protocadherin 19), a member of the cadherin superfamily, is involved in the pathogenic mechanism of a X-linked model of Epilepsy [1,2]. The biological function of PCDH19 in human neurons and during embryogenesis is currently unknown. Therefore, we decided to use the model of the induced pluripotent stem cells (iPSCs) to characterize the location and timing of expression of PCDH19 during cortical neuronal differentiation. We used human iPSCs, obtained from skin fibroblasts of

healthy subjects, and iPSC-derived neurons to recapitulate the role of PCDH19 in neuronal differentiation. In particular, we developed adherent culture protocols leading to the formation of Neuronal Stem Cells (NSC) arranged radially around a lumen termed "neural rosettes". Importantly the structure of the neural rosette resembles the polar organization of the neural tube in the developing human embryo (with a lumen indicating the basal part of the polarized neuronal progenitor cell) [3]. Our data show that PCDH19 is present in pluripotent cells before differentiation in a homogeneous pattern, while following differentiation its localization is polarized. The proper control of the cell orientation ensures a fine balancing between symmetric (giving rise to two progenitor sister cells) versus asymmetric (giving rise to one progenitor cell and one newborn neuron) division. This process results in the polar organization of the neural tube with a lumen indicating the basal part of the polarized neuronal progenitor cell; in the iPSC model the cells are organized in the 'neural rosette' and interestingly, PCDH19 is located at the center of the rosette, with other well-known markers of the lumen (N-cadherin and ZO-1). These data suggest that PCDH19 has a role in instructing the apico-basal polarity of the progenitor cells, thus regulating the development of a properly organized human brain.

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### UNVEILING A NEW ROLE FOR PLATELET DERIVED GROWTH FACTOR B DURING EMBRYONIC DEVELOPMENT

D. Corsinovi,<sup>1,2</sup> K. Giannetti,<sup>1</sup> A. Cericola,<sup>1</sup> V. Naef,<sup>1</sup> M. Ori<sup>1</sup>

<sup>1</sup>Unit of Cell and Developmental Biology, Department of Biology, University of Pisa; <sup>2</sup>Department of Translational Research and of New Surgical and Medical Technologies, University of Pisa, Italy  
E-mail: michela.ori@unipi.it

The PDGF family consists of four ligands (PDGF-A to -D) and two tyrosine kinase receptors (PDGFR  $\alpha$  and  $\beta$ ). In vertebrates, PDGF signaling exerts multiple functions during embryonic development, driving cellular responses including proliferation, survival, migration, deposition of extracellular matrix and tissue remodeling and an up-regulation of PDGF signaling is also implicated in the etiology of human gliomas.<sup>1</sup> Despite all these evidences the exact role of each member of the family during embryonic development is still incomplete and partially controversial. In *Xenopus* pdgf-a and pdgfr- $\alpha$  are essential for gastrulation and in Zebrafish and mouse the pdgfr signaling is required for the formation of craniofacial structures derived from neural crest cells (NCC)<sup>2,3</sup>. As this receptor could be activated by different combination of ligands with different affinity and the knockout of *pdgf-a* did not show craniofacial alteration, we investigated the possible role of *pdgf-b* during embryogenesis and in particular during NCC development. The working hypothesis of our project is based on our recent data that unveiled the presence of *pdgf-b* mRNA during embryonic development in territories adjacent to the NCC4, which express receptor *pdgfr- $\alpha$* . To test our hypothesis we obtained *pdgf-b* morphants by injecting a splice-blocking morpholino in *Xenopus* embryos. Preliminary results suggested that *pdgf-b* may play a role in the cranial neural crest segregation in distinct migratory streams by interfering with the expression of key molecules involved in this process such as neuropilin and ephrin family members. These data proposed a new role for *pdgf-b* during vertebrate development and *pdgf-b* morphants as a new model to study the many

different functions of pdgf signaling *in vivo*.

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### HIPPOCAMPAL NEURONS OF DYSTROPHIC MDX MICE HAVE REDUCED SENSITIVITY TO ACUTE CORTICOSTERONE TREATMENT IN VIVO COMPARED TO WILD TYPE

F. Cosmi,<sup>1,2</sup> P. Fragapane,<sup>3</sup> I. Bozzoni,<sup>1</sup> M.E. De Stefano<sup>1,2</sup>

<sup>1</sup>Dipartimento di Biologia e Biotecnologie "C. Darwin", Università di Roma Sapienza; <sup>2</sup>Laboratorio affiliato all'Istituto Pasteur Italia-Fondazione Cenci Bolognetti, Roma; <sup>3</sup>Istituto di Biologia e Patologia Molecolari Consiglio Nazionale delle Ricerche, Roma, Italy

Duchenne muscular dystrophy (DMD) is a lethal X-linked disease characterized by progressive muscular wasting due to lack of dystrophin (Dp427), a cytoskeletal protein expressed in muscle and selected brain regions, as the hippocampus. For this reason, DMD patients also experience neurological disturbances. These could be aggravated by the glucocorticoid (GC) treatments to which they are exposed, as hippocampus is one major GC target. In this study we analyzed whether acute *in vivo* treatment with corticosterone (CORT) affected hippocampal neuron physiology. We evaluated: mRNA and protein levels of the GC receptor (GR), directly modulated by GC genomic activity; activation (phosphorylation) of ERK, a rapid non-genomic intracellular pathway operating through membrane-associated GRs via caveolin-1 (Cav1). Cav1 is a lipid raft and GR-associated protein, part of a protein complex linked to Dp427 and indispensable for non-genomic GR activity. Wild type (WT) and dystrophic *mdx* mice, a DMD animal model, were acutely injected with 40 mg/kg of CORT. Mice were killed 90 min and 6 h after injection, and rapid and long-lasting effects of GC activity were evaluated in hippocampal tissue extracts. Protein levels of intracellular and membrane bound phosphorylated (active) pGRs, significantly increased in both WT and in *mdx* mice compared to the respective controls (non injected mice) 90 min after injection. Differently, GR mRNA and protein levels remained unaltered in WT mouse hippocampi, and significantly decreased in *mdx* mice. Therefore, although GRs were activated, the genomic effects were not yet evident, and fast receptor degradation was possibly triggered in *mdx* mouse neurons. This could depend on a reduced non-genomic signaling in the dystrophic genotype compared to WT. CORT short treatment, in fact, induced a significant increase in Cav1, ERK and pERK protein levels in WT mice. Differently, in *mdx* mouse hippocampus, Cav1 was reduced compared to control and, although ERK and pERK protein levels increased after treatment, they were significantly lower compared to WT. As expected, 6 h after CORT administration, GR protein level in the WT genotype significantly increased, compared to control, while those of pGR remained unaltered. No changes were seen in *mdx* mouse hippocampi. These results, although to be completed, suggest that response of *mdx* hippocampal neurons to GCs is reduced compared to WT, with consequent alteration in both early and long-lasting effects.

## CXCR1/2 PATHWAYS IN PACLITAXEL-INDUCED NEUROPATHIC PAIN

M. d'Angelo,<sup>1</sup> L. Brandolini,<sup>2</sup> E. Benedetti,<sup>1</sup> P.A. Ruffin,<sup>3</sup> R. Russo,<sup>4</sup> L. Cristiano,<sup>2</sup> A. Antonosante,<sup>2</sup> V. Castelli,<sup>2</sup> A. Giordano,<sup>5,6</sup> M. Allegretti,<sup>2</sup> A. Cimini,<sup>1,6,7</sup>

<sup>1</sup>Department of Life, Health and Environmental Sciences, University of L'Aquila, Italy; <sup>2</sup>Dompé Farmaceutici SpA, L'Aquila, Italy; <sup>3</sup>Dompé Farmaceutici SpA, Milan, Italy; <sup>4</sup>Department of Pharmacy, University of Naples Federico II, Italy; <sup>5</sup>Department of Medicine, Surgery and Neuroscience, University of Siena, Italy; <sup>6</sup>Sbarro Institute for Cancer Research and Molecular Medicine and Center for Biotechnology, Temple University, Philadelphia, PA, USA; <sup>7</sup>National Institute for Nuclear Physics (INFN), Gran Sasso National Laboratory (LNGS), Assergi, Italy  
E-mail: dangelomichele@hotmail.com

Chemotherapy-induced peripheral neuropathy is a type of neuropathic pain that represents a frequent and serious consequence of chemotherapy agents. Over the last years, significant progress has been achieved in elucidating the underlying pathogenesis of CIPN. The interference of taxanes with microtubule has been proposed as a mechanism that leading to altered axonal transport and to permanent neurological damages. The inflammatory process activated by chemotherapeutic agents has been considered as a potential trigger of nociceptive process in CIPN. In this study we investigated the effect of an inhibitor of CXCR1/CXCR2, reparixin, in suppressing the development of paclitaxel-induced nociception in rats. Moreover, reparixin activity in reversing the neurotoxic effects induced by paclitaxel or GRO/KC in F11 cells was also analyzed. Reparixin administered by continuous infusion ameliorated paclitaxel-induced mechanical and cold allodynia in rats. In F11 RDG cells, reparixin was able to inhibit the increase of acetylated  $\alpha$ -tubulin induced both by paclitaxel and by GRO/KC. The subsequent experiments were performed in order to dissect the signal transduction pathways under GRO/KC control, eventually modulated by paclitaxel and/or reparixin. We found that reparixin significantly counteracted p-FAK, p-JAK2/p-STAT3, and PI3K-p-cortactin activation induced either by paclitaxel or GRO/KC. Overall the present results have identified CXCL8/CXCR1/2 pathway as a mechanism involved in paclitaxel-induced CIPN. In particular, the obtained data suggest that the inhibition of CXCR1/2 combined with standard taxane therapy, in addition to potentiating the taxane anti-tumor activity can reduce chemotherapy-induced neurotoxicity, thus giving some insight for the development of novel treatments.

## FUNCTIONAL STUDY OF LIN28B PROTO-ONCOGENE DURING THE DEVELOPMENT OF NEURAL CREST CELLS IN XENOPUS AND ZEBRAFISH EMBRYOS

M. De Sarlo,<sup>1</sup> D. Corallo,<sup>2</sup> S. Aveic,<sup>2</sup> G.P. Tonini,<sup>2</sup> S. Candiani,<sup>3</sup> M. Ori<sup>1</sup>

<sup>1</sup>Department of Biology, University of Pisa; <sup>2</sup>Neuroblastoma Laboratory, Pediatric Research Institute, Città della Speranza, Padua; <sup>3</sup>Department of Earth, Environmental and Life Sciences, (DISTAV), University of Genoa, Italy  
E-mail: michela.ori@unipi.it

Neuroblastoma is a neuroendocrine tumor and it is one of the most common pediatric malignant tumors. Experimental evidence suggests that it arises during embryonic development. An altered differentiation and/or abnormal migration of trunk neural crest cells (NCC), the precursors of chromaffin cells of the sympathetic neurons in the adrenal glands may be the cause of

the disease.<sup>1</sup> High levels of genomic amplification and aberrant overexpression of *LIN-28B* was observed in patients with *MYCN* gene amplification (clinically classified as high risk neuroblastoma).<sup>2</sup> The correlation between *lin28b* and neuroblastoma is also documented by GWAS.<sup>3</sup> Furthermore, previous studies on murine models showed that *lin28b* overexpression induces the epithelial to mesenchymal transition (EMT) and promotes metastasis in other types of cancer.<sup>4</sup> Despite these evidences the role of *lin28b* during NCC development is far to be elucidated. We investigated the role of *lin28b* during *Xenopus laevis* and *Danio rerio* embryos development showing that it is expressed in trunk NCC. *lin28b* gene gain of function experiments suggested a putative role of *lin28b* in trunk neural crest migration and differentiation in both animal models. These data lay the foundation for the subsequent study of *lin28b* function during NCC development and possible generation of a new *in vivo* model for the study of the etiology of infantile neuroblastoma.<sup>5</sup>

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## ANALYSIS OF ANTIOXIDANT RESPONSE ACTIVATION IN HUMAN ASTROGLIAL/NEURONAL CULTURE MODEL

V. D'Ezio, R Mastrantonio, M. Colasanti, T. Persichini  
Laboratory of Cell Biology, University of "Roma Tre", Rome, Italy  
E-mail: tiziana.persichini@uniroma3.it

Oxidative stress is a common hallmark of neurotoxicity. To maintain a redox balance, many cells may up-regulate the expression of endogenous antioxidant enzymes through the activation of Nrf2 transcription factor. During oxidative stress, astrocytes are able to activate Nrf2-mediated antioxidant response by increasing the expression of several ARE genes.<sup>1</sup> Here, we investigated the antioxidant response activation in human astrocyte/neuron co-culture system following treatment with amyloid- $\beta$  ( $A\beta$ ), an important mediator of neurotoxicity in Alzheimer's disease.<sup>2</sup> In particular, we used U373 and SH-SY5Y cells as astroglial and neuronal cell models, respectively, and evaluated the activation of Nrf2-mediated antioxidant response, separately, in both cell types. After treatment with  $A\beta$  (50  $\mu$ M) for 0,5 h, 2 h and 4 h, we observed Nrf2 activation in astrocytes with a peak at 2 h. We therefore analyzed the expression of ARE genes treated with  $A\beta$  (50  $\mu$ M) at 4, 8, 16 h post-treatment. As expected, we observed increased levels of several ARE genes in astrocytes at 8-16 h post-treatment. Among the Nrf2-dependent genes we particularly focused our study on those involved in GSH synthesis and homeostasis such as gamma-GlutamylCysteine Ligase (GCL) and Cystine/Glutamate antiporter (xCT subunit). We also evaluated the expression of Superoxide dismutase-1 (SOD1), Catalase (CAT) and heme oxygenase-1 (HO1). Activation of the antioxidant response in astrocytes as well as modification in their gene expression profile may greatly affect the role of astrocytes in establishing neuroprotective or detrimental conditions for neuronal functions and viability. Our results may contribute to a better understanding of molecular and cellular pathways involved in cell survival during chronic oxidative stress.

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## INDUCTION OF NEUROSTEROID SYNTHESIS BY D-ASPARTIC ACID

M.M. Di Fiore, G. Chieffi Baccari, S. Falvo, F. Di Giacomo Russo, A. Santillo

*DiSTABiF, University of Campania "L. Vanvitelli", Caserta, Italy*  
E-mail: mariamaddalena.difiore@unicampania.it

Neurosteroids are defined as neuroactive steroids synthesized de novo in the central nervous system (CNS). Many studies have revealed important roles of these steroids in mediating several brain functions, such as sexual differentiation and reproductive behavior. D-Aspartate (D-Asp) is an endogenous amino acid occurring in CNS of various animal phyla. In rat D-Asp seems to play an important role either in the development of CNS or in different neuronal activities including learning and memory processes. Our previous studies have provided evidence that D-Asp plays a role in steroid synthesis in gonads.<sup>1</sup> To verify the effects of D-Asp in brain steroid synthesis we performed in vivo and in vitro experiments. Specifically, we carried out 1) a chronic experiment in which adult rats were allowed to drink a solution of 20 mM D-Asp for 15 days, and 2) an acute experiment in which rats were injected with 2  $\mu$ mol/g bw of D-Asp. We found that exogenous D-Asp accumulated in the brain. Following D-Asp oral administration, brain progesterone (P) and 17 $\beta$ -estradiol (E2) increased about 1.3-fold over basal level whereas testosterone (T) of 2.1-fold. In acute experiment, 30' after D-Asp injection, brain P, T and E2 concentrations increased about 1.3- fold over basal levels. After 2h P, T and E2 reached about 1.5-fold; after 8h P, T and E2 increased about 2.0-fold. Further, to verify whether D-Asp had a direct effect on the activity of neurosteroidogenic enzymes, brain homogenates were incubated with different substrates (cholesterol, P and T) with or without the addition of D-Asp. Enzyme activities were measured by evaluating the in vitro conversion rate of: 1) cholesterol to P, T and E2; 2) P to T and E2; 3) T to E2. We found a significant increase of P, T and E2 after addition of cholesterol+D-Asp. The addition of P+D-Asp induced a significant increase of both T and E2. Finally, the addition of T+D-Asp induced a significant increase of E2. These results indicated that D-Asp affects the activity of specific enzymes of the steroidogenic cascade in rat brain revealing a novel function of this amino acid in the brain, i.e. local activation of synthesis of steroids.

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## THE EARTHWORM *DENDROBAENA VENETA*: SOIL TOXICOLOGY, WOUND HEALING, AND OTHER

S. Ferrando,<sup>1</sup> A. Amaroli,<sup>2</sup> L. Gallus,<sup>1</sup> E. Roccotiello,<sup>1</sup> S. Rosatto,<sup>1</sup> M. Rottigni<sup>1</sup>

<sup>1</sup>DISTAV, University of Genoa, <sup>2</sup>DISC, University of Genoa, Italy  
E-mail: sara131274@gmail.com

According to the Krogh's principles, it is worth to search for species on which a given biological process can be most conveniently studied. In vertebrate and in the branch of Lophotrochozoa, gene loss and sequence divergence occurred at a relatively low rate than in the branch of Ecdysozoa, suggesting that more species from Lophotrochozoa should be considered as animal models in laboratory research. In the frame of the 3R principle (Replacement, Reduction, and Refinement), and for the interesting phylogenetic position of Annelida, we are attempting to employ and validate the use of the earthworm *Dendrobaena veneta* (Rosa, 1886) in our laboratory. Earthworm are well e biological processes. We immediately had

the possibility to include *D. veneta* in active research lines. We determined the growing curve for *D. veneta* newborn in standard condition; that is allowing us to perform, for the first time, long-term toxicological experiments on newborn earthworm. First results on nickel long-term effects are showing that growing curve of newborn *D. veneta* is sensitive for a wide range of concentrations (from 25 to 1000 mg Ni/Kg of soil) and correlates to alterations of epidermal and calciferous glands. We are also using for the first time, as a model of wound healing, thirty caudal segments (30CS) excised from adult earthworms. The percentage of surviving of the animals after segments excision (accomplished after brief cold anaesthesia) is 100% and they are not involved in the experimental part at all. The 30CS are vital for more than 10 days and, in the first 4 days, they activate healing processes. In particular we are now using this model in studies about photobiomodulation by laser sources, and we observed that it can affect muscle contraction, pathogens proliferation, and immune response. Other studies are planned, in collaboration with other research groups. The promising results and the rich potential of this species encourage us to deep our knowledge of its biology.

