5° Congresso annuale

20-21 Dicembre 2007

INDICE DEI LAVORI

A phosphate-controlled regulator for the biosynthesis of the dalbavancin precursor A40926

Alduina R., Lo Piccolo L., D'Alia D., Barrile R., Gunnarsson N., Donadio S. and Puglia A. M.
Dipartimento di Biologia Cellulare e dello Sviluppo "A.Monroy", University of Palermo, Italy
valduina@unipa.it

The actinomycete *Nonomuraea* sp. Strain ATCC 39727 produces the lycopeptides A40926, the precursor of the novel antibiotic dalbavancin. Previous studies have shown that phosphate limitation results in enhanced A40926 production. The A40926 biosynthetic gene (*dbv*) cluster, which consists of 37 genes, encodes two putative regulators, Dbv3 and Dbv4, as well as the response regulator (Dbv6) and the sensor-kinase (Dbv22) of a putative two-component system. Reverse transcription-PCR (RT-PCR) and real-time RT-PCR analysis revealed that the *dbv14-dbv8* and the *dbv30-dbv35* operons, as well as *dbv4*, were negatively influenced by phosphate. Dbv4 shows a putative helix-turn-helix DNA-binding motif and shares sequence similarity with StrR, the transcriptional activator of streptomycin biosynthesis in *Streptomyces griseus*. Dbv4 was expressed in *Escherichia coli* as an N-terminal His6-tagged protein. The purified protein bound the *dbv14* and *dbv30* upstream regions but not the region preceding *dbv4*. Bbr, a Dbv4 ortholog from the gene cluster for the synthesis of the lycopeptides balhimycin, also bound to the *dbv14* and *dbv30* upstream regions, while Dbv4 bound appropriate regions from the balhimycin cluster. Our results provide new insights into the regulation of lycopeptides antibiotics, indicating that the phosphate-controlled regulator Dbv4 governs two key steps in A40926 biosynthesis: the biosynthesis of the nonproteinogenic amino acid 3,5-dihydroxyphenylglycine and critical tailoring reactions on the heptapeptide backbone.

Poster presentation

.....

Silencing RB, Brca1 and Mad2 genes in human cells… what's up there?

Amato A., Barra V., Insalaco L., Lentini L., Schillaci T. and Di Leonardo A.
Dipartimento di Biologia Cellulare e dello Sviluppo "A.Monroy", University of Palermo, Italy

Aneuploidy is a hallmark of most human cancers, but whether it is a cause or consequence of cellular transformation remains a subject of debate. The spindle assembly checkpoint (SAC) functions to prevent aneuploidy and plays a central role in this process. Mad2 is a central component of SAC that controls the anaphase onset and insures the fidelity of chromosome segregation.

Previously we showed that *RB* acute loss in human primary fibroblasts associates with aneuploidy and reduced levels of *MAD2* mRNA.

To investigate if this differential expression of *MAD2* gene could account for chromosomal instability after RB acute loss, we evaluated the effects of *MAD2* transient depletion by RNAi in human fibroblasts. *MAD2* knock-down cells arrested in G1 as suggested by the presence of high levels of $p21^{waf1}$ mRNA, very low expression levels of mitotic genes and absence of mitotic cells. Moreover *MAD2* depleted cells showed multiple centrosomes, premature sister chromatid segregation and endoreduplicated chromosomes. Similar effects we observed after *MAD2* RNAi in human tumor cells as HCT116, even if in these cells RB acute loss promote increased expression of *MAD2* gene.

Recently, Brca1 was shown to control *MAD2* expression at transcriptional levels and we showed that its levels increases after *RB* acute loss in HCT116 cells. Brca1 is a tumor suppressor involved in the DNA damage response, but how it's involved in G2/M transition is not well understood. In the attempt to answer this question we simultaneously silenced *RB* and *BRCA1* in HCT116 cells by RNAi showing that modulating *BRCA1* levels we could reduce centrosome amplification and MAD2 transcription levels, but we couldn't revert chromosomal instability phenotype.

These findings together suggest that *BRCA1* depletion couldn't