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## NEW INSIGHTS INTO THE REPAIR PHASE OF ECHINODERM ARM REGENERATION

C. Ferrario,<sup>1,2</sup> Y. Ben Khadra,<sup>3</sup> A. Czarkwiani,<sup>4</sup> A. Zakrzewski,<sup>4</sup> M. Ascagni,<sup>5</sup> F. Bonasoro,<sup>1</sup> M.D. Candia Carnevali,<sup>1</sup> P. Oliveri,<sup>4</sup> M. Sugni<sup>1,2</sup>

<sup>1</sup>Dipartimento di Scienze e Politiche Ambientali, Università degli Studi di Milano, Italy; <sup>2</sup>Center for Complexity & Biosystems, Dipartimento di Fisica, Università degli Studi di Milano, Italy; <sup>3</sup>Laboratoire de Recherche, Génétique, Biodiversité et Valorisation des Bioressources, Institut Supérieur de Biotechnologie de Monastir, Université de Monastir, Monastir, Tunisia; <sup>4</sup>Department of Genetics, Evolution and Environment, University College London, UK; <sup>5</sup>Dipartimento di Bioscienze, Università degli Studi di Milano, Italy

E-mail: cinzia.ferrario@unimi.it

Regeneration is a complex post-embryonic developmental process during which lost body parts are completely reformed. The first post-injury events are overall known as repair phase and include wound closure, inflammatory and immune response, extracellular matrix and tissue remodelling. These are fundamental for the effectiveness of the subsequent regeneration. Indeed, their impairment can limit or prevent the regenerative phenomena. Echinoderms are deuterostomes invertebrates which possess amazing regenerative abilities. Here, the brittle star *Amphiura filiformis* and the starfish *Echinaster sepositus* were selected as valid experimental models to study the main events of the repair phase using microscopy and molecular techniques and to provide a comparative perspective with mammals, which are known for their limited regenerative abilities. Our results showed that re-epithelialisation is faster and more effective in echinoderms than in mammals, whereas the inflammatory response, analysed through TNF- $\alpha$ -like immunodetection, is comparable between them. Gene expression pattern of *fibrinogen-like* and *ficolin* in echinoderms is a promising starting point to deepen immune system response after injury. Moreover, differently from mammals, delayed and limited collagen deposition characterise echinoderms' repair phase. Overall, the higher effectiveness of echinoderms' repair processes suggests that this step is crucial for the success of the subsequent regenerative process. Further analyses are necessary to better understand



similarities and differences between animals able or unable to regenerate.

### MUSCLE PERIVASCULAR STEM CELLS FOR ADVANCED SKELETAL MUSCLE TISSUE REGENERATIVE APPROACHES

E. Fornetti,<sup>1</sup> S. Testa,<sup>1</sup> F. Tamiro,<sup>1</sup> S. Bernardini,<sup>1</sup> S. Cannata,<sup>1</sup> C. Gargioli<sup>1</sup>

<sup>1</sup>Department of Biology, University of "Rome Tor Vergata", Italy

Skeletal muscle tissue engineering represents a revolutionary approach for the treatment of musculoskeletal tissue pathologies.<sup>1</sup> However, there are some limitations related to the complex muscle architectural organization and to the difficulty to find a reliable and appropriate source of myogenic progenitors able to sustain swift vascularization. Recent studies have led to the identification of a new muscle progenitor cell population namely pericytes.<sup>2</sup> These are perivascular muscle progenitors able to undergo a robust myogenic differentiation beside a preserved angiogenic ability.<sup>3,4</sup> Human derived pericytes (hPeri) were isolated from human muscle biopsies by enzymatic digestion and selected for low confluence plastic adhesion and alkaline phosphatase (AP) expression<sup>5</sup>, showing colony forming capability and a remarkable spontaneous myogenic activity. hPeri were characterized *in vitro* for mesodermic differentiation and *in vivo* by intramuscular injection, revealing their astonishing myogenic capability. Moreover, for skeletal muscle tissue engineering purpose, it was evaluated the combination of hPeri and PEG-Fibrinogen (PF) based biomimetic scaffold<sup>6</sup> for building a human derived artificial muscle *in vivo* upon subcutaneous implantation. The results obtained showed that PF based matrix encapsulating hPeri promoted the generation of an engineered human derived muscle, moreover presenting an important vascularization upon hPeri angiogenic action. Hence the perivascular compartment with hPeri can be considered a remarkable reliable source for myogenic stem/progenitor cells.

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### POLYMERS OF Z ALPHA-1 ANTITRYPSIN ARE SECRETED IN CELL MODELS OF DISEASE

A. Fra,<sup>1</sup> F. Cosmi,<sup>2\*</sup> A. Ordoñez,<sup>3\*</sup> R. Berardelli,<sup>1</sup> J. Perez,<sup>4</sup> N.A. Guadagno,<sup>2</sup> L. Corda,<sup>5</sup> S.J. Marciniak,<sup>3</sup> D.A. Lomas,<sup>6</sup> E. Miranda<sup>2</sup>

<sup>1</sup>Department of Molecular and Translational Medicine, University of Brescia, Italy; <sup>2</sup>Department of Biology and Biotechnology "Charles Darwin", "Sapienza" University, Rome, Italy; <sup>3</sup>Department of Medicine, CIMR, University of Cambridge, UK; <sup>4</sup>Department of Cell Biology, Genetics and Physiology, University of Malaga, Spain; <sup>5</sup>Division of Internal Medicine, Spedali Civili, Brescia, Italy; <sup>6</sup>Wolfson Institute for Biomedical Research, UCL, London, UK \*Equal contributions

E-mail: mariaelena.mirandabanos@uniroma1.it

Alpha-1 antitrypsin (AAT) is a glycoprotein secreted from hepatocytes to blood that protects the lungs from excessive proteolysis by neutrophil elastase. AAT deficiency (AATD) is due to mutations in AAT (particularly the Z allele, E342K), which lead to

aberrant polymers that accumulate in the endoplasmic reticulum (ER), causing low levels of circulating AAT and emphysema.<sup>1</sup> Extracellular polymers are found in the culture medium of cells *in vitro*<sup>2</sup> and in plasma of carriers of the Z allele.<sup>3</sup> These polymers originate in the liver, but it is unknown if they are secreted from hepatocytes or form extracellularly from secreted monomeric Z-AAT. We confirmed that extracellular polymers bear mature N-glycans, so they are not released from the ER after cell death. Adding the polymerisation-blocking monoclonal antibody (mAb) 4B12<sup>4</sup> to the culture medium of Z-AAT expressing cells did not decrease extracellular polymer levels, indicating they were secreted from cells. Polymers have never been shown to colocalise with Golgi-resident proteins. We cultured Z-AAT expressing cells at 20°C for 4 h to reduce protein exit from the Golgi and found the majority of polymers (positive to mAb 2C1<sup>2</sup>) in colocalisation with BiP in the ER, but also in partial colocalisation with giantin and TGN-46 in the Golgi. Finally, we transiently transfected myc- and HA-tagged versions of M- and Z-AAT and performed immunoprecipitation of the culture media with an anti-HA antibody, followed by western blot with an anti-myc antibody. Only cells cotransfected with Z<sub>HA</sub> and Z<sub>myc</sub> showed a band positive to the anti-myc antibody, demonstrating the presence of Z<sub>HA</sub>/Z<sub>myc</sub> heteropolymers in their culture medium. In contrast, cells transfected separately with Z<sub>HA</sub> and Z<sub>myc</sub> that were afterwards cultured together showed no signal with the anti-myc antibody. Our results indicate that polymers formed within the ER can traffic and be secreted *via* the Golgi compartment, supporting a contribution of intracellular origin to plasma polymers in AATD.

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### OXIDATIVE STRESS AND ANTIOXIDANT RESPONSE IN THE FRONTAL CORTEX OF DEMENTED AND NON - DEMENTED INDIVIDUALS WITH ALZHEIMER'S NEUROPATHOLOGY

A. Fracassi,<sup>1,2</sup> S. Moreno,<sup>1</sup> G. Tagliatalata<sup>2</sup>

<sup>1</sup>Department of Science-LIME, University of Rome "Roma Tre", Italy; <sup>2</sup>Mitchell Center for Neurodegenerative Diseases, UTMB, Galveston, TX, USA

E-mail: sandra.moreno@uniroma3.it

Age is a major risk factor for several neurodegenerative disorders, including Alzheimer's disease (AD). This is histopathologically characterized by extracellular senile plaques, composed of amyloid  $\beta$  (A $\beta$ ) peptide, and intracellular neurofibrillary tangles, formed by hyperphosphorylated Tau protein<sup>1</sup>. The widely accepted correlation between A $\beta$  plaques and clinical manifestation has recently been challenged by the emergence of a group of individuals, referred to as "Non-Demented with Alzheimer's Neuropathology" (NDAN), showing severe neuropathological signs, though remaining cognitively intact<sup>2</sup>. The existence of these individuals suggests that some unknown mechanisms are triggered to resist A $\beta$ -mediated neurotoxic effects, preventing cognitive decline. Noteworthy, A $\beta$  accumulation affects mitochondrial redox balance increasing oxidative stress, *i.e.*, a primary culprit in AD pathogenesis<sup>3</sup>. To clarify the relationship linking A $\beta$ , oxidative stress and cognitive decline, we performed a comparative immunofluorescence study on AD, NDAN and normally aged *post-mortem* frontal cortices. To assess the cellular localization of oxidative damage, we evaluated 8-oxo-dG occurrence in neurons and astrocytes. We also investigated the expression of SOD2, PGC1 $\alpha$  and PPAR $\alpha$  as key factors in

antioxidant response and energy metabolism. We could confirm the crucial role of redox imbalance in AD pathogenesis, correlated with an impairment of antioxidant defenses. By contrast, NDAN subjects displayed low oxidative damage and high content of scavenging systems, suggesting the ability to activate an efficient PGC1 $\alpha$ -dependent antioxidant response, preventing A $\beta$ -mediated detrimental effects. Moreover, the analyses conducted in a comparative manner in neurons and astrocytes highlighted cell-specific mechanisms to counteract redox imbalance. These emerging concepts may help envisioning neuroprotective strategies aimed at potentiating antioxidant response in age-related cognitive impairment.

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### MASS CYTOMETRY POTENTIAL TO LOOK INTO SKELETAL MUSCLE PHYSIOLOGY AND PATHOLOGY

C. Fuoco, L.L. Petrilli, F. Spada, A. Reggio, L. Castagnoli, Gargioli, G. Cesareni

*Department of Biology, University of Rome "Tor Vergata", Italy*  
*E-mail: c.fuoco@yahoo.it*

The skeletal muscle has a robust, finely orchestrated and sophisticated capacity to regenerate. The regeneration process is governed by the interplay between different populations of resident mononuclear cells, which directly or indirectly contribute to restoring tissue homeostasis.<sup>1</sup> Pathological conditions, such as muscular dystrophies, ageing or cancer (as rhabdomyosarcoma) lead to an alteration in the regeneration capability which reflects in a variation of the relative abundance and the functional integrity of muscle mononuclear populations. Describing how the profile of mononuclear cell populations changes in the different conditions is essential to elucidate the physiological processes that govern muscle regeneration. In addition this information could help to comprehend how the pathological states originate and evolve in time. We have used mass cytometry, a high content single cell technology to monitor the kinetic of changes of the mononuclear cell populations in the muscle.<sup>2</sup> By this approach, we have confronted muscle from wild type with those of a mouse model of Duchenne muscular dystrophy (*mdx*) which undergoes chronic damage and regeneration cycles. In both systems, we have monitored how the profile of mononuclear cell populations changes with ageing. In addition, in both systems we have also studied the kinetic of regeneration after an acute damage caused by injection of a cardiotoxin (CTX). Finally, we investigate how the mononuclear population profile varies in cancer condition in order to identify the cell type that initiate the tumor. To this end, we have compared the cell populations of healthy muscles with that of a rhabdomyosarcoma, a common type of soft tissue sarcoma in children and adolescents, under 20 years of age.<sup>3</sup> To achieve our objective, we adopt the *Kras*<sup>G12D/+</sup> *Trp53*<sup>F1/F1</sup> 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<sup>4</sup>.

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### MODELING HUMAN INTELLECTUAL DISABILITY AND AUTISM: ROLE OF THE CHROMATIN REGULATOR SETD5 DURING ZEBRAFISH BRAIN DEVELOPMENT

C. Gabellini,<sup>1</sup> D. Martini,<sup>1</sup> C. Pucci,<sup>1,2</sup> C. Di Lauro,<sup>1</sup> W. Norton,<sup>3</sup> A. Zippo,<sup>4</sup> V. Broccoli,<sup>5,6</sup> A. Sessa,<sup>5</sup> M. Andreazzoli<sup>2</sup>

<sup>1</sup>*Department of Biology, University of Pisa, Italy;* <sup>2</sup>*Sant'Anna School of Advanced Studies, Pisa, Italy;* <sup>3</sup>*Department of Neuroscience, Psychology and Behaviour, University of Leicester, UK;* <sup>4</sup>*National Institute of Molecular Genetic;* <sup>5</sup>*Stem Cell and Neurogenesis Unit, Division of Neuroscience, San Raffaele Scientific Institute, Milan, Italy;* <sup>6</sup>*CNR Institute of Neuroscience, Milan, Italy*

*E-mail: chiara.gabellini@biologia.unipi.it*

SETD5 loss-of-function (LoF) mutations in humans have been recently associated to intellectual disability (ID) and autistic spectrum disorders.<sup>1-7</sup> Interestingly, SETD5 gene encodes for a putative histone H3 methyltransferase highly expressed in the brain and it falls within the critical interval deleted in the 3p25.3 microdeletion syndrome,<sup>8</sup> characterized by ID, microcephaly and congenital heart defects. The aim of this study is to generate and characterize zebrafish models in which *setd5* has been knocked down or knocked out. Antisense morpholino oligonucleotides-mediated targeting of *setd5* in zebrafish embryos determined microcephaly, cardiac edema and reduced locomotor behavior. *setd5* knockdown reduces the expression domain of central nervous system specification markers and *setd5* LoF brains show a reduced size compared to embryos injected with a control morpholino. Through bromodeoxyuridine pulse-chase experiment we are currently evaluating if a possible delay in mitosis may be responsible of the increased cell death in developing brain areas of *setd5* morphants. Furthermore, we generated stable *setd5* mutant zebrafish lines through Crispr/Cas9 strategy to analyze the effect of *setd5* gene knockout on the phenotype of zebrafish larvae. These animal models will be extremely useful to identify the molecular mechanisms underlying SETD5 LoF phenotype. The future perspective is to screen for compounds able to rescue the developmental defects, to identify novel promising drugs exerting therapeutic efficacy on individuals affected by autism and intellectual disability due to SETD5 haploinsufficiency.

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### TISSUE REGENERATION IN THE DEMOSPONGE *CHONDROSIA RENIFORMIS*

L. Gallus,<sup>1</sup> M. Pozzolini,<sup>1</sup> S. Ferrando,<sup>1</sup> C. Gagliani,<sup>2</sup> M. Bertolino,<sup>1</sup> M. Giovine,<sup>1</sup> S. Scarfi<sup>1</sup>

<sup>1</sup>*Department of Earth, Environment and Life Sciences, DiSTAV, University of Genoa;* <sup>2</sup>*Department of Experimental Medicine, University of Genoa, Italy*

*E-mail: galluslorenzo@gmail.com*

*Chondrosia reniformis* is a demosponge lacking a typical skeleton of siliceous spicules or spongin fibres. The skeleton is a collagenous scaffold characterized by structural and mechanical adaptability that allows impressive morphological changes interpreted as adaptive strategies related to environmental factors, asexual

reproduction or localized locomotor phenomena. Moreover *C. reniformis* is able to incorporate exogenous silica, selectively dissolving the crystalline forms.<sup>1,2,3</sup> We decided to investigate the morphological aspects of tissue regeneration from mechanical injuries in this sponge with a particular focus on collagen distribution and cell migration in the damaged tissue. We observed the regeneration process by SEM/TEM, light microscopy and epifluorescence. Experiments were performed on cylindrical samples (fragmorphs) obtained from different specimens collected in the Ligurian Sea. The fragmorphs were maintained in the aquarium and sampled at 0, 24, 48 and 72h, fixed in paraformaldehyde (PAF) or glutaraldehyde/PAF and embedded in paraffin or resin. We used the lectin Wheat Germ Agglutinin (WGA) to highlight exopinocytes and spherulose cells (SC). At 24h the surface on the cut side showed exposed collagen, rare cells and vesicles from SC. At 48h the SC were brought to the cut surface, and apparently migrating WGA-positive cells populations were observable. At 72h the surface showed collagen fibers partially covered by an incomplete layer of exopinocytes, bacteria and vesicles from the SC. Concluding, the reconstitution of the exopinocyte layer is ongoing and not completed at 72h. This data are in agreement to our preliminary results regarding the expression of genes involved in the tissue regeneration and collagen synthesis. We will extend the morphological observations over the 72h, improving the identification of the cells observed, and test inhibitors of the signal molecules involved in the regeneration process.

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#### A NEW ISSUE OF CRYPTIC SPECIES IN A MODEL ASCIDIAN: DEVELOPMENTAL STAGES AND DNA BARCODE FOR *BOTRYLLUS SCHLOSSERI* CLADE A

F. Gasparini,<sup>1</sup> C. Gissi,<sup>2</sup> F. Mastrototaro,<sup>3</sup> R. Brunetti,<sup>4</sup> L. Manni<sup>1</sup>  
<sup>1</sup>Department of Biology, University of Padua; <sup>2</sup>Department of Biosciences, Biotechnology and Biopharmaceutics, University of Bari "A. Moro"; <sup>3</sup>Department of Biology, University of Bari "A. Moro"; <sup>4</sup>Natural History Museum of Venice, Italy  
 E-mail: fabio.gasparini@unipd.it

*Botryllus schlosseri* is a colonial ascidian considered cosmopolitan and amply used as model for the study of sexual and asexual reproduction, developmental biology and immunobiology.<sup>1,2</sup> Recently, molecular data lead to hypothesize that the species named *B. schlosseri* may consist of more than a single taxon, each one genetically identified as a clade (namely, clades A-E) and referred as cryptic species.<sup>3</sup> In this context, and lacking both a type and a detailed morphological description, we believe that it was necessary, as taxonomic reference point, to designate a neotype and re-describe the species. Therefore, samples from the Lagoon of Venice (Adriatic Sea, Italy) have been described. The provided morphological description takes into account several developmental stages (oozoid, zooid of first asexual generations, and mature zooid) and was carefully compared with the previous descriptions of samples coming from other localities. We associated our sample description to a "DNA barcode", consisting of a long fragment of the mitochondrial COI gene, which resulted associated to clade A. We believe that the present study represents a prerequisite to investigate the existence of morphological and developmental differences between the other "cryptic species" hidden under the name *B. schlosseri*. This will clarify if the research community is running into a second case of nomenclature division model organism, similar to the recently one occurred in the solitary ascidian *Ciona intestinalis*.<sup>4</sup> Indeed,

in the latter case, the presence of a number of public databases with -omics information involved developmental biologists, bioinformatics, bio-curators and ecologists in a revision of their data, according to the new taxonomic status of their model species. *B. schlosseri* could represent a new challenge.

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#### NEUROSERPIN POLYMERS CAUSE OXIDATIVE STRESS IN A NEURONAL MODEL OF DEMENTIA FENIB

N.A. Guadagno,<sup>1#</sup> C. Moriconi,<sup>1</sup> V. Licursi,<sup>1</sup> E. D'Acunto,<sup>1</sup> P.S. Nisi,<sup>1</sup> E. Cacci,<sup>1</sup> A. De Jaco,<sup>1</sup> R. Negri,<sup>1</sup> G. Lupo,<sup>2</sup> E. Miranda<sup>1</sup>

<sup>1</sup>Department of Biology and Biotechnologies "Charles Darwin", Sapienza University of Rome; <sup>2</sup>Department of Chemistry, Sapienza University of Rome, Italy

<sup>#</sup>Current address: Department Biosciences, Centre for Immune Regulation, University of Oslo, Norway

E-mail: mariaelena.mirandabanos@uniroma1.it

The serpinopathies are human pathologies caused by mutations that promote polymerisation and intracellular deposition of proteins of the serpin superfamily, leading to a poorly understood cell toxicity.<sup>1</sup> The dementia FENIB is caused by polymerisation of the neuronal serpin neuroserpin (NS) within the endoplasmic reticulum (ER) of neurons.<sup>2,3</sup> We have generated transgenic neural progenitor cell (NPC) cultures from mouse foetal cerebral cortex, stably expressing the control protein GFP (green fluorescent protein), or human wild type, G392E or delta NS.<sup>4</sup> We have characterised these cell lines in the proliferative state and after differentiation to neurons. Our results show that wild type NS was correctly secreted into the culture medium but G392E NS formed polymers that were mostly retained within the ER. DeltaNS was absent at steady state due to rapid degradation, but it was detected upon proteasomal block. Looking at their intracellular distribution, wild type NS was found in partial co-localisation with ER and Golgi markers, while G392E NS was localised with the ER only. Furthermore, polymers of NS were detected in G392NS cells by ELISA and immunofluorescence. We used control GFP and G392E NPCs differentiated to neurons to investigate which cellular pathways were modulated by intracellular polymers by performing RNA sequencing. We identified 747 genes with significant up- (623) or downregulation (124) in G392E NS-expressing cells, and focused our attention on several genes involved in the anti-oxidant defence that were upregulated in cells expressing G392E NS (*Aldh1*, *ApoE*, *Gpx1*, *Gstm1*, *Prdx6*, *Scara3*, *Sod2*). Inhibition of intracellular antioxidants by specific pharmacological reagents uncovered the damaging effects of NS polymers. Our results support a role for oxidative stress in the cellular toxicity underlying the neurodegenerative dementia FENIB.

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## ALS-ASSOCIATED FUS MUTATION ALTERS MOTONEURON RNA EXPRESSION AND CIRCUITS

P. Laneve,<sup>1</sup> D. Capauto,<sup>1</sup> S. Biscarini,<sup>1</sup> L. Lu,<sup>2</sup> A. Colantoni,<sup>3</sup> G. Peruzzi,<sup>1</sup> T. Santini,<sup>1</sup> B. Blanco Redondo,<sup>2</sup> E. Caffarelli,<sup>4</sup> N.A. Shneider,<sup>2</sup> I. Bozzoni<sup>3</sup>

<sup>1</sup>Italian Institute of Technology, Center for Life Nano Science, Rome, Italy; <sup>2</sup>Department of Neurology, Center for Motor Neuron Biology and Disease, Columbia University, New York City, NY, USA; <sup>3</sup>Department of Biology and Biotechnology, Sapienza University of Rome, Italy; <sup>4</sup>Institute of Molecular Biology and Pathology, CNR, Rome, Italy

Amyotrophic Lateral Sclerosis (ALS) is a neurological disease characterized by degeneration of upper and lower motoneurons (MNs), leading to progressive muscle atrophy and death.<sup>1</sup> Several mutations have been associated with familiar ALS, a fraction of which targets RNA binding proteins with multiple functions in RNA metabolism, like as FUS/TLS.<sup>2,3</sup> The pleiotropic role played by FUS on RNA drives the speculation that aberrant RNA pathways might underlie crucial mechanisms of neurodegeneration in ALS, and raises the fascinating hypothesis of ALS as an RNA disorder. To get novel insights into the molecular implications of FUS-dependent neurotoxicity in the cell type specifically targeted in ALS, we *in vitro* differentiated HB9::GFP mouse Embryonic Stem Cells (mESCs)<sup>4</sup> into wild type or mutant MNs reproducing the aggressive human FUS mutation P525L (FUSP517L in mice). GFP(+) MNs were then purified and RNA was sequenced for global analysis of long and short transcripts. By combining the potential of stem cell-based cellular biology with high-throughput screenings and integrative molecular analyses, we are investigating from innovative perspectives the gene pathways orchestrated by FUS in healthy or affected MNs. Changes in mRNA and microRNA expression profiles suggest how specific deregulated genes impinge on ALS pathogenesis, whereas following mechanistic characterizations identify novel FUS-dependent RNA circuits in MNs. Revealing altered expression of long non-coding and circular RNAs,<sup>5</sup> both emerging as essential players in nervous system physiopathology, is propaedeutic to on-going functional studies aimed to shed light on yet unexplored noncoding sides of neurodegeneration.

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## NON-NEUROGENIC STRUCTURAL PLASTICITY REVEALED BY DOUBLECORTIN IN MAMMALS: IS THERE A TREND?

C. La Rosa,<sup>1,2</sup> A. Pecora,<sup>1</sup> I. Amrein,<sup>3</sup> L. Bonfanti<sup>1,2</sup>

<sup>1</sup>Neuroscience Institute Cavalieri Ottolenghi (NICO), Orbassano, TO, Italy; <sup>2</sup>Department of Veterinary Sciences, University of Turin, Italy; <sup>3</sup>Division of Functional Neuroanatomy, Institute of Anatomy, University of Zürich, Switzerland.

E-mail: chiara.larosa@unito.it

Brain structural plasticity, including neurogenic and non-neurogenic processes, is an exception to the genetically-determined structure of the nervous system. It is essential for adaptation to the changing environment and potentially useful for brain repair. New neurons functionally integrate in the neural circuits through adult neurogenesis coming from stem cell niches. Yet, such process decreases in large-brained, long-living species (e.g., dolphins, humans;<sup>1,2</sup>) and does not provide reparative out-

comes. Other brain regions host non-newly generated, immature cells,<sup>3</sup> which express Doublecortin (DCX; a cytoskeletal protein used as a marker of structural plasticity) and appear to vary in mammals.<sup>4</sup> Current knowledge about these cells is largely incomplete, most studies having been conducted in small-brained, short-living species (laboratory rodents), hosting these cells only in the paleocortex. A hypothesis has been advanced that non-neurogenic plastic events increase their importance in large-brained, long-living mammals.<sup>5</sup> The aim of this study is to systematically assess the occurrence and features of such "parenchymal plasticity" in 14 mammalian species, belonging to the orders carnivore, primates, chiroptera, macroscelidae, lagomorpha, perissodactyla and artiodactyla, which are endowed with different neuroanatomy, brain size, lifespan, ecological niche. Preliminary results indicate remarkable presence of DCX+ cell populations in all species analyzed with respect to laboratory rodents. Extra-cortical regions (white matter and pericapsular structures) are also involved. Carnivora host DCX+ cells in more brain areas with respect to other species. These cells can have different morphologies (e.g., bipolar, multipolar) and spatial organization (e.g., isolated cells, clusters). The linear density of DCX+ cells in the cortical layer II was measured: preliminary data indicate that such density highly vary, with a 30-fold range from bats to cats. The proliferation of DCX+ cells was evaluated using Ki-67 antigen and never found to occur. Hence, non-neurogenic structural plasticity appears to be highly heterogeneous in mammals, in terms of neuroanatomical location, cell morphology and spatial organization. Further analyses might clarify the possible link between structural plasticity and species-specific factors (neuroanatomy, lifespan, ecological niche, food habits), as well as the possible occurrence (or not) of a phylogenetic trend.

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## DOSE-DEPENDENT RESPONSE OF NEURAL STEM/PROGENITOR CELLS TO IONIZING RADIATION

V. Licursi,<sup>1</sup> S. Anzellotti,<sup>1</sup> J. Favaro,<sup>1</sup> N. Carucci,<sup>1</sup> E. Cacci,<sup>1</sup> E. Cundari,<sup>2</sup> M. Fiore,<sup>2</sup> P.S. Nisi,<sup>1</sup> R. Amendola,<sup>3</sup> F. Verni,<sup>1</sup> S. Biagioni,<sup>1</sup> G. Lupo,<sup>4</sup> R. Negri<sup>1</sup>

<sup>1</sup>Department of Biology and Biotechnology "C. Darwin", Sapienza University of Rome; <sup>2</sup>Institute of Molecular Biology and Pathology, CNR National Research Council, Rome; <sup>3</sup>ENEA, SSPT, TECS, Rome; <sup>4</sup>Department of Chemistry, Sapienza University of Rome, Italy

During mammalian neural development, neuronal and glial cell types of the central nervous system are generated by neural stem/progenitor cells (NSPCs) that are found in the ventricular and subventricular zones of the neural tube. The ability of NSPCs to self-renew and generate new neurons is known to be affected by a variety of intrinsic and extrinsic factors, such as ageing, neurodegenerative diseases and environmental agents, leading to decreased neurogenesis and potentially contributing to cognitive decline. Ionizing Radiation (IR) is a genotoxic agent that has been shown to deplete the NSPC pool and impair neurogenesis, but may also cause oncogenic transformation of irradiated NSPCs. Under appropriate conditions IR can also be employed in a clinical setting for cancer radiotherapy. Therefore, the effects of IR on NSPCs need to be thoroughly investigated. We have studied the response of mouse NSPCs to low (0.2 Gy), moderate (1 Gy) and high (10 Gy) doses of X-rays IR at differ-

ent time points after treatment (4, 24 and 48 hours (h) or 8 days (d)). During the first 24-48 h post-irradiation, we found that 10 Gy IR caused considerable NSPCs mortality, although roughly 70% of irradiated cells were still viable at these time points. Furthermore, we detected a marked delay of cell cycle progression and significant upregulation of cell cycle inhibitors, apoptotic markers and glial differentiation markers in irradiated cells. Surprisingly, when 10 Gy IR-treated NSPCs were cultured for 8d, these effects were largely recovered, leading to expansion of cultures with similar viability, cell cycle rate and gene expression profiles to untreated cultures. NSPCs treated with lower IR doses showed lesser effects that appeared to be recovered by 24h post-irradiation. These results suggest that NSPCs are resistant to IR-dependent toxicity, since they can continue to grow in spite of exposure to IR levels known to be generally lethal for mammalian cells. This raises the question of whether NSPCs are capable of fully repairing IR-dependent DNA damage, retaining features of untransformed NSPCs, or whether radiation-induced genomic instability may instead lead to NSPC conversion into cancer-like stem cells and to expansion of transformed cultures. Further analysis of DNA damage and repair in irradiated NSPCs are underway to address this key issue.

### EPIGENOMIC PROFILING OF AGED MOUSE NEURAL STEM/PROGENITOR CELLS IDENTIFIES DBX2 AS A CANDIDATE REGULATOR OF AGE-ASSOCIATED NEUROGENIC DECLINE

G. Lupo,<sup>1</sup> P.S. Nisi,<sup>2</sup> P. Esteve,<sup>3</sup> Y.-L. Paul,<sup>4</sup> S. Biagioni,<sup>2</sup> P. Bovolenta,<sup>3</sup> E. Cacci,<sup>2</sup> P. J. Rugg-Gunn<sup>4</sup>

<sup>1</sup>Department of Chemistry, Sapienza University of Rome, Italy; <sup>2</sup>Department of Biology and Biotechnology "C. Darwin", Sapienza University of Rome, Italy; <sup>3</sup>Centro de Biología Molecular "Severo Ochoa", Consejo Superior de Investigaciones Científicas-Universidad Autónoma de Madrid, Spain; <sup>4</sup>Epigenetics Programme, The Babraham Institute, Cambridge, UK

Adult neurogenesis declines with ageing, but the underlying molecular mechanisms are poorly understood. To investigate the intrinsic molecular changes that occur upon neural stem/progenitor cell (NSPC) ageing, we compared the transcriptional, histone methylation and DNA methylation signatures of NSPCs derived from the subventricular zone of young adult (3 months old) and aged (18 months old) mice. Surprisingly, the genome-wide transcriptional and epigenetic profiles of SVZ-derived NSPCs were largely unchanged in aged cells. Despite the global similarities, we identified robust age-dependent changes at several genes and their regulatory elements. Among them, the homeobox gene *Dbx2* was upregulated, and its promoter region was DNA hypomethylated, in aged NSPCs. Using functional *in vitro* assays, we show that elevated *Dbx2* expression in adult NSPCs promotes age-related phenotypes, including the reduced growth of NSPC-derived neurospheres and the altered expression levels of genes implicated in NSPC self-renewal and differentiation. Taken together, these results provide new insights into the molecular programmes that are altered on NSPC ageing, and uncover a new functional role for *Dbx2* in promoting age-associated neurogenic decline.

### A COMPARATIVE STUDY ON THE EFFECTS OF IMIDACLOPRID IN ZEBRAFISH AND JAPANESE MEDAKA

M. Maisano,<sup>1</sup> T. Cappello,<sup>1</sup> C. Vignet,<sup>2</sup> K. Lajoie,<sup>3</sup> Q. Fu,<sup>4</sup> G. De Marco,<sup>1</sup> H. Mottaz,<sup>2</sup> C. Clérandeau,<sup>3</sup> J. Hollender,<sup>4</sup> J. Cachot,<sup>3</sup> K. Schirmer<sup>2,5,6</sup>

<sup>1</sup>Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Italy;

<sup>2</sup>Department of Environmental Toxicology, and <sup>4</sup>Department of Environmental Chemistry, Eawag, Dübendorf, Switzerland;

<sup>3</sup>EPOC, UMR CNRS, University of Bordeaux, Pessac, France;

<sup>5</sup>Department of Environmental Systems Science, ETH Zürich, Switzerland; <sup>6</sup>School of Architecture, Civil and Environmental Engineering, EPF Lausanne, Switzerland

E-mail: maria.maisano@unime.it

Neonicotinoids are the most widely used class of insecticides, frequently found in freshwater at levels up to µg/L. Despite the impact of these compounds on non-target invertebrates is well-documented, the potential toxicity of neonicotinoids on fish still needs to be clarified. To this aim, the effects of imidacloprid, one of the most used neonicotinoids, were investigated focusing on the development of two common fish model species: zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*). Fish were exposed for 5 (zebrafish) and 14 (medaka) days to 0, 0.2, 2, 20, 200 and 2000 µg/L imidacloprid by aqueous exposure, matching the respective time from fertilization to emerged larvae for the two species. At similar developmental stage, after evaluating the uptake of imidacloprid, survival, hatch, growth, morphology, and histology were examined. No impact on survival was found. A delay for 2 µg/L imidacloprid exposures was observed for hatch in both zebrafish and Japanese medaka. A striking difference was found in morphology, as well as in histology, since a high number of alterations were noticed for medaka whereas almost none were found in zebrafish. Specifically, yolk and bone oedemas started to be noticed at 20 µg/L after hatch, jaw deformity and lordosis/scoliosis increased with concentration level, and an increase of haemorrhage was found at the highest concentration (2000 µg/L). Also at histological level, changes in eye and muscle structure were seen at the higher imidacloprid doses. Therefore, a metabolomics investigation was applied on medaka, revealing alteration in energy metabolism and neurotransmission. Overall, these findings demonstrate the toxic potential of imidacloprid, which differentially interferes with the development of the two tested fish model species.

### THE COMPLETE ANNOTATION OF *CIONA* DEVELOPMENT AND ANATOMY: THE LARVAL AND METAMORPHOSIS STAGES

L. Manni,<sup>1</sup> D. Dauga,<sup>2</sup> K. Hotta<sup>3</sup>

<sup>1</sup>Department of Biology, University of Padua, Italy; <sup>2</sup>Bioself Communication, Marseille, France; <sup>3</sup>Department of Biosciences and Informatics, Faculty of Science and Technology, Keio University, Yokohama, Japan

E-mail: lucia.manni@unipd.it, contact@bioself-communication.com, khotta@bio.keio.ac.jp

The use of model species in any field of Biology requires the management of huge amount of information of different kind (molecular, anatomical, evolutionary, etc.). Public scientific databases represent indispensable tools to manage and relate each other information. A specific way to correlate anatomical and developmental data is represented by ontologies. An ontology is a defined vocabulary related to a model species, listing a number of anatomical entities in relationship to developmental stages. Ontologies are very important, since they facilitate data standardization, sharing of both data and protocols among different laboratories, compar-

isons between different kinds of data. Among ascidians, *Ciona* is a model in a number of biological fields, such as Evo-Devo, genomics and proteomics. For this taxon, the embryonic development has been described in high detail and the ontology covers all stages of embryogenesis according to cell lineage. However, no standardized ontology of post hatching larva, including stages of swimming larva, metamorphosis and early juvenile, is available. In this study, we present a new ontology, covering the following 12 developmental stages: Larva, subdivided in 4 stages (Hatching Larva, Early-, Mid- and Late-swimming Larva); Adhesion; Tail Absorption, subdivided in 3 stages (Early-, Mid- and Late-Tail Absorption); Body Axes Rotation, subdivided in 3 stages (Early-, Mid- and Late-Body Axes Rotation), and Juvenile (in particular, the Early Juvenile I stage). These stages are all easily recognizable at stereomicroscope, analyzing *in vivo* animals or fixed whole mount specimens. Then, combining time-lapse movies, 3D confocal microscopy and complete series of histological sections, we annotated more than 200 anatomical entities. For each of them, we determined the start stage (also referring to the existing ontology of embryogenesis), the end stage, and the structures from which they develop. We manually annotated a definition for each anatomical entity and the possible synonyms. Moreover, we associated each entity to bibliographic references, considering both classical anatomical texts and the more updated and technologically advanced publications. These data will be interactively manageable as TunicAnatO (Tunicate Anatomical and developmental Ontology) database. We think that this ontology will represent an important tool for sharing results and experimental protocols within the scientific community.

#### CELLULAR AND MOLECULAR MECHANISMS ACTIVATED IN SEA URCHIN EMBRYOS EXPOSED TO GADOLINIUM

C. Martino,<sup>1,2</sup> R. Chiarelli,<sup>1</sup> C. Costa,<sup>2</sup> D. Koop,<sup>3</sup> R. Scudiero,<sup>4</sup> M. Byrne,<sup>3</sup> V. Matranga,<sup>2</sup> M.C. Roccheri<sup>1</sup>

<sup>1</sup>Department STEBICEF, University of Palermo, Italy; <sup>2</sup>CNR, IBIM, Palermo, Italy; <sup>3</sup>Department of Anatomy and Histology, University of Sydney, Australia; <sup>4</sup>Department of Biology, University of Naples Federico II, Italy

E-mail: chiara.martino@unipa.it

Gadolinium (Gd) concentration is constantly increasing in the aquatic environment, becoming an emergent environmental pollutant. We investigated the consequences of sea urchin embryo exposure to sublethal Gd concentrations, comparing the effects 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 rogersii*. Measures of the Gd and Ca content inside embryos by ICP-MS showed a time- and dose-dependent Gd increase, in parallel with a reduction of the Ca amount, suggesting that Gd is able to compete 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 48h, with species-specific threshold levels of sensitivity. Skeleton growth was found partially resumed in recovery experiments. The mesodermal cells designated to biomineralization were found correctly migrated at 24 hpf, but not at 48 hpf. Western blot analysis showed an increase of the LC3-II autophagic marker at 24 and 48 hpf. Confocal microscopy studies confirmed the increased number of autophagolysosomes and autophagosomes. RT-PCR gene expression analysis showed the misregulation of several genes implicated both in the skeletogenic and the left-right axis specification networks, including: transcription factors, signaling molecules and skeletal matrix proteins. Preliminary experiments showed that the stunting effect of Gd exposure was partially counterbalanced by a modest temperature increase (+3°C), while the effect of a major

increase (+6°C) was synergic. Taken together, the results pose serious questions on the hazard of Gd in the marine environment and indicate that Gd is able to affect three different levels of the stress response in sea urchin embryos: morphogenesis, survival strategies such as autophagy, and gene expression.

#### THE INDUCTION OF THE Nrf2/ARE PATHWAY THROUGH SPERMINE OXIDASE ACTIVATION ELICITED BY THE NMDA RECEPTOR IN HUMAN NEURONAL CELLS

R. Mastrantonio,<sup>1</sup> M. Cervelli,<sup>2</sup> S. Pietropaoli,<sup>2</sup> V. D'Ezio,<sup>1</sup> P. Mariottini,<sup>2</sup> M. Colasanti,<sup>1</sup> T. Persichini<sup>1</sup>

<sup>1</sup>Laboratory of Cell Biology, University "Roma Tre", Rome;

<sup>2</sup>Laboratory of Molecular Biology, Department of Science, University "Roma Tre", Rome, Italy

E-mail: tiziana.persichini@uniroma3.it

Oxidative stress refers to elevated intracellular levels of reactive oxygen species (ROS), as well as reactive nitrogen species (RNS), that can cause neurotoxicity. Chronic oxidative stress is involved in HIV-associated dementia and can be induced by HIV-1-secreted proteins. However, ROS and RNS can trigger an antioxidant cell response through the transcriptional induction of oxidative stress-responsive genes regulated by Nrf2. Here, we report the role of polyamine catabolism in the antioxidant response of HIV-1 Tat-induced human neuronal (SH-SY5Y) cells. In particular, we have demonstrated that the treatment of SH-SY5Y cells with Tat induces Nrf2 activation and the subsequent antioxidant gene expression (catalase, heme oxygenase-1, NADPH:quinone oxidoreductase, superoxide dismutase isoforms 1 and 2). This up-regulation is mediated by the enzyme spermine oxidase (SMO), an effect being reversed by chlorhexidine (CHL), a competitive SMO inhibitor.<sup>1</sup> Furthermore, we have observed that Nrf2 activation is mediated by the NMDA receptor (NMDAR), as demonstrated by using the antagonist MK801. These findings suggest that the NMDAR/SMO/Nrf2 pathway can represent an important target for protection against HIV-associated neurotoxicity.<sup>2</sup> A goal of future research may be to spatially and temporally modulate the molecular pathways involved in the potentiation of the antioxidant response versus oxidative stress.

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#### THE Ambra1a, Ambra1b AND Epg5 KNOCK OUT LINES: THREE ZEBRAFISH MODELS TO STUDY AUTOPHAGY

G. Meneghetti,<sup>1</sup> T. Skobo,<sup>1</sup> N. Facchinello,<sup>1</sup> C. Fontana,<sup>1</sup> M. Chrisam,<sup>2</sup> F. Cecconi,<sup>3</sup> P. Sabatelli,<sup>4</sup> P. Bonaldo,<sup>2</sup> L. Dalla Valle<sup>1</sup>

<sup>1</sup>Department of Biology, University of Padua; <sup>2</sup>Department of Molecular Medicine, University of Padua; <sup>3</sup>Department of Biology, University of Rome "Tor Vergata"; <sup>4</sup>Institute of Molecular Genetics, National Research Council of Italy, Bologna, Italy

E-mail luisa.dallavalle@unipd.it

Ambra1 (Ambra1a and 1b in zebrafish) and Epg5 are two proteins involved in autophagy: Ambra1 as a positive regulator of autophagy induction and Epg5 as a Rab7 effector required for fusion of autophagosomes with late endosomes and lysosomes. To study their functions, we generated, by means of CRISPR/Cas9 technology, the corresponding zebrafish mutant lines bearing premature stop codons. *epg5*<sup>-/-</sup> mutants develop

normally and reach sexual maturity without any overt phenotype. In agreement with Epg5 roles on autophagy, 20-dpf *epg5*<sup>-/-</sup> mutants display a higher number of Lc3:RFP dots and western blot analysis shows a higher level of Lc3-II under both fed and fasted conditions, together with a significant reduction of birefringence in skeletal muscles. Ultrastructural analysis shows a higher accumulation of degradation-defective autolysosomes in muscles of starved *epg5*<sup>-/-</sup> larvae. The number of mucus-secreting goblet cells is statistically reduced in 5-dpf *epg5*<sup>-/-</sup> mutants, suggesting a role of Epg5 in intestine physiology. Interestingly, morphology of adult *epg5*<sup>-/-</sup> mutant intestine is severely affected. Caudal fin regeneration, a process that requires autophagy, is reduced in *epg5*<sup>-/-</sup> but not in *ambra1a*<sup>-/-</sup> and *ambra1b*<sup>-/-</sup> mutants. At difference from results obtained with the corresponding ATG-morpholinos, *ambra1a*<sup>-/-</sup> and *ambra1b*<sup>-/-</sup> mutant embryos do not display developmental defects. Up-regulation of *ambra1b* transcripts in *ambra1a*<sup>-/-</sup> mutants point at a potential compensatory effect between the two paralogous genes. Moreover, *ambra1a*<sup>-/-</sup> mutants are weakly sensitive to *ambra1a*-MO but strongly sensitive to *ambra1b*-MO knockdown. In agreement with the high levels of *ambra1b* expression in the zebrafish ovary, no female was found among *ambra1b*<sup>-/-</sup> adults, suggesting an involvement of Ambra1b in ovary development. In conclusion, the three zebrafish mutant lines we generated are valuable models to study autophagy and for the functional dissection of the specific functions of the two zebrafish *ambra1* paralogous genes.

#### **IN VITRO EMBRYO EXPOSURE TO RETINOIC ACID, VALPROIC ACID, ETHANOL AND TO THE ANTIFUNGAL PROCHLORAZ ALONE OR IN MIXTURE: EFFECTS ON CRANIO-FACIAL MORPHOGENESIS**

E. Menegola,<sup>1</sup> R. Bacchetta,<sup>2</sup> M. Battistoni,<sup>1</sup> F. Di Renzo,<sup>2</sup> F. Metruccio,<sup>4</sup> A. Moretto<sup>3,4</sup>

<sup>1</sup>Department of Environmental Science and Policy, University of Milan; <sup>2</sup>Department of Biosciences, University of Milan; <sup>3</sup>Department of Biomedical and Clinical Sciences University of Milan; <sup>4</sup>ICPS, ASST Fatebenefratelli Sacco, Milan, Italy  
E-mail: elena.menegola@unimi.it

Among human abnormalities, oral cleft (cleft lip and/or palate alone or associated with other cranio-facial deformities) are one of the most frequent (1:700 live births) both as isolated anomalies and in syndromic conditions.<sup>1</sup> Cranio-facial defects show increasing interest because of their demonstrated multifactorial origin, involving both genetic and environmental risk factors. Environmental agents inducing stage-dependent cranio-facial malformations in experimental models include drugs (specially isotretinoine and antiepileptics) and pesticides (specially azole fungicides), alcohol consumption and hypervitaminosis A.<sup>1</sup> We compared, by using the postimplantation rat whole embryo culture method (WEC), the effects of retinoic acid (RA), ethanol (Eth), the antiepileptic drug sodium valproate (VPA), the azole antifungal prochloraz (PCZ) alone or in mixture. In the case of a possible multiple aetiology of an adverse outcome, in fact, the effects of a multiple exposure to different factors should be taken into account.<sup>2</sup> Embryos were exposed to the selected molecules during the whole culture period and then morphologically examined or processed for the immunolocalization of proteins involved in early cranio-facial morphogenesis. In addition, some embryos were co-exposed to the lowest tested concentration levels of RA in combination with Eth or VPA or PCZ. All the tested molecules induced defects of the cranio-facial structures similar to those induced by RA. Mixture evaluation showed a possible synergistic effect. This suggests that the tested molecules

act on the same developmental pathway (RA-related morphogenetic events).

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#### **AUTOSOMAL RECESSIVE RETINITIS PIGMENTOSA: TARGETING IMPG2**

A. Messina,<sup>1</sup> M.E. Castellini,<sup>1</sup> A. Speccher,<sup>1</sup> X. Zhang,<sup>1</sup> S. Casarosa<sup>1,2</sup>

<sup>1</sup>Neural Development and Regeneration Laboratory, Centre for Integrative Biology (CIBIO), University of Trento; <sup>2</sup>CNR Neuroscience Institute, National Research Council (CNR), Pisa, Italy

E-mail: andrea.messina@unitn.it

The InterPhotoreceptor Matrix (IPM) consists of the extracellular matrix localized between photoreceptors outer segments and retinal pigmented epithelium (RPE). It is thought to play a role in the communication of photoreceptors with the RPE, and as such, in their function. The IPM consists of proteins (opsin, the alpha-subunit of transducin, interphotoreceptor retinoid-binding protein, and others), carbohydrates and proteoglycans. This composition may suggest multiple functions, such as 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 due to IMPG2, a proteoglycan unique 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 protein. 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, the possible role of IMPG2 in vertebrate retinal development and function, and how a truncated IMPG2 could lead to RP degeneration is not yet known. Zebrafish genetically modified lines represent a powerful tool to study human neurodegenerative diseases and to identify candidates for therapeutic approaches. We have started to study IMPG2 during zebrafish retinal development and to generate zebrafish knockout lines by CRISPR/Cas9 genome editing. We plan to introduce the human mutations in zebrafish IMPG2, to truncate IMPG2 mimicking the alteration found in patients. This new animal model will allow us to study the cellular and molecular biology of IMPG2-related retinopathies. With this animal model, it will also be possible to perform large-scale testing of new therapeutic compounds.

#### **EFFECTS OF BISPHENOL A ON EARLY DEVELOPMENT OF TWO ASCIDIAN SPECIES**

S. Messinetti, S. Mercurio, G. Linguanti, F. De Bernardi, R. Pennati

Dipartimento di Scienze e Politiche Ambientali (DISPA), Università degli Studi di Milano, Italy

E-mail: silvia.messinetti@unimi.it

Bisphenol A (BPA) is an organic compound present in plastic products that is released into the environment after degradation. BPA is both a teratogenic substance and an endocrine disruptor.<sup>1</sup> The phylogenetic position of tunicates as sister group of vertebrates and their cosmopolitan distribution in marine ecosystems make them reliable model organisms for ecotoxicol-

ogy bioassays.<sup>2</sup>

We tested the effects of different concentration of BPA (0.1, 0.5, 1, 5, 10, and 20  $\mu\text{M}$ ) on sperm viability, fertilization and embryogenesis of two ascidian species, *Phallusia mammillata* and *Ciona intestinalis*. We evaluated the type and the incidence of induced malformations. Then we focused on the effects on the nervous system performing an immunostaining of central nervous system (CNS) and whole mount *in situ* hybridization (WISH) with neural specific markers. Exposure of sperm to BPA did not influence fertilization rate. Co-exposure of eggs and sperm to concentrations higher than 5 $\mu\text{M}$  caused incomplete division of zygote that did not develop further. In *P. mammillata*, embryonic development was altered by 20 $\mu\text{M}$  BPA causing a severe phenotype with malformed sensory organs in almost all treated larvae. In *C. intestinalis* 20  $\mu\text{M}$  BPA was lethal, while 10  $\mu\text{M}$  concentration caused alteration to the sensory organs, indicating that *C. intestinalis* is less tolerant to BPA. Nervous system is a target of BPA action that caused an altered pattern of neural fibers. WISH with Ci-GAD and Ci-TH showed an alteration of dopaminergic and GABAergic cells after exposure to 10  $\mu\text{M}$  BPA. These results showed that the most sensitive process to BPA is the first cell division. After 2-cell stage, higher concentrations are required to alter the development. BPA confirmed its teratogenic effects on ascidians<sup>3,4</sup> and its interference with CNS development.

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### ROLE OF INNER MEMBRANE POTENTIAL IN TARGETING MITOCHONDRIA TO PRIMORDIAL GERM CELLS

L. Milani, V. Franceschini

Department of Biological, Geological and Environmental Sciences (BiGeA), University of Bologna, Italy

E-mail: liliانا.milani@unibo.it

Which mitochondria are transmitted to progeny? A subset of inactivated mitochondria, or the most active ones? The need for a functional mitochondrial genome, protected from oxidative damage induced by mitochondrial activity, is at the base of the "inactive theory".<sup>1,2</sup> By contrast, for the 'active theory' mitochondrial activity is an indication of correct functioning, and it may be the phenotype under selection for the inheritance mechanism.<sup>3,4</sup> Inherited mitochondria appear to be a specific organelle subset. Mitochondria of the Balbiani body (Bb), a distinctive structure found in oocytes of many organisms, are taken up by embryo primordial germ cells (PGCs) together with germ line determinants.<sup>5</sup> The selective accumulation in the Bb of mitochondria with high inner membrane potential ( $\Delta\psi\text{m}$ ) is consistent with the active theory.<sup>6</sup> Mitochondrial trafficking is tightly associated with the ability of mitochondria to produce ATP,<sup>7</sup> so mitochondria with high  $\Delta\psi\text{m}$ , indicating mtDNA integrity, would have a better chance to attach to microtubules, be recruited into the Bb, and be preferentially carried into PGCs.<sup>4</sup> Moreover, it appears that the timing of germ line specification can influence this process. In animals with an early mechanism of germ line specification, the segregation of the most active mitochondria in gonadic presumptive blastomeres has to take place contextually, in some cases allowing sperm mitochondria segregation into PGCs.<sup>4</sup> In animals in which germ line specification is driven by inductive signals at a later developmental stage, if sperm mitochondria are not degraded they are segregated early into specific blastomeres, preventing their spread in the embryo.<sup>4</sup> In sum-

mary,  $\Delta\psi\text{m}$  may allow the most active mitochondria to reach specific locations, but the different timing of germ line specification can influence the outcome of the segregation mechanism.

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### ALTERATIONS OF VISUAL STRUCTURES IN ARTEMIA SALINA: A NEW BIOMARKER FOR ENVIRONMENTAL CONTAMINATION

C.M. Motta,<sup>1</sup> M. Tizzano,<sup>1</sup> R. Panzuto,<sup>1</sup> P. Simoniello,<sup>2</sup> L. Rosati,<sup>1</sup> R. Di Maro,<sup>1</sup> V. Marchesano,<sup>3</sup> B. Avallone<sup>1</sup>

<sup>1</sup>Dipartimento di Biologia, Università degli Studi di Napoli "Federico II", Naples; <sup>2</sup>Dipartimento di Scienze e Tecnologie, Università di Napoli Parthenope, Naples; <sup>3</sup>TIFPA - Infn Trento Institute for Fundamental Physics and Applications, Italy

Contamination of coastal marine areas is a matter of major concern: many big cities are located along flat shores or protected bays where contaminants accumulate, in sediments and in the water column. Therefore, it has become essential to know all the possible targets of the different xenobiotic so to assess associated environmental risk. From this point of view, the larval stages of marine organisms represent very useful model organisms to test toxicity responses. Among those, *Artemia salina* nauplii are a perfect choice since easy to breed and observe and their widespread use in lab toxicity tests is a confirm. Mortality and mobility are the two common end-point used; however, others might be equally cheap, sensitive and simple. For this reason, in this study we focus on the possibility of introducing pigmented naupliar and paired eyes as a new end-point and tested this hypothesis by investigating responses to two heavy metals (nickel and cadmium) and two antibiotics (amoxicillin and gentamicin). Results indicate that naupliar eyes morphology is affected by the four contaminants (asymmetry and/or hypopigmentation) and that evidences can be collected as early as 12 hours following contamination. At the same time, the experiments revealed that the two heavy metals induce a delay in paired eyes formation; the information, however, is only available from day 5 post hatching. In conclusion, these preliminary experiments indicate that naupliar eyes are a potentially good new end-point in toxicity test even though further researches are required to clarify specificity and sensitivity. Results also indicate that naupliar visual structures are a target for common xenobiotic, as already demonstrated in other terrestrial<sup>1</sup> and aquatic<sup>2,3</sup> organism. Therefore, the environmental contamination might severely impair visual performances and, eventually, threaten animal survival.

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## USING INDUCED PLURIPOTENT STEM CELLS (iPSCs) TO STUDY A RARE HUMAN DEVELOPMENTAL DISEASE ASSOCIATED WITH RIBOFLAVIN DEFICIENCY

A. Niceforo,<sup>1,2</sup> F. Colasuonno,<sup>1</sup> M. Muzzi,<sup>1</sup> R. Borghi,<sup>1,2</sup> A. Di Giulio,<sup>1</sup> E. Bertini,<sup>2</sup> S. Moreno,<sup>1</sup> C. Compagnucci<sup>2</sup>

<sup>1</sup>Department of Science-LIME, University "Roma Tre", Rome, Italy; <sup>2</sup>Department of Neuroscience, Laboratory of Molecular Medicine IRCCS, Children's Research Hospital Bambino Gesù, Rome, Italy

E-mail: claudia.compagnucci@opbg.net

Riboflavin (vitamin B2) is indispensable for normal cellular function, growth and redox reactions, not synthesized by mammalian cells. In humans, this micronutrient is absorbed through three Riboflavin Transporters (hRFTs).<sup>1</sup> Mutations in *SLC52A3* and *SLC52A2* genes, encoding for hRFT2 and hRFT3, are causative for the childhood-onset Brown Vialetto Van Laere (BVVL) syndrome, a rare neurodegenerative disorder characterized by progressive sensorineural deafness and selective degeneration of ponto-bulbar motor neurons (MNs).<sup>2</sup> Induced pluripotent stem cells (iPSCs), derived from reprogramming of somatic cells are increasingly used to reproduce rare diseases.<sup>3</sup> In this study, we explored the potential of iPSCs to model BVVL syndrome, aiming to both characterize cellular/molecular aspects of this developmental disease, and test possible therapeutic effects of riboflavin and other antioxidants. Specifically, iPSCs obtained from fibroblasts of three BVVL patients were examined by light and electron microscopy and compared with controls. Diseased cells showed abnormal cell-to-cell contact and displayed aberrant mitochondrial features, compatible with the concept that riboflavin deficiency affects energy metabolism. When iPSCs were differentiated into MNs, deranged organization of cytoskeletal components were observed. Indeed, both immunofluorescence and qPCR showed altered expression and distribution of  $\alpha$ - and  $\beta$ -tubulin, actin and NFH intermediate filaments in patients-derived MNs. Interestingly, supplementation with riboflavin and other antioxidants (N-acetyl-cysteine, glutathione) significantly improved cytoskeletal organization. Overall, our data support the use of iPSCs for *in vitro* modeling of BVVL syndrome, highlighting the cellular effects of riboflavin deficiency and possible treatments to ameliorate cytoskeletal abnormalities and energy dysmetabolism.

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## FUNCTIONAL ANALYSIS OF THE EXTRA-CELLULAR PROTEIN TSUKUSHI IN NEURAL STEM/PROGENITOR CELLS FROM THE MOUSE EMBRYONIC CEREBRAL CORTEX

P.S. Nisi,<sup>1</sup> V. Licursi,<sup>1</sup> E. Cacci,<sup>1</sup> M.E. Miranda Banos,<sup>1</sup> N. Carucci,<sup>1</sup> R. Negri,<sup>1</sup> S. Biagioni,<sup>1</sup> K. Ohta,<sup>2</sup> G. Lupo<sup>3</sup>

<sup>1</sup>Department of Biology and Biotechnology "Charles Darwin", Sapienza University of Rome, Italy; <sup>2</sup>Department of Developmental Neurobiology, University of Kumamoto, Japan; <sup>3</sup>Department of Chemistry, Sapienza University of Rome, Italy

Tsukushi (TSK) belongs to the small leucine-rich proteoglycan family of extra-cellular proteins.<sup>1</sup> It is expressed in the cerebral cortex and in the subventricular zone in the embryonic and post-natal mouse brain. TSK regulates essential signaling pathways, such as TGF $\beta$ , Wnt, Notch and Receptor Tyrosine Kinase (RTK), during gastrulation and neurulation in frog and chick embryos.<sup>1,2</sup> Knock-out mutant mice lacking TSK function display abnormal

brain development,<sup>3</sup> but the underlying molecular mechanisms remain unclear. Using neural stem/progenitor cells (NSPCs) derived from the mouse embryonic cerebral cortex, we generated transgenic NSPCs with stable expression of a membrane-tethered form of TSK (TSK-TM), which retains comparable biological activity to that of the soluble protein, along with control NSPCs expressing Green Fluorescent Protein (GFP). We compared the transcriptome of TSK-TM and GFP NSPCs by microarray analysis and validated differentially expressed genes by real-time RT-PCR. This approach identified 17 genes showing consistent transcriptional changes in TSK-TM NSPCs. In particular, components of the Notch and RTK pathways were downregulated in TSK-TM samples, along with modulation of genes implicated in cell adhesion, motility or survival. Since Notch and RTK signaling play key roles in the regulation of NSPC self-renewal and differentiation, the above described gene expression data prompted us to investigate the effects of TSK overexpression on the proliferation and differentiation of NSPCs. TSK-TM NSPCs showed comparable cell viability and proliferation to GFP-expressing cells, based on the analysis of growth curves, flow cytometry of propidium iodide-stained cells and trypan blue exclusion assays. Preliminary data also suggest that early stages of neuronal and astrocyte differentiation are not affected in cells expressing TSK-TM. Altogether, our results suggest that TSK overexpression modulates specific gene expression programmes in NSPCs, but it is not sufficient to alter their proliferative and differentiative properties *in vitro*, possibly owing to exogenous growth factors compensating for TSK-dependent gene expression changes. Further experiments are underway to analyse the effects of TSK overexpression in more detail and to evaluate the consequences of TSK knockdown in NSPCs.

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## IN VITRO EXPOSURE TO CLORPHYRIFOS: EFFECTS ON FERTILITY AND GENE METHYLATION PATTERN IN BOS TAURUS SPERMATOZOA

M.M. Pallotta,<sup>1</sup> V. Barbato,<sup>1</sup> S. Braun,<sup>1</sup> M. Di Nardo,<sup>1</sup> A. Pinton,<sup>2</sup> R. Gualtieri,<sup>1</sup> R. Talev,<sup>1</sup> T. Capriglione

<sup>1</sup>Department of Biology, University of Naples Federico II, Italy;

<sup>2</sup>GenPhysSE INRA, Toulouse, France

E-mail: teresa.capriglione@unina.it

Chlorpyrifos (CPF) is an organophosphate insecticide used primarily to control foliage and soilborn insect pests on a variety of food and feed crops. The main mechanism of CPF action is the inhibition of acetylcholinesterase (AChE), which results in accumulation of acetylcholine and subsequent hyperactivity in the cholinergic system.<sup>1</sup> However, multiple developmental studies on animal models have reported that chronic CPF exposure can alter brain development and neuronal morphogenesis even in the absence of significant AChE inhibition. In addition, several studies demonstrated that CPF can affect endocrine system, inhibiting the secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH), and determining a decrease of testosterone level.<sup>2,3</sup> Based on these evidence, we evaluated *in vitro* the effects of CPF on bovine sperm. Frozen semen samples were incubated with different concentrations of CPF (5,10,25,50  $\mu$ g/mL) for 2 h, and various spermatozoa functional parameters were assessed: motility, *in vitro* fertilization rates, DNA fragmentation, chromatin alterations, methylation pattern. Progressive forward motility was significantly ( $p < 0.05$ ) reduced in spermatozoa exposed to the 10, 25, 50  $\mu$ g/mL of CPF while DNA fragmentation and putative chromatin deconstruction

appeared to increase significantly ( $p < 0.05$ ) at CPF higher concentrations ( $> 10 \mu\text{g/ml}$ ). The *in vitro* fertilization capabilities and the percentage of 8 cell embryos were significantly reduced at  $50 \mu\text{g/ml}$  of chlorpyrifos when compared to control. Evaluation of the global sperm DNA methylation level, carried out on samples exposed to 10 and  $25 \mu\text{g/ml}$  of CPF, showed no statistically significant differences between the treated groups and the control. To confirm the results, we selected two genes, Xist and GNAS, known to be imprinted in human and mice. In particular, we analyzed ten CpG islands XIST promoter and twentyone CpG islands for GNAS promoter. While GNAS regulative region did not show any significant variations in methylation level, XIST promoter showed an increase of DNA methylation in sperm cells exposed to  $10 \mu\text{g/ml}$  CPF.

These results suggest that exposure to low doses of chlorpyrifos can have adverse effects on male reproductive system of mammals, impairing sperm quality and, then, reducing male fertility potential.

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## DO AGING-MEDIATED EPIGENETIC CHANGES CAUSE CARDIAC AGING?

R. Papait,<sup>1,2</sup> R. Gornati,<sup>3</sup> G. Bernardini<sup>3</sup>

<sup>1</sup>Genetic and Biomedical Research Institute, National Research Council of Italy; <sup>2</sup>Department of Cardiovascular Medicine, Humanitas Research Hospital, Rozzano, MI; <sup>3</sup>Department of Biotechnology and Life Sciences, University of Insubria, Varese, Italy

E-mail: roberto.papait@humanitasresearch.it

Cardiac aging is a complex multifactorial biological process characterized by a gradual decline of normal physiological function in a time-dependent manner. Although vascular abnormalities associated with aging (e.g., hypertension and atherosclerosis) contribute to aging-related cardiac malfunctioning, myocardium-specific processes are also involved (e.g., apoptosis necrosis and the oxidative stress of cardiomyocytes).<sup>1</sup> Recent papers support the idea that underlying myocardium aging there are gene expression changes occurring in a time-dependent manner. However, the molecular mechanisms that cause these changes are not completely understood. The epigenome plays a key role in defining the transcription program at the base of cardiomyocyte differentiation and heart homeostasis in the adult.<sup>2,3</sup> Moreover, alterations of some epigenetic mechanisms (e.g., DNA methylation and histone acetylation and methylation) have been described during aging in different tissues.<sup>4</sup> These studies support the hypothesis that alteration of the epigenome due to aging leads to modifications of the gene expression program in the cardiomyocyte that could be responsible for cardiac aging. To test this hypothesis, we will investigate the cause-effect relationships between some epigenetic markers, gene expression and ultrastructure of cardiomyocytes during aging, through the study of the epigenome and transcriptome with genome-wide approaches, such as ChIP-seq and RNA-seq, and ultrastructural observations with transmission electron microscopy (TEM).

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## THE EXPRESSION OF IMMUNE-RELATED GENES IS INVOLVED IN ASCIDIAN DEVELOPMENT

N. Parrinello, D. Parrinello, M.G. Parisi, M. Cammarata

Marine Immunobiology Laboratory, Università degli Studi di Palermo, Italy

The upregulation of innate immunity genes is an evolutionarily conserved form of fast host defense against microbes and infectious metazoans. In addition, the transcription of immune-related genes is known to be necessary for the developmental program.<sup>1</sup> In ascidians, which belong to the sister group of vertebrates,<sup>2</sup> growing findings raise the question if the expression of some innate immunity genes in reproduction and during development is merely the programmed maturation of the adult immune system or it is required to developmental phases from gametogenesis to metamorphosis.<sup>3</sup> The cosmopolitan species *Ciona intestinalis* is a research model for evolutionary developmental and immunological studies. In this ascidian, upregulation of galectin, cytokine and phenoloxidase genes has been shown to be involved in innate immunity.<sup>4</sup> The transcriptional events of the corresponding genes and protein production are examined (*in situ* hybridization and immunohistochemistry) in developmental programmed phases, supporting the hypothesis of a differential regulation of immune-related genes that may be associated to development.<sup>5</sup>

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## MUSCARINIC RECEPTOR ACTIVATION MODULATES NEUROTROPHIC FACTORS PRODUCTION IN RAT SCHWANN-LIKE CELLS DERIVED FROM ADIPOSE MESENCHYMAL STEM CELLS

R. Piovesana,<sup>1</sup> A. Faroni,<sup>2</sup> M. Soligo,<sup>3</sup> L. Manni,<sup>3</sup> A.J. Reid,<sup>2</sup> A.M. Tata<sup>1</sup>

<sup>1</sup>Department of Biology and Biotechnology "Charles Darwin", "Sapienza" University, Rome, Italy; <sup>2</sup>Blond McIndoe Laboratory, University of Manchester, UK; <sup>3</sup>Institute of Translational Pharmacology-CNR, Rome, Italy

E-mail: roberta.piovesana@uniroma1.it

Peripheral nerve injury is commonly caused by direct mechanical trauma. Regeneration depends on the ability of Schwann cells (SCs) to create a favourable environment, by producing neurotrophic factors. Although SCs are effective in promoting nerve regeneration, they are not a convenient source of transplantable cells to improve outcomes after injury. Mesenchymal Stem Cells derived from adipose tissue (ASCs) seem to be a promising alternative source considering their ability to differentiate towards SC phenotype (Schwann-like). SCs express different receptors for neurotransmitters. In particular cholinergic stimulation of M2 muscarinic receptor decreases SCs proliferation whilst upregulating myelination. Previously, we demonstrated that Schwann-like cells express muscarinic receptors; in particular the M2 receptor activation resulted in decreased proliferation and reduced migration. In present work, we have characterised the effects mediated by muscarinic receptors on neurotrophic factors (NFs) expression and production. The selective activation of M2 receptors by arecaidine propargyl ester (APE) caused a significant decrease of the transcript levels for NFs (NGF, BDNF and GDNF), while the non-selective agonist muscarine did not influence NFs mRNA expression. By custom made

Elisa Assay, we analysed the production of two different NGF forms, precursor (proNGF) and mature NGF (mNGF). APE treatment induced a decreased release of both NGF forms, whereas muscarine treatment stimulated an increased release of mNGF. Western blot analysis indicated that both agonists caused a significant decrease in the expression of the proNGF isoform at 25 kDa, which is likely involved in the modulation of apoptotic processes. The data obtained suggest a relevant role of muscarinic receptors in the modulation of NFs production in Schwann-like cells. In particular the ability of both muscarinic agonists to negatively modulate the proNGF isoform, thereby suggesting a neuroprotective role of muscarinic receptors towards regenerating axons.

### THE ROLE OF *CYYR1* GENE DURING ZEBRAFISH DEVELOPMENT IN HH-MEDIATED MYOGENESIS AND NEUROMASTS DIFFERENTIATION

F. Pizzetti,<sup>1</sup> G. Deflori,<sup>2</sup> A. Pistocchi,<sup>3</sup> L. Ferrari,<sup>2</sup> M.C. Pelleri,<sup>1</sup> R. Casadei,<sup>4</sup> F. Frabetti<sup>1</sup>

<sup>1</sup>DIMES Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna; <sup>2</sup>The FIRC Institute of Molecular Oncology (IFOM) Foundation, Milan; <sup>3</sup>Department of Medical Biotechnology and Translational Medicine, University of Milan; <sup>4</sup>Department for Life Quality Studies, University of Bologna, Italy

E-mail: fabrizio.pizzetti2@unibo.it

*CYYR1* (Cysteine/tyrosine-rich 1) cloned on human chromosome 21 defines a new family of highly conserved vertebrate-specific genes.<sup>1,2</sup> The analysis of the human locus revealed the presence of a multitranscript-system that includes alternative spliced isoforms and one ncRNA gene overlapping *CYYR1* in antisense orientation.<sup>3</sup> Original results suggest the need of further investigations in order to verify a putative role of *CYYR1* in the tumorigenic process, caused by dysfunction of cell differentiation and possibly related to the Hh pathway;<sup>4</sup> to date, the specific function of the *CYYR1* product is still unknown. The zebrafish *cyrr1* is present in single copy and maintains almost 58% of identity with human protein therefore, we decided to perform a full characterization of *cyrr1* expression and function using zebrafish as model system. WISH approach defined a broad expression in central nervous system (CNS), somites and muscles during somitogenesis and at 24-48 hpf. The *cyrr1* knock-down with two different MOs targeting the ATG and the first splice-site of the transcript, affected both CNS and muscle development with a significant rescue in embryo co-injected with *cyrr1* mRNA. Defects were also evident in ciliated cells of neuromast of the lateral line. Morphologically, the *cyrr1*-MOs injected embryos display some features of embryos inhibited for the Hh pathway through injection of the *lefty* mRNA and *cyrr1* expression was significantly inhibited following Hh inhibition. Interestingly, the injection of *cyrr1* mRNA was able to partially rescue Hh-defective phenotype in embryos at 24 hpf. Results obtained through immunofluorescent staining, qPCR and western blotting, support a role for *cyrr1* in primary myogenesis probably downstream of Hh pathway.

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### GENE STRUCTURE, DIFFERENTIAL EXPRESSION AND PHYLOGENETIC ANALYSIS OF THE METALLOTHIONEIN GENE FAMILY OF THE SEA URCHIN *PARACENTROTUS LIVIDUS*

M.A. Ragusa,<sup>1</sup> A. Nicosia,<sup>2</sup> S. Costa,<sup>1</sup> A. Cuttitta,<sup>2</sup> F. Gianguzza<sup>1</sup>

<sup>1</sup>Department of Biological, Chemical, and Pharmaceutical Sciences and Technologies, University of Palermo, Italy,

<sup>2</sup>Laboratory of Molecular Ecology and Biotechnology, National Research Council-Institute for Marine and Coastal Environment (IAMC-CNR) Detached Unit of Capo Granitola, Trapani, Italy

E-mail: maria.ragusa@unipa.it

Metallothioneins (MT) are small and cysteine-rich proteins that bind metal ions such as zinc, copper, cadmium, and nickel.<sup>1</sup> Several functions were proposed for these peptides, ranging from toxic metal protection to physiological metal homeostasis, free radical scavenging, oxidative stress protection, antiapoptotic defense, control of the redox status of the cell and also roles during development. Previously, we reported the identification of five different MT homologues (P1MT4-8) from the Mediterranean sea urchin species *P. lividus*.<sup>2</sup> In order to shed some light on MT gene structure and evolution, we cloned seven *P. lividus* MT genes, comparing them to Echinodermata and Chordata genes.<sup>3</sup> Moreover, we performed a phylogenetic analysis of newly identified MTs from different classes of echinoderms and from the most ancient chordates, highlighting the relationships between them. Since MTs have multiple roles in the cells, we performed RT-qPCR and *in situ* hybridization experiments to understand better MT functions in sea urchin embryos. Results showed that the expression of MTs is regulated throughout development in a cell type-specific manner and in response to various metals.<sup>3</sup> The MT7 transcript is expressed in all tissues, especially in the stomach and in the intestine of the larva, but it is less metal-responsive. In contrast, MT8 is ectodermic and rises only at relatively high metal doses. MT5 and MT6 expression is highly stimulated by metals in the mesenchyme cells. Our results suggest that the *P. lividus* MT family originated by gene duplications and evolved developmental and environmental sub-functionalization. A preliminary *in silico* promoter analysis showed interesting similarities and differences among these genes.

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### HIGH CONTENT SCREENING IDENTIFIES AZATHIOPRINE AS A NEGATIVE MODULATOR OF THE INTRINSIC ADIPOGENIC POTENTIAL OF MUSCLE FIBRO/ADIPOGENIC PROGENITORS. DISRUPTING MUSCLE CELL DIFFERENTIATION TRAJECTORIES BY SMALL MOLECULES

A. Reggio, A. Zuccotti, F. Spada, A. Calderone, A. Cerquone, Perpetuini, S. Pirrò, M. Rosina, C. Fuoco, C. Gargioli, L. Castagnoli, G. Cesareni

Department of Biology, University "Tor Vergata", Rome, Italy

Muscle regeneration is governed by a complex interplay between several resident and circulating cell types. Although the main and irreplaceable contribution is provided by muscle satellite cells, additional resident cell populations, with a stem potential, play a crucial role in muscle recovery. Satellite cell activation to form new myofibers is positively regulated by stimulatory signals in the muscle niche. A population of mesenchymal stem

cells, Fibro/Adipogenic Progenitors (FAPs), are also activated after damage to support healthy muscle regeneration by providing pro-myogenic stimuli that promote satellite cells proliferation and differentiation. The tight control of FAPs proliferation and differentiation is progressively lost in pathological conditions, as for instance in muscular dystrophies, where they contribute to the deposition of fat and scar infiltrates. Blocking fibro/adipogenic conversion of FAPs could ameliorate the condition of ageing dystrophic patients offering, at the same time, a wider window in which gene therapy and cell-based strategies could be successfully employed. Here we report the results of a small-molecule phenotyping screening that identifies the immunosuppressant azathioprine, as a negative modulator of the intrinsic adipogenic potential of muscle FAPs. In addition, we show that azathioprine does not affect satellite cells commitment and differentiation in *in vitro* experiments. We will describe the molecular mechanisms underlying this activity.

### THE EUKARYOTIC INITIATION FACTOR 6 (eIF6) IS RATE-LIMITING FOR CD4<sup>+</sup> T LYMPHOCYTES DEVELOPMENT

S. Ricciardi,<sup>1</sup> N. Manfrini,<sup>1</sup> A. Miluzio,<sup>1</sup> A. Scagliola,<sup>1</sup> S. Biffo<sup>1,2</sup>

<sup>1</sup>National Institute of Molecular Genetics "Romeo ed Enrica Invernizzi"-INGM; <sup>2</sup>Department of Biosciences, University of Milan, Italy

The Eukaryotic Initiation Factor 6 (eIF6) is required for 60S ribosomal subunit biogenesis and for efficient initiation of translation. Recently, we have demonstrated that eIF6 is a translational regulator of metabolism, acting upstream of transcription. Several lines of evidence have emerged indicating that changes in nutrient uptake and cellular metabolism support many aspects of CD4<sup>+</sup> T cells differentiation and acquisition of effector function. On this basis, we hypothesized that eIF6 may also have a role in the regulation of the immune system. Following T cell receptor (TCR) activation, CD4<sup>+</sup> T cells rapidly divide and differentiate into CD4<sup>+</sup> T helper (Th) cells, such as Th1, Th2 or Th17. In this study, we found that eIF6 expression is triggered upon TCR activation in human CD4<sup>+</sup> T cells cultured in the absence of differentiating cytokines (Th0) or polarized as Th1 or Th2 cells. We further demonstrated that eIF6 is specifically involved in Th2 polarization *in vitro*. Indeed, shRNA knockdown of eIF6 in CD4<sup>+</sup> T cells polarized as Th2 reduced the expression of IL4 mRNA, whereas the expression of IFN $\gamma$  mRNA was not affected in eIF6 downregulated Th1 cells. Accordingly, eIF6 depleted Th2 cells secreted less IL-4 than did wild-type Th2 cells, whereas eIF6 depleted Th1 cells secreted amounts of IFN- $\gamma$  similar to those secreted by wild-type Th1 cells. Upon activation, CD4<sup>+</sup> naive T cells undergo a precise and fast metabolic reprogramming, necessary for proper acquisition of effector functions. Th2 cells upregulate glycolysis for anabolic purpose. We found that eIF6 down modulation blocks the glycolytic reprogramming in Th2 cells, as determined by reduction of lactate secretion and ATP production. Together, these results are compatible with a scenario in which eIF6 controls the Th2 differentiation program regulating the activation-driven metabolic changes, *in vitro*. Moreover, we found a reduction of peripheral CD4<sup>+</sup> T cells in eIF6 heterozygous mice, accompanied by increased mortality during viral infection. Together with the analysis of CD4<sup>+</sup> T helper (Th) cell subsets in eIF6 het mice conducted in parallel, the study of a mouse model of OVA-induced allergic asthma that promotes strictly Th2 responses and results in lung pathology will definitively clarify the contribution of eIF6 in the orchestration of CD4<sup>+</sup> T lymphocytes differentiation program.

### RTN-1C PARTICIPATES IN THE ER-DEPENDENT BIOGENESIS OF AUTOPHAGOSOMES

A. Risuglia,<sup>1</sup> S. Oliverio,<sup>1</sup> R. Nardacci,<sup>2</sup> M. Piacentini,<sup>1,2</sup> F. Di Sano<sup>1</sup>

<sup>1</sup>Department of Biology, University of Rome "Tor Vergata";

<sup>2</sup>National Institute for Infectious Diseases IRCCS "L. Spallanzani", Rome, Italy

The endoplasmic reticulum (ER) is a key organelle fundamental for the maintenance of cellular homeostasis and for the determination of cell fate under stress conditions.<sup>1</sup> Among the proteins known to regulate ER structure and function is Reticulon-1C (RTN-1C), a member of the reticulon family proteins localized primarily on the outer ER membrane.<sup>2</sup> We previously demonstrated that RTN-1C expression affects ER function under stressful condition.<sup>3</sup> Although ER is an essential site for the regulation of apoptotic pathways, it has also been recently recognized as an important component of autophagic signalling.<sup>4</sup> Based on these findings, we have investigated the impact of RTN-1C modulation on autophagy induction. Interestingly we found that RTN-1C overexpression is able to activate the autophagic machinery, and its silencing results in a significant inhibition of both basal and induced autophagic response. Considering the role of reticulon proteins in the control of ER membranes shaping and homeostasis<sup>5</sup> we speculated that the observed phenomena might depend on RTN-1C ability to influence the autophagic vesicles biogenesis from the ER compartment. Our data indicate a possible mechanism by which this structural ER protein may modulate cellular stress at the basis of different autophagy-related cellular processes.

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### HUMAN INDUCED PLURIPOTENT STEM CELLS (iPSCs) FROM JOUBERT SYNDROME AS A NOVEL *IN VITRO* MODEL TO ELUCIDATE CILIOPATHY-ASSOCIATED MOLECULAR MECHANISMS

J. Rosati,<sup>1</sup> F. Altieri,<sup>1</sup> S. Tardivo,<sup>2</sup> E.M. Valente,<sup>2</sup> A. Vescovi<sup>1</sup>

<sup>1</sup>IRCCS Ospedale Casa Sollievo della Sofferenza–Mendel Institute; <sup>2</sup>IRCCS Fondazione Santa Lucia, Rome, Italy

Joubert syndrome (JS) is a rare autosomal recessive condition characterized by a peculiar midbrain-hindbrain malformation, known as the molar tooth sign (MTS). To date, most of the identified causative genes of JS encode proteins involved in cilia function or assembly. We focused our attention on a point mutation of AHI1 (H896R), a gene encoding a protein named Joubertin. hiPSCs from a healthy donor and a JS patient were produced and fully characterized. In order to study how AHI1 mutation could affect neural differentiation at morphological and molecular level, iPSCs were differentiated in neural cells. Our results show that: 1) The H896R AHI1 mutation leads to a constitutive hyperactivation of Wnt pathway, 2) this mutation causes an incorrect differentiation both in cells morphology and in the balance among neural populations during development. To date, the functions of primary cilia in hiPSCs and their changes during neural differentiation remain largely vague, the next goal will be to investigate how the impairment of cilia could influence the neural development.

## MYTILUS GALLOPROVINCIALIS: A SENTINEL SPECIES FOR THE ENVIRONMENTAL BIOMONITORING IN THE NAPLES BAY

L. Rosati, M. Agnese, M. Verderame, P. Andreuccetti, M. Prisco  
*Department of Biology, University of Naples Federico II, Naples, Italy*

Mussels represent ideal sentinel organisms to investigate the toxicological effects induced by the anthropogenic activities as they are suspension feeders, and filter large volumes of water to collect huge amounts of particulate to be used for growth and reproduction, so they accumulate pollutants in their tissues.<sup>1,2</sup> More recently, we have investigated the oocyte growth<sup>3</sup>, as well as the reproductive cycle in males<sup>4</sup> and females<sup>5</sup> of *Mytilus galloprovincialis* with new insights regarding the mechanism of reproduction control in mussels.<sup>4,5</sup> Now, we report an investigation on specimens collected from two mussel farms located in two areas, respectively A-area and B-area, next to Castel dell'Ovo, an historical complex located in the Naples Bay. Such areas are different for water quality, as mussels from A-area are sale without purification, whereas mussels from B-area have to be purified before sale. The goal of the present investigation is to extend the observations at parameters that are usually utilized to evaluate the pollution and its possible effects in *Mytilus galloprovincialis*. At this regard, we have investigated the condition and gonad-somatic indices (CI and GSI), the modifications in the structural organization of female gonad, the presence of apoptosis, as well as the expression of estrogen receptor (ERs) during the non-reproductive and reproductive period. Significant differences in CI, GSI, structural organization of the ovary, apoptosis events, as well as in expression of estrogen receptors were observed between the individuals collected in the two areas during the two periods, probably as a consequence of a different quality water. In particular, it has been observed a considerable modification in the expression of ER2, so this receptor could be considerate as biomarker in environmental biomonitoring investigations.

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## IMPACT OF ACUTE INFLAMMATION ON NEUROGENESIS IN RAT SUBVENTRICULAR ZONE

P. Rosso,<sup>1,2</sup> V. Carito,<sup>1</sup> S. Moreno,<sup>2</sup> P. Tirassa<sup>1</sup>

<sup>1</sup>*Institute of Cell Biology & Neurobiology, CNR, Rome;*

<sup>2</sup>*Department of Science, University "Roma Tre", Rome, Italy*

*E-mail: paola.tirassa@cnr.it*

Subventricular zone (SVZ) is a major neurogenic niche in mammalian brain, and contains three cell types able to generate glia and neurons: type B cells, displaying an astrocyte-like phenotype; type C cells, which differentiate into either neuroblasts (type A cells) or oligodendrocytes depending on microenvironmental conditions.<sup>1</sup> Growth factors, including neurotrophins and cytokines, modulate the survival, proliferation, differentiation and migration of the above cell types, so that the presence of immune cells and inflammation may have a great impact on tissue damage recovery following injury.<sup>2</sup> To investigate the effects of brain inflammation on SVZ, adult rats immunised by complete Freund's adjuvant (CFA) alone, or added with MBP (acute experimental autoimmune encephalitis model, aEAE) received ocular treatment with NGF eye drops (eNGF) during the first

two weeks after immunisation.<sup>3</sup> Confocal analysis and cell count were performed to evaluate the effects of inflammation, EAE, and eNGF on the distribution of SVZ cell types. Our results show that the severity of inflammation and brain levels of chemokines and cytokines influence the topographic organisation and distribution of type B and type C cells in the SVZ, thus affecting oligodendrocyte and neuroblast survival. Specifically, the number of cells expressing type C and immature oligodendrocyte markers are increased in SVZ and along the corpus callosum of rats treated with CFA alone, when compared to aEAE, suggesting that these cells might be targets of inflammatory mediators, and contribute to demyelination during disease. In addition, our findings demonstrate anti-inflammatory effects of eNGF resulting in increased type A and C cells, and oligodendrocyte precursors, thus counteracting the effects of CFA and aEAE. In conclusion, our study confirms that inflammation impacts on brain self-renewal ability, and suggests that the increase of pro-inflammatory factors might generate inhibitory environmental conditions. These events contribute to the failure of recovery process during neurodegenerative diseases.

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## REVEALING NEW PATHS OF MUSCLE STEM CELLS OSTEOGENIC TRANS-DIFFERENTIATION: A POSSIBLE INVOLVEMENT OF DNA DAMAGE RESPONSE

M. Rosina, F. Langone, A. Cerquone Perpetuini, G. Giuliani, A. Calderone, S. Pirrò, A. Reggio, C. Fuoco, C. Gargioli, L. Castagnoli, G. Cesareni

*Department of Biology, University "Tor Vergata", Rome, Italy*

Skeletal muscle is a complex and organized tissue with high regenerative capacity. Regeneration can fail in pathological conditions, as in Duchenne Muscle Dystrophy (DMD). In this case aberrant trans-differentiation of muscle resident stem cells can occur. Aside from fibrotic and fat infiltrations, old *mdx* mice can show heterotopic ossification of skeletal muscles. The cellular origin of this phenomenon is not well understood. Mesoangioblasts are muscle mesenchymal stem cells capable of osteogenic differentiation *in vitro* and *in vivo* when stimulated by Bone Morphogenic Protein 2 (BMP2). BMP2 signaling is the main pathway controlling osteogenesis in physiological conditions but the contribution of alternative pathways is still unclear. With a High-content screening approach we selected the antiviral drug Idoxuridine (IdU) as an inducer of osteogenic differentiation of mesoangioblasts. IdU has been widely used to treat *Herpes simplex* virus infections since it is incorporated inside the virion DNA and yields non-infective viruses. Moreover, IdU is used as a radio-sensitizer in radiotherapy treated cancer patients since it induces DNA damage in actively proliferating cells. We are currently aiming at characterizing the mechanism of action of IdU. Preliminary data suggest that IdU acts by a BMP2 independent mechanism, opening the possibility of alternative pathways controlling osteogenesis. Conversely, IdU treatment results in p38 activation which is also a pro-osteogenic signal. We hypothesize that IdU induces a DNA damage response after its incorporation into MABs DNA. Further analysis confirms that the osteogenic conversion of MABs is dependent on the incorporation of IdU in the genomic DNA. DNA damage response has found to be critical for the correct differentiation of stem cell precursors. We propose that this phenomenon could drive not only uni-potent stem cells differentiation but also influence the multi-potent stem cell potential. Chronic DNA damage response could be at the basis of aberrant trans-differ-

entiation, common feature of many degenerative diseases such as DMD.

### THE ROLE OF TRP RECEPTORS IN THE SIGNALLING PATHWAYS IN *HYDRA VULGARIS*

V. Russo,<sup>1</sup> V. Malafoglia,<sup>1,2</sup> M. Scalici,<sup>1</sup> C. Muscoli,<sup>3</sup> W. Raffaeli,<sup>2</sup> T. Persichini,<sup>1</sup> M. Colasanti<sup>1</sup>

<sup>1</sup>Department of Science, University "Roma Tre", Rome, Italy; <sup>2</sup>Institute for Research on Pain, ISAL-Foundation, Torre Pedrera (RN), Italy; <sup>3</sup>IRC-FSH, Department of Health Science, University of "Magna Graecia", Catanzaro, Italy  
E-mail: marco.colasanti@uniroma3.it

The ability of mammals to feel stimuli lies in a heterogeneous group of primary somatosensory neurons termed nociceptors, which express specific membrane receptors, such as the Transient Receptor Potential (TRP) family. Since their discovery, the TRP family of proteins have now grown significantly, and to date consist of six sub-families with 28 mammalian members, categorized as canonical (TRPC), vanilloid (TRPV), ankyrin (TRPA), melastatin (TRPM), polycystin (TRPP), and mucolipin (TRPML).<sup>1</sup> In general, TRP channels are primary transducers of most known sensory modalities such as vision, hearing, olfaction, taste and touch, to a wide range of innocuous-to-noxious stimuli.<sup>2</sup> Interestingly, *Hydra vulgaris* expresses TRPM3, a nociceptive calcium channel involved in the detection of noxious heat in mammals.<sup>3</sup> TRPM3 activation induces the expression of HSP70 and nitric oxide synthase (NOS), two genes induced by TRP-mediated heat noxious stimuli in mammals as well as Nrf2 and SOD, known as markers of the oxidative stress during pain conditions.<sup>3</sup> Another excellent candidate as a mediator of oxidative/nociceptive-like system in *Hydra* is TRPA1, a nociceptive receptor involved in nitro-oxidative stress associated with different types of pain as well as innate immunity.<sup>4</sup> Understanding the TRP features in the context of the life histories and habitats of cnidarians would be of great interest to demonstrate the importance of a oxidative/nociceptive-like system in low invertebrates.

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### MOTHER EXPOSED TO BPA CAUSE EPIGENETIC CHANGES IN F1 ZEBRAFISH EMBRYOS: A MOLECULAR APPROACH

S. Santangeli,<sup>1</sup> C. Consales,<sup>2</sup> F. Maradonna,<sup>1,3</sup> M. Spanò,<sup>2</sup> O. Carnevali<sup>1,3</sup>

<sup>1</sup>Dipartimento Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Ancona; <sup>2</sup>Laboratory of Toxicology, Unit of Radiation Biology and Human Health, ENEA Casaccia Research Center, Rome; <sup>3</sup>INBB Consorzio Interuniversitario di Biosistemi e Biostrutture, Rome, Italy

Bisphenol A is a phenol with a worldwide distribution due to its use in the manufacturing of plastic materials. Recently, increasing evidence suggests that BPA may alter the epigenetic regulation of gene expression having the ability to cause DNA hypermethylation/hypomethylation at CpG (cytosine-guanine dinucleotide) islands near gene promoter regions.<sup>1</sup> Nowadays several studies suggest that exposure to endocrine-disrupting chemicals (EDCs) may have cumulative adverse effects on future generations, and that these effects could be mediated by epigenetic

mechanism.<sup>2</sup> To determine BPA effects on the subsequent generation, zebrafish females were treated with two doses of BPA ( $10^{-7}$  M and  $10^{-5}$  M) for four weeks and then crossed with untreated males. 24 hours post fertilization (hpf) embryos were collected and the effects of maternal exposure to BPA on epigenetic changes were assessed by Real-Time PCR (q-PCR) and bisulphite pyrosequencing. The results showed that both BPA doses interfere with the CpG island methylation level of *amh* promoter region. The lowest BPA dose decreases the expression of *dnmt1*, involved in the maintenance of methylation process and increases the expression of *dnmt3* and *dnmt7*, both involved in the novo methylation supporting the hypothesis that this BPA dose impair the normal methylation patterns occurring in embryo development, both interfering with de novo and the maintenance of methylation. Concluding, these data clearly suggested that the changes due to BPA exposure of adult females can be inherited by F1 generation and interfere with the general methylation status of the developing embryos.

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### AMPA RECEPTOR AND EXCITATORY AMINO ACID-INDUCED MOLECULAR PATHWAYS IN SPERMATOGONIAL GC-1 CELLS

A. Santillo, S. Falvo, M.M. Di Fiore, F. Di Giacomo Russo, G. Chieffi Baccari

DiSTABiF, University of Campania "L. Vanvitelli", Caserta, Italy  
E-mail: alessandra.santillo@unicampania.it

AMPA ( $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and NMDA (N-methyl-D-aspartate) receptors are multi-subunit transmembrane ionchannels, largely expressed in Central Nervous System. Actually, AMPA receptors (AMPA) are tetrameric assemblies of two dimers of four potential subunits (GluA1–GluA4) encoded by distinct genes. NMDA receptors (NMDAR) comprise the obligatory GluN1 subunit and the modulatory GluN2 subunits (GluN2A–GluN2D), each encoded by a separate gene. Previous studies have demonstrated that rat and mouse testes express GluN1,<sup>1,2,3</sup> GluA1 and GluA2/3 (unpublished data). In this study, the protein expression of GluN1, GluA1 and GluA2/3 subunits was investigated in a cell line derived from immortalized type-B mouse spermatogonia retaining markers of mitotic germ cells (GC-1). The results demonstrated that in GC-1 cells GluA1 and GluA2/3 subunit expressions were higher than GluN1. Therefore, GC-1 cells were incubated with medium containing excitatory amino acids (100/200  $\mu$ M D- aspartate, 10-50  $\mu$ M NMDA) to determine, at intervals during incubation, GluA1, GluA2/3, p-ERK, p-Akt and PCNA expressions. At 30' and 2h of incubation either with D-Asp or with NMDA, GluA1, GluA2/3, p-ERK, p-Akt and PCNA expressions were significantly higher as compared with controls. The results of this study firstly demonstrate that spermatogonia GC -1 express GluN1 and GluA1–GluA2/3 subunits and that both D-Asp and NMDA induce an increase of GluA1 and GluA2/3 expressions. Further, we propose that the response of GC-1 cells to the amino acids depends on AMPAR-mediated activation of the ERK and Akt pathways. Finally, the increased PCNA levels could suggest an enhanced proliferative activity.

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## IN VITRO GENOTOXIC EFFECTS OF TITANIUM DIOXIDE NANOPARTICLES (TiO<sub>2</sub> NPS) IN HUMAN SPERM CELLS

M. Santonastaso,<sup>1</sup> F. Mottola,<sup>2</sup> N. Colacurci,<sup>1</sup> C. Iovine,<sup>2</sup> S. Pacifico,<sup>2</sup> M. Ferrara,<sup>2</sup> V. Feola,<sup>2</sup> M. Cammarota,<sup>3</sup> F. Cesaroni,<sup>4</sup> L. Rocco<sup>2</sup>

<sup>1</sup>Dipartimento DBC, Università della Campania "Luigi Vanvitelli"; <sup>2</sup>Dipartimento DiSTABiF, Università della Campania "Luigi Vanvitelli"; <sup>3</sup>Dipartimento Medicina Sperimentale, Università della Campania "Luigi Vanvitelli"; <sup>4</sup>Centro PMA del Cassinate, Italy

E-mail: lucia.rocco@unicampania.it

Among the varieties of engineered nanoparticles (NPs) being used today, titanium dioxide (TiO<sub>2</sub>) nanoparticles are one of the most widely used in consumer products, such as plastics, paper, sunscreens, cosmetics, drugs and foods. As regards the massive presence in the environment, the attention of the study has been focused on the genotoxic effects of TiO<sub>2</sub>-NPs on human spermatozoa *in vitro*. It is known that in mice TiO<sub>2</sub> NPs are able to cross the blood-testis barrier induced inflammation, cytotoxicity and gene expression changes that lead to impairment of male reproductive system. The study presents new data on the DNA damage in human sperm exposed *in vitro* to two concentrations of n-TiO<sub>2</sub> (1 µg/L and 10 µg/L) for different times (15, 30, 45 and 90 minutes) and the role of ROS as mediators of n-TiO<sub>2</sub> genotoxicity. Primary n-TiO<sub>2</sub> characterization was performed by TEM. The dispersed state of the n-TiO<sub>2</sub> in media was spectrophotometrically determined at 0, 24, 48 and 72 h from the initial exposure, collecting samples of n-TiO<sub>2</sub>-enriched culture media. n-TiO<sub>2</sub> concentration value was extrapolated from the calibration curve obtained from n-TiO<sub>2</sub> standard solutions. The genotoxicity have been highlighted by using different experimental approaches (Comet Assay, TUNEL test, DFC Assay, RAPD-PCR). The Comet Assay showed a statistically significant loss of sperm DNA integrity after 30 min of exposure. The results of the TUNEL test showed an increase in sperm DNA fragmentation after 30 minutes of exposure. The RAPD-PCR analysis showed a variation of the polymorphic profiles of the sperm DNA exposed to n-TiO<sub>2</sub> respect to the control sperm DNA. The evidences from DFC Assay showed statistically significant increase of intracellular oxidative stress caused by n-TiO<sub>2</sub>. This research provides the first data on the evaluation of the potential genotoxicity of n-TiO<sub>2</sub> on human sperm that occurs through the production of intracellular ROS. The results provide a starting point for investigations on the potential effects that nanomaterials could have on infertility rate.

## EFFECTS OF NANOPARTICLE TREATMENTS ON THE DEVELOPMENT OF THE WATER FROG, *PELOPHYLAX SINKL ESCULENTUS*

G. Scillitani,<sup>1</sup> G. Bernardini,<sup>2</sup> R. Rossi,<sup>3</sup> M. Mastrodonato<sup>1</sup>

<sup>1</sup>Department of Biology, Laboratory of Histology and Comparative Anatomy and <sup>3</sup>Department of Emergency and Organ Transplants, University of Bari "Aldo Moro", Bari; <sup>2</sup>Department of Biotechnology and Life Sciences, University of Insubria, Varese, Italy

E-mail: giovanni.scillitani@uniba.it

The increasing use of nanoparticles (NP) in industrial and medical applications renders necessary to understand how they affect both biological and environmental systems. Therefore, we tested the effects of three NP (cobalt, nickel, iron) on developing embryos of the water frog *Pelophylax sinkl. esculentus*. This frog is commonly found in Italian wetlands and is regarded as a

good biomarker. Samples came from an artificial tank in the University Botanic Garden in Bari. Embryos at the developmental stage 10 (earliest involution of blastopore dorsal lip) were treated with iron, nickel or cobalt NP from IoLiTec (Heilbronn, Germany). A control group and three treatments per NP at increasing concentrations (LC50/2, LC50, and 2xLC50, according to literature) were considered, for a total of ten groups. Each group included about 20 individuals. Groups were monitored for the following ten days. A low mortality was observed and it was similar throughout the groups. Total length (LT) and eye diameter (LO) were significantly higher in the control (mean LT in control = 7.8 mm, mean LT in treatments = 6.3 mm; mean LO in control = 0.3 mm, mean LO in treatments = 0.2 mm). Significant differences in proportions were observed between controls and treatments in developmental stages (30% of controls reached stage 23 and 66% stage 21, lower values were observed in treatments). Malformations were observed in about 30% of controls, whereas in treatments they reached 60%. The most common malformations observed were abnormally large ventral mass and bent body axe, followed by abnormal development of the head and eye malformations. Investigations of integument at light, SEM and TEM microscopy revealed that in the epidermis of the treatments muciparous cells were hypertrophic, ciliated cells showed a higher number of cilia in respect to controls and ionocytes presented several swelled mitochondria, indicating a stressed condition. Besides, treatments were infested by the chytrid fungus *Batrachochytrium dendrobatidis*, as indicated by the several zoospores and zoosporangia observed in the epidermis. It is concluded that NP treatments are responsible of reduced growth, developmental delay and malformations, increase of secretion and oxidative stress in the cells of the integument, as well as and reduced resistance to fungal infections.

## THE ROLE OF PEROXISOMES DURING ADULT NEUROGENESIS IN A MOUSE MODEL OF ALZHEIMER'S DISEASE

C. Scopa,<sup>1,2</sup> A. Fracassi,<sup>1</sup> R. Scardigli,<sup>2,3</sup> S. Moreno<sup>1</sup>

<sup>1</sup>Department of Science-LIME, University "Roma Tre", Rome; <sup>2</sup>Laboratory of Neurotrophic Factors and Neurodegenerative Diseases, European Brain Research Institute (EBRI), Rome; <sup>3</sup>CNR-Institute of Translational Pharmacology, Rome, Italy

E-mail: c.scopa@ebri.it

In adult mammals, neurogenic niches include the subventricular zone (SVZ) of lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus.<sup>1</sup> Neurogenesis has been reported to be altered in neurodegenerative disorders, particularly Alzheimer's disease (AD).<sup>2</sup> Our group described peroxisomal changes in the brain of a transgenic mouse AD model (Tg2576), at the onset of disease.<sup>3-5</sup> On the other hand, Peroxisome Proliferator Activated Receptors (PPARs) have been suggested to play an important role in neural stem cell (NSCs) fate determination.<sup>6,7</sup> To address the role of peroxisomes during adult neurogenesis in early AD, we investigated the expression of specific markers in neurogenic niches both *in vivo* and *in vitro*. Immunohistochemical analysis of SVZ and SGZ in 3-month-old mice reveals stronger staining for PMP70 - a major peroxisomal membrane protein - and PPAR in Tg2576, as compared to WT. Accordingly, western blotting analyses of neurospheres lysates from 1.5-month-old Tg2576 demonstrate significantly higher levels of both markers than in WT. Also, confocal microscopy of differentiated neurospheres shows enhanced immunoreactivity in Tg2576 cells, as compared to their WT counterparts. Interestingly, while PMP70 appears equally expressed in neurons and astrocytes, PPAR preferentially colocalizes with GFAP,

rather than the neuronal marker Tuj1, supporting the emerging concept that this transcription factor regulates glial lineage, during adult neurogenesis.<sup>6,7</sup> On the other hand, the overexpression of the two peroxisomal markers in Tg2576 neurogenic niches suggests peroxisomal involvement at pre-symptomatic stages of the disease, related to neuronal and/or glial generation.

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### **BRD4 MODULATES AUTOPHAGY BY REGULATING OXIDATIVE STRESS IN SKELETAL MUSCLE: PHYSIOPATHOLOGICAL IMPLICATIONS IN DUCHENNE MUSCULAR DYSTROPHY**

M. Segatto,<sup>1,2</sup> R. Fittipaldi,<sup>2</sup> R. Szokoll,<sup>2</sup> P. Filippakopoulos,<sup>3</sup> G. Caretti<sup>2</sup>

<sup>1</sup>CNR-IBCN, Rome, Italy; <sup>2</sup>Department of Bioscience, University of Milan, Italy; <sup>3</sup>Nuffield Department of Clinical Medicine, University of Oxford, UK

E-mail: marco.segatto@uniroma3.it

BRoMoDomain-containing protein 4 (BRD4) is an epigenetic reader that recognizes acetylated histones through its bromodomains. This protein plays a crucial role in the transcriptional modulation of several genes implicated in different cellular processes, such as chromosome segregation, cell cycle progression, cell growth and differentiation.<sup>1,2</sup> In this work, we evaluated the role of BRD4 in the modulation of autophagy in skeletal muscle. To reach this aim, we took advantage of a pharmacological approach by using the small BRD4 inhibitor JQ1. Our main results demonstrate that BRD4 regulates autophagy in cultured skeletal muscle myotubes under oxidative stress conditions. Oxidative stress is known to play a causative role in autophagy impairment in the mdx mouse model of Duchenne muscular dystrophy.<sup>3</sup> Importantly, BRD4 inhibition by JQ1 restores autophagy alterations caused by oxidative stress in the mdx mouse, thus ameliorating the pathological outcomes of the dystrophic phenotype. In particular, BRD4 blockade determines a significant decrease in the expression of NADPH oxidase subunits, and this effect is accompanied by the induction of autophagic proteins. Taken together, our data indicate that BRD4 controls muscle autophagy by affecting oxidative stress, suggesting that BRD4 blockade could represent a new therapeutic avenue for the management of neuromuscular disorders.

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### **DAPHNIA MAGNA AND XENOPUS LAEVIS AS IN VIVO MODELS TO PROBE TOXICITY AND UPTAKE OF QUANTUM DOTS FUNCTIONALIZED WITH GH625**

A. Siciliano, M. Tussellino, E. Galdiero, R. Carotenuto

Department of Biology, University of Naples "Federico II", Naples, Italy

E-mail: antosiciliano@outlook.it

The use of quantum dots (QDs) for nanomedicine is hampered

by their potential toxicologic effects and difficulties with delivery into the cell interior.<sup>1</sup> We accomplished an *in vivo* study exploiting *Daphnia magna* and *Xenopus laevis* to evaluate both toxicity and uptake of QDs coated with the membranotropic peptide gH625 derived from the glycoprotein H of herpes simplex virus and widely used for drug delivery studies.<sup>2,3,4,5</sup> *X. laevis* embryos were reared starting from stage 4/8 in Frog Embryo Teratogenesis Assay-*Xenopus*<sup>6</sup> (FETAX) solution containing QDs (10nM) or gH625-QDs (10nM). All embryos were harvested until stage 45/46. *D. magna* neonates (24 h old) were isolated for exposures and used in all experiments of this study. We evaluated and compared the effects of QDs and gH625-QDs on the survival, uptake, induction of several responsive pathways and genotoxicity in *D. magna*, and we found that QDs coating plays a key role. Moreover, studies on *X. laevis* embryos allowed to better understand their cell/tissue localization and delivery efficacy. *X. laevis* embryos showed that both nanoparticles localized in the gills, lung and intestine, but they showed different distributions, indicating that the uptake of gH625-QDs was enhanced; the functionalized QDs had a significantly lower toxic effect on embryos' survival and phenotypes. We observed that *D. magna* and *X. laevis* are useful *in vivo* models for toxicity and drug delivery studies.

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### **RBBP6 AND CRYL1 GENES ARE UPREGULATED IN RESPONSE TO CADMIUM EXPOSURE IN ZEBRAFISH EMBRYOS AND ADULTS**

P. Simoniello,<sup>1</sup> R. Panzuto,<sup>2</sup> C.M. Motta,<sup>2</sup> R. Scudiero<sup>2</sup>

<sup>1</sup>Department of Sciences and Technology, University of Naples Parthenope, Naples; <sup>2</sup>Department of Biology, University of Naples "Federico II", Naples, Italy

E-mail: palma.simoniello@uniparthenope.it

The marked toxicity of cadmium (Cd) ions on aquatic organisms with respect to the terrestrial counterparts is commonly attributed to the higher bioavailability in water and to the multiple routes of uptake (skin, gills, gut). In both marine and freshwater habitats, Cd exerts its toxicity even at traces environmental concentrations causing cell toxicity and posing a severe threat to wildlife. The zebrafish (*Danio rerio*) is one of the most commonly used animals in the investigation of environmental cadmium toxicity in aquatic vertebrates. In this study, we identified two cadmium-responsive genes, the RetinoBlastoma Binding Protein 6 (RBBP6) and the Crystallin-Lambda 1 (CRYL1), that are up-regulated in the early phases of zebrafish development, at the gastrula stage. RBBP6 is associated with increased protein degradation and cell proliferation; CRYL1 is a lens protein with redox activity. Since zebrafish eyes are particularly sensitive to cadmium toxicity, we decided to investigate the expression of these 2 genes in zebrafish adult eyes by using *in situ* hybridization analysis, in natural condition and after 30 days cadmium-exposure. In particular, three sub-lethal concentrations (1.5, 20 and 40 µM) were tested. The results confirmed the ability of cadmium to up-regulate the expression of both genes in retinal cells in a dose-dependent manner. The over-expression was transient, being switched off when cadmium was removed. Our data suggest an involvement of RBBP6 and CRYL1 proteins in the



onset of morphological alterations previously observed in the zebrafish retina after exposure to cadmium.

### ENVIRONMENTAL SAFE HANDLING OF NANOTECHNOLOGY: INFLUENCE OF AgNPs SIZE, CONCENTRATION AND TIME

B.A. Tenuzzo,<sup>1</sup> E. Carata,<sup>1</sup> S. Mariano,<sup>1</sup> E. Panzarini,<sup>1</sup> L. Dini,<sup>1,2</sup>

<sup>1</sup>Department DiSTeBA, University of Salento; <sup>2</sup>CNR Nanotech, Lecce, Italy

In the article entitled *Safe handling of nanotechnology* Maynard *et al.*<sup>1</sup> discussed on the tremendous opportunities for new or improved products offered by nanotechnology, but also on the concerns raised among the public and the scientific community regarding human health and environmental hazards. More than 2000 products, containing engineered nanoparticles (NPs) are currently in use with a constant growth environmental release. Nevertheless more than a decade of intensive research has produced considerable knowledge regarding NPs behaviour, fate and ecotoxicity, the need to "get nanotechnology right" without causing a major environmental time bomb is critical. Maynard *et al.*<sup>1</sup> identified five "grand challenges" to address these concerns in a rational and systematic way: 1- Develop instruments to assess the exposure to NPs in air and water; 2- Develop and validate methods to evaluate the toxicity of NPs; 3- Develop models for predicting the potential impact of NPs on the environment and human health; 4- Develop robust systems for evaluating the health and environmental impact of NPs over their entire life; 5- Develop strategic programs that enable relevant risk-focused research. However, is still virtually impossible to relate the effects of classes of NPs to their physicochemical properties (categorization and function);<sup>3</sup> and toxic effects seem to be NP- and species-dependent.<sup>4</sup> Indeed, the wide variety in NP properties, size, morphology, chemical composition and types of surface coatings,<sup>5</sup> and the lack of standard reference and test protocols are key factors in the slow scientific progress, despite intensive multidisciplinary research. Here we present our nanoecotoxicology data, by focusing on environmental fate and behaviour of silver nanoparticles (AgNPs) of different sizes from 10 nm to 100 nm (10, 20, 40, 60, 100 nm diameter), different concentrations from 1ng/ml-1 to 100 ng/ml<sup>-1</sup> (1, 10, 100 ng/ml<sup>-1</sup>) and different exposure times (24 and 48 h and 1 week) on sea urchins and *Chlorella vulgaris*.

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### THE MOLECULAR IDENTITY OF MOUSE ES CELL-DERIVED NEURAL CELLS AFFECTS THEIR PROJECTION PATTERNS *IN VIVO*

M. Terrigno,<sup>1</sup> C. Alia,<sup>2</sup> I. Busti,<sup>2</sup> M. D'Onofrio,<sup>3</sup> M. Caleo,<sup>2</sup> F. Cremisi<sup>1,4</sup>

<sup>1</sup>Scuola Normale Superiore di Pisa; <sup>2</sup>Istituto di Neuroscienze, CNR di Pisa; <sup>3</sup>Istituto Eбри di Roma; <sup>4</sup>Istituto di Biofisica, CNR di Pisa, Italy

E-mail: federico.cremisi@sns.it

The capability of generating neural precursor cells with distinct types of regional identity *in vitro* has recently opened new opportunities of cell replacement in animal models of neurodegenera-

tive diseases. However, the extent and specificity of long range projections generated by such cells was not systematically compared in experiments of grafting in different brain regions. By finely manipulating Wnt and BMP signaling, we steered the differentiation of mouse ES cells towards isocortical or hippocampal molecular identity. These two types of cells showed different capacity of axonal projection and targeted different regions when co-transplanted in the healthy or lesioned isocortex, or in hippocampus. Following transplantation within the hippocampal dentate gyrus (DG), only precursor cells with hippocampal molecular identity sent axons to CA3. After grafting in the intact motor cortex, hippocampal -but not isocortical-like cells - were capable of extending axonal projections. Precursor cells with isocortical molecular identity were able to generate long range projections only when transplanted after the induction of a photothrombotic stroke in the motor cortex, sending fibers toward thalamic nuclei and motor tracts. We transduced isocortical cells with lentiviral vector encoding an evolved M3-muscarinic receptor that allows to evoke non-invasively burst firing of the grafted neurons via administration of the otherwise inert small molecule clozapine-N-oxide (CNO). When transplanting these cells into the ischemic lesioned motor cortex, the forelimb asymmetry induced by the stroke (as measured in the Schallert cylinder test) was corrected in rehabilitated animals treated with CNO to activate the transplanted neurons. Our results indicate that: 1) neural precursor cells generated *in vitro* by mouse ES cells carry intrinsic signals guiding their projections, 2) the damaged adult brain provides signals supporting axonal extension by isocortical-like cells and 3) isocortical cell functional integration is sufficient to improve the motor performance after stroke.

### PERICYTES FROM ANORECTAL MALFORMATIONS: POTENTIALITY AND APPLICATIONS

S. Testa, E. Fornetti, S. Bernardini, R. Belli, S. Cannata, C. Gargioli

Department of Biology, University of Rome "Tor Vergata", Italy

Fecal incontinence (FI) is a major public health issue that heavily affects the life quality of a large percentage of adult people in western countries.<sup>1</sup> In particular adults suffering from post-surgery consequences or trauma<sup>2,3</sup> while children affected by anorectal malformations (ARM) are the main categories of patients suffering of FI due to defective sphincter complex, which an ARM incidence rate ranging from 1:1500 to 1:5000 live births.<sup>4,5,6</sup> Despite the technical advancement and the better knowledge of these conditions by clinicians and surgeons, the long-term outcome involves the loss of normal continence in up to 85% of cases.<sup>7</sup> Regenerative medicine based on *in vitro*-generated muscle constructs represents a promising approach for the treatment of FI, and few initial studies using non-structured cellularized matrices have been reported.<sup>8</sup> Here we propose to create for the first time an *in vitro* engineered muscular sphincter, starting from human pericytes. These cells, in fact, have demonstrated great capability to generate skeletal muscle tissue *in vivo*, while also supporting blood vessel formation.<sup>9</sup> For our experiments we have isolated pericytes from ARM biopsies; their differentiation potential were tested in cellular differentiation analysis and moreover, these cells were embedded in a matrix of Poly-ethylene-glycol-Fibrinogen (PF), in order to create a 3D circular muscle construct mirroring sphincter shape and architecture for artificial tissue construction.

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## RHOMBOMERE 4-DERIVED AUDITORY AND VESTIBULAR SYSTEMS IN *Hoxb1* MUTANTS

M. Tizzano,<sup>1</sup> M. Di Bonito,<sup>2</sup> M. Studer,<sup>2</sup> B. Avallone<sup>1</sup>

<sup>1</sup>Department of Biology, University of Naples "Federico II", Naples, Italy; <sup>2</sup>Institute of Biology Valrose, iBV, University of Nice Sophia Antipolis, Nice, France

Rhombomere r4 (r4) and *Hox* associated genes *Hoxb1* and *Hoxb2* contribute to the formation of specific auditory and vestibular subcircuits. In particular, sensory and motor components of the sound transmission pathway, sensorimotor reflex circuits, as well as the hindlimb vestibulospinal reflex, derive from r4 and are strongly affected in *Hoxb1* mutants.<sup>1,2</sup> Inner ear efferent (IEE) neurons also originate from r4 and form vestibular (VEN) and cochlear (CEN) efferent neuron subpopulations. The CEN is further subdivided into medial (MOC) and lateral (LOC) olivocochlear motoneurons. MOC neurons inhibit the motility of outer hair cells (OHCs), which amplify low intensity sounds, while LOCs innervate the afferent terminations on the inner hair cells (IHC) modulating the excitability of the cochlear nerve, thus protecting the cochlea from acoustic damage. *Hoxb1*<sup>null</sup> mutants lack MOC and LOC efferent neurons leading to defects in OHCs and in cochlear amplification, and mice have increasing auditory thresholds.<sup>1</sup> A hypothesis is that MOC neuron endings play a trophic function on OHCs and that the physical interaction between MOC efferents and OHCs is essential for proper maturation and functioning of OHCs. Further genetic intersectional studies impinging either on motor or sensory components of r4-derived auditory subcircuits are needed to understand the involvement of efferent innervations for proper functioning of the cochlea. Regarding the vestibular system, *Hoxb1*<sup>null</sup> mutant mice also fail to form the VEN at early developmental stages.<sup>2</sup> However, transmission electron microscopy (TEM) in adult mice reveals the presence of both afferent and efferent neuronal endings on receptor cells. To understand whether projections are missing at birth and new connections gradually appear during the first month by compensatory plasticity mechanisms, we are in the process of testing newborn mutant pups for the presence of efferent endings on hair cells by TEM. We also aim to use retrograde labelling in 3-month old mutant mice to assess their eventual presence and identify their origin.

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## EFFECT OF THERMAL STRESS ON *DANIO RERIO* BEHAVIOUR: A PROTEOMIC STUDY TO UNDERSTAND THE MOLECULAR MECHANISM

M. Toni,<sup>1</sup> G. Miccoli,<sup>1</sup> C. Cioni,<sup>1</sup> E. Angiulli,<sup>1</sup> E. Alleva,<sup>2</sup> F. Frabetti,<sup>3</sup> F. Pizzetti,<sup>3</sup> F. Santagata,<sup>4</sup> F. Grassi Scalvini,<sup>4</sup> G. Tedeschi<sup>4,5</sup>

<sup>1</sup>Department of Biology and Biotechnology "Charles Darwin", Sapienza University, Rome; <sup>2</sup>Section of Behavioural Neuroscience, Department of Cell Biology and Neuroscience, Istituto Superiore di Sanità, Rome; <sup>3</sup>Department of Experimental, Diagnostic and Specialty Medicine, University of

Bologna; <sup>4</sup>Department of Veterinary Medicine University of Milan; <sup>5</sup>Fondazione Filarete, Milan, Italy

Environmental temperature variations affect many properties and functions of biomolecules and structural components at the cellular level, and influence the animal physiology and behavior at the organism level. It is well accepted that the biochemical changes induced by temperature stress are attributed to gene expression modulations. The aim of this work was to understand the molecular mechanism and the behavioural responses correlated to temperature stress in zebrafish (*Danio rerio*), which is a widely used animal model for environmental genomics researches. Adult specimens of wild type zebrafish were kept at three different temperatures: 18°C, 34°C and 26°C (used as a control) for 21 days and then subjected to behavioural tests using a Y-Maze task to evaluate the response to novelty and the spatial memory. Proteomic analysis were carried out on brains to evaluate the thermal effect at the central nervous system level. Briefly, for each temperature 9 brains were lysed and after reduction and derivatization, the proteins were digested with trypsin. LC-ESI MS/MS analysis was performed on a Dionex Ultimate 3000 HPLC. The eluate was electrosprayed into an LTQ Orbitrap Velos. Four technical replicate analyses of each sample were performed. Mass spectra were analyzed using MaxQuant and Peak Studio software. Finally, the bioinformatic analysis was carried out by DAVID and PANTHER softwares to evaluate enriched categories filtered for biological processes, molecular function (MF), cellular component (CC) and pathways involved. Preliminary results suggest that thermal stress at the cellular level influences the CC organization, biogenesis, structural morphogenesis and MF and at the organism level affects the interest for the new environment and the spatial memory.

## ENDOPLASMIC RETICULUM STRESS IN AUTISM SPECTRUM DISORDERS: *IN VITRO* AND *IN VIVO* EVIDENCE

L. Trobiani,<sup>1</sup> F. Lietta Favaloro,<sup>1</sup> M.A. Di Castro,<sup>2</sup> M. Cariello,<sup>1</sup> M. Di Mattia,<sup>1</sup> A. De Jaco<sup>1</sup>

<sup>1</sup>Department of Biology and Biotechnology "Charles Darwin", and Sapienza University, Rome; <sup>2</sup>Department of Physiology and Pharmacology, Sapienza University, Rome, Italy

E-mail: antonella.dejaco@uniroma1.it

Autism Spectrum Disorders (ASDs) are neurodevelopmental syndromes, characterized by behavioral deficits and a strong genetic background. In the last decades, an increasing number of mutations have been reported in genes encoding for proteins involved in synapse formation and maintenance such as the post-synaptic adhesion proteins neuroligins. The autism-linked R451C substitution in neuroligin3 (NLGN3) affects locally the folding of the extracellular protein domain, causing the retention of most of the mutant protein in the endoplasmic reticulum (ER).<sup>1</sup> Misfolded proteins accumulating in the ER can lead to an ER stress condition and to the activation of the unfolded protein response (UPR), a signaling pathway that aims to restore ER homeostasis. Lately UPR has been implicated in pathological and physiological conditions of the nervous system.<sup>2,3</sup> We have shown that the over-expression of R451C NLGN3 in a PC12 Tet-ON cell system leads to the activation of the UPR, both in proliferative and neuronal-differentiating conditions.<sup>4</sup> Our recent work has focused on investigating UPR activation *in vivo*, in the R451C NLGN3 knock-in (KI) mouse expressing endogenously the mutant protein. This mouse strain is characterized by a consistent reduction in the brain of NLGN3 R451C levels, in spite of unchanged mRNA levels.<sup>5</sup> We have found that the residual mutant protein is also trapped in the ER, *in vivo*, as previously shown by *in vitro* evidence. The activation of the UPR

in the KI mouse brain has been evaluated by studying the expression of classic UPR targets. Interestingly we have observed the activation of UPR at embryonic and in adult stages only in the cerebellum, in comparison to the parental mouse strain. Patch clamp recordings of excitatory transmission from Purkinje cells showed alterations of the excitatory neurotransmission in the cerebellum of the KI mice. The inhibition of the PERK-mediated UPR branch in cerebellar slices restored the frequency of the miniature excitatory currents to control values, indicating a possible link between UPR and alterations of presynaptic neurotransmitter release. In conclusion, our work provides evidences of a role played by the UPR in neurodevelopmental disorders characterized by the retention of misfolded proteins in the ER.

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### ACTIVITY DEPENDENT MODULATION OF GRANULE CELL SURVIVAL IN THE ACCESSORY OLFACTORY BULB AT PUBERTY

S. Trova,<sup>1,2</sup> L. Oboti,<sup>3</sup> R. Schellino,<sup>1,2,4</sup> M. Marraudin,<sup>2,4</sup> N.R. Harris,<sup>5</sup> O.M. Abiona,<sup>5</sup> M. Stampar,<sup>6</sup> W. Lin,<sup>6</sup> P. Peretto<sup>1,2</sup>

<sup>1</sup>Department of Life Sciences and Systems Biology, University of Turin, Italy; <sup>2</sup>NICO - Neuroscience Institute Cavalieri Ottolenghi, Italy; <sup>3</sup>Center for Neuroscience Research, Children's National Health System, USA; <sup>4</sup>Department of Neurosciences "Rita Levi Montalcini", University of Turin, Italy; <sup>5</sup>Department of Biological Sciences, University of Maryland Baltimore County, USA; <sup>6</sup>Research Center for Genetic Medicine, Children's National Health System, USA

E-mail: sara.trova@unito.it

The vomeronasal system (VNS) is specialized in the detection of salient chemical cues triggering social and neuroendocrine responses. Such responses are not always stereotyped, instead, they vary depending on age, sex and reproductive state, yet the mechanisms underlying this variability are unclear. Here, by analyzing neuronal survival in the first processing nucleus of the VNS, namely the accessory olfactory bulb (AOB), through multiple bromodeoxyuridine (BrdU) birthdating protocols, we show that exposure of female mice to male soiled bedding material affects the integration of newborn granule interneurons mainly after puberty. This effect is induced by urine compounds produced by mature males, as bedding soiled by younger males was ineffective. The granule cell increase induced by mature male odor exposure is not prevented by pre-pubertal ovariectomy, indicating a lesser role of circulating estrogens in this plasticity. Interestingly, the intake of adult male urine-derived cues by the female vomeronasal organ (VNO) increases during puberty, suggesting a direct correlation between sensory activity and AOB neuronal plasticity. Thus, as odor exposure increases the responses of newly born cells to the experienced stimuli, the addition of new GABAergic inhibitory cells to the AOB might contribute to the shaping of vomeronasal processing of male cues after puberty. Consistently, only after puberty, female mice are capable to discriminate individual male odors through the VNS.

### MOLECULAR ANALYSIS OF A SMITH-MAGENIS SYNDROME PATIENT DERIVED CELL LINE

E.M. Turco,<sup>1,2</sup> A. De Jaco,<sup>3</sup> E. Vinci,<sup>1,3</sup> G. Mazzoccoli,<sup>2</sup> L. Bernardin,<sup>2</sup> L. Trobiani,<sup>3</sup> M. Della Monica,<sup>4</sup> A.M. Tata,<sup>3</sup> A. Vescovi,<sup>1,2</sup> J. Rosati<sup>1</sup>

<sup>1</sup>Cellular Reprogramming Unit, Mendel Institute-IRCCS; <sup>2</sup>IRCCS "Casa Sollievo della Sofferenza", San Giovanni Rotondo; <sup>3</sup>Department of Biology and Biotechnologies "Charles Darwin", "Sapienza" University of Rome; <sup>4</sup>Medical Genetics Unit, Ospedale Pediatrico Meyer, Florence, Italy

Smith-Magenis syndrome (SMS, MIM 182290) is a complex developmental disorder characterized by behavioral deficits, craniofacial alterations, intellectual disability and circadian rhythm dysregulation.<sup>1</sup> About 90% of SMS cases are due to the 17p11.2 deletion containing retinoic acid induced 1 (*RAI1*) gene, the remaining 10% is due to heterozygous mutation within the *RAI1* coding region. *RAI1* is a nuclear protein with transcription factor activity, however little is known about its role in development. Here we characterized a newly described deletion, NM\_030665.3: c.1194del,<sup>3</sup> creating a premature stop codon and generating a truncated form of *RAI1*. We have over-expressed wild type and mutant *RAI1* encoding plasmids in HEK293 cells and we showed that the mutant truncated *RAI1* protein is not found in the nucleus but rather accumulates in the cytoplasm. Skin biopsies were collected from a SMS patient carrying this *RAI1* deletion, in order to generate primary human fibroblasts expressing endogenously the *RAI1* deletion (COL04). We have also originated control primary fibroblasts derived from a healthy relative (COL03). Fibroblasts were reprogrammed to induce pluripotent stem cells (iPSCs) to investigate the cellular and molecular effects due to *RAI1* haploinsufficiency. We found that in fibroblasts and iPSCs derived from the SM-patient there is a down-regulation of *RAI1* messenger and a different response to treatments with retinoic acid. Many studies support *RAI1* as a transcriptional regulator of the circadian cycle genes.<sup>3</sup> We have investigated the expression of *CLOCK* gene and downstream targets, such as *CRY1* and *PER1*, in COL03 and COL04 fibroblasts by quantitative RT-PCR. Our data show that the reduced function of *RAI1* in SMS results in an altered expression of critical genes involved in circadian rhythm.

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### H<sub>2</sub>O<sub>2</sub> RESISTANT MESOANGIOBLAST CLONE ISOLATION WITH A DISTINCT SURVIVAL ADVANTAGE IN VITRO AND IN VIVO

G. Turturici,<sup>1</sup> C. Gargioli,<sup>2</sup> M.M. Barreca,<sup>1</sup> C. Fuoco,<sup>2</sup> S. Testa,<sup>2</sup> S. Feo,<sup>1</sup> S.M. Cannata,<sup>2</sup> G. Cossu,<sup>3</sup> G. Sconzo,<sup>1</sup> F. Geraci<sup>1</sup>

<sup>1</sup>STEBICEF Department, University of Palermo, Italy; <sup>2</sup>Department of Biology, University of Rome "Tor Vergata", Italy; <sup>3</sup>Division of Cell Matrix Biology and Regenerative Medicine, University of Manchester, UK

E-mail: gabriella.sconzo@unipa.it

The release of molecules from damaged tissues stimulates both resident and circulating stem cells to initiate a tissue repair programme.<sup>1</sup> However, during transplantation procedures the therapeutic efficacy of stem cells is compromised by reduced homing capability towards the target site.<sup>2,3</sup> Furthermore, cell survival is very low and many studies focused on improving cell viability upon implantation. In this study, we performed *in vitro*

severe oxidative stress to select some more resistant mouse mesoangioblasts, vessel-associated progenitor stem cells endowed with the ability of multipotent mesoderm differentiation. We found that the selected subpopulation retains self-renewal and myogenic differentiation capabilities under physiological growth conditions, showing, also, an enhancement in cell survival and migration capabilities under stress conditions respect to the unselected cells. In fact, following oxidative stress treatment the isolated cell subpopulation showed more resistance, survival and recovery properties. To evaluate whether or not the isolated cell clone showed selective advantages over the parental mesoangioblasts, we carried out *in vivo* experiments using immunocompromised dystrophic mice. We injected intramuscularly the *Tibialis Anterior* with both the selected cells and the parental cells. Actually resistant mesoangioblasts displayed markedly enhanced survival and integration capabilities into the host damaged skeletal muscle, displaying more than 70% increase in integration compared to the non-selected mesoangioblast cell population. In conclusion, the positive effects of sorting on mesoangioblast cells suggest that a selection step using oxidative stress preconditioning may provide a novel methodology to select for resistant cells that can be used in regenerative tissue applications to prevent high mortality rates upon transplantation.

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#### **GLYPHOSATE INDUCES HEPATIC AND TESTICULAR TOXICITY IN THE NON-TARGET LIZARD *PODARCIS SICULA***

M. Verderame, R. Scudiero

*Department of Biology, University of Naples Federico II, Naples, Italy*

*E-mail: ilaria.verderame@unina.it*

Exposure to environmental contaminants represents a growing concern due to the interference of the pollutants on developmental and reproductive functions. The potential toxicity of Glyphosate (Gly), a broad spectrum herbicide widely used, is currently a great matter of debate. Although classified by the EPA as "non-toxic and not an irritant", some evidence exists on adverse effects of Gly-based herbicides (GBH) on non-target wildlife.<sup>1,2</sup> Many studies on Gly are aimed to evaluate the effects of Roundup (Monsanto), the most used GBH in the world, in mammals and fish.<sup>3,4</sup> So, we decided to investigate the effects of Gly exposure on the lizard *Podarcis sicula*, a suitable bioindicator of terrestrial environmental pollution. To exclude the possible interference of the adjuvants, we exposed the animals to pure Gly. Adult *P. sicula* specimens were divided in 3 groups (n=6): animals of group 1 and 2 were exposed to Gly 0.1 and 1 µg/L, respectively, via gavage every other day for 3 weeks; animals of group 3 received by gavage the same dose of tap water (100 µL). Our results demonstrate that both Gly doses induced histopathological and molecular damages in liver and testis, representing typical changes in the androgen/estrogen balance and signalling. In liver, we observed an increase of melanocytes degranulation and the appearance of nodular/cystic formations typical of hepatic fibrosis. In addition, the liver of Gly-treated males displayed the hepatic biosynthetic alterations typical of an estrogenic contamination: hepatocytes, in fact, contained vitellogenin and estrogen receptors transcripts, detected by ISH. At reproductive level, the ovary was not affected by Gly exposures, also at higher dose; on the contrary, the testis showed clear signs of morphological alterations. Spermatogenesis was slightly slower, at low dose of Gly scattered spermatocytes II

fused to form rosette were found, at high dose the amount of rosette-shaped arrangement of spermatocytes II increased; spermatids were damaged and in the lumen of the tubules some cells in degeneration were evident.

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#### **HMGA AND PATZ GENES IN XENOPUS NEURAL CREST DEVELOPMENT**

R. Vignali

*Laboratory of Cell and Developmental Biology, University of Pisa, Italy*

*E-mail: robert.vignali@unipi.it*

HMGA and PATZ1 are chromatin remodeling factors involved in several forms of human cancer. HMGA1 and HMGA2 are expressed during embryogenesis, but not in adult differentiated tissues; they are however reactivated in several forms of tumours. Both HMGAs elicit epithelial-mesenchymal transition (EMT) in cancer cell models; PATZ1 may likely play a similar role. In *Xenopus* only *hmga2* and *patz1*, but not *hmga1*, are present. During development, *hmga2* and *patz1* are expressed in the developing central nervous system (CNS) and in neural crest cells (NCCs), with a rather similar spatio-temporal pattern. We have studied *hmga2* role in NCCs by gain and loss-of function approaches. We found that *hmga2* knock-down impairs NCCs specification and their subsequent EMT and migration, leading to severe disruption of the pharyngeal skeleton. Knock-down of *hmga*-related *AT-hook* genes results in much milder phenotypes. We are currently studying *Xenopus patz1* function in a similar functional approach.

#### **INTERACTIONS OF METAL NANOPARTICLES WITH THE PLASMA MEMBRANE**

D. Zanella, E. Bossi, R. Gornati, G. Bernardini

*Department of Biotechnology and Life Sciences, University of Insubria, Varese, Italy*

*E-mail: giovanni.bernardini@uninsubria.it*

The ability of nanoparticles (NPs) to be promptly uptaken by the cells makes them both dangerous and useful to human health. It has been recently postulated that some NPs might cross the plasma membrane also by a non-endocytotic pathway gaining access to the cytoplasm. Notwithstanding its possible importance in nanomedicine, little attention has been given to this pathway perhaps because it challenges the idea of non-permeability of cytomembranes to large hydrophilic molecules. We tested the hypothesis of a direct crossing of the plasma membranes by NPs using mature *Xenopus* oocytes that we filled with Calcein, whose fluorescence is strongly quenched by divalent metal ions. We exposed them to different cobalt NPs and quantified quenching as evidence of the increase of the concentration of Me<sup>2+</sup> released by the NPs that entered into the cytoplasm. We demonstrated that metal oxide NPs, but not zerovalent metal NPs, can cross plasma membranes.<sup>1</sup> However, a direct crossing of the plasma membrane by NPs implies a disarrangement of the lipid bilayer structure to let NPs pass through it. This disarrangement can be followed by a lipid reorganization to reseal the plasma membrane. These modifications of the structure of the lipid bilayer should cause a change in the plasma

membrane electrical properties that can be investigated with electrophysiological techniques. Therefore, we expect that a NP that is capable to pass through the plasma membrane open a conductance that will be soon repaired by the cell. Conversely, a NP that does not cross the plasma membrane should not cause a change in membrane resistance. Indeed, we have demonstrated by two electrodes voltage clamp that NP crossing of plasma membrane is paralleled by a small and transient increase of membrane conductance.

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### **NEURAL NETWORK-BASED IDENTIFICATION OF DEVELOPMENTALLY COMPETENT MOUSE ANTRAL OOCYTES**

M. Zuccotti,<sup>1</sup> T.T.H. Bui,<sup>2</sup> M. Belli,<sup>2,3</sup> L. Fassina,<sup>4</sup> G. Vigone,<sup>2,5</sup> F. Cavalera,<sup>1,2</sup> V. Merico,<sup>2</sup> S. Garagna<sup>2</sup>

<sup>1</sup>Department of Medicine and Surgery, University of Parma, Italy; <sup>2</sup>Department of Biology and Biotechnologies "Lazzaro Spallanzani", University of Pavia, Italy; <sup>3</sup>Department of Reproductive Medicine, University of California, USA;

<sup>4</sup>Department of Electrical, Computer and Biomedical Engineering, University of Pavia, Italy; <sup>5</sup>Department of Cell Biology, University of Connecticut, USA

In the mouse, fully-grown antral oocytes are classified based on their chromatin organisation into surrounded nucleolus (SN) and not-surrounded nucleolus (NSN). Whilst SN oocytes may develop to term, NSN oocytes cease development at the 2-cell stage. Here, for the first time, coupling time-lapse observation with the Particle Image Velocimetry method, we analysed the cytoplasmic movements (CMs) occurring during the GV-to-MII transition of SN and NSN oocytes.<sup>1</sup> Then, CM profiles were analysed with an artificial neural network that, after training, classified the oocytes as SN or NSN. This procedure allows the identification of competent oocytes with a mean probability of 92.23% and incompetent oocytes with a mean probability of 88.68%. This study shows that behind a different pattern of CMs occurring during meiosis resumption lies a different developmental competence of the female gamete.

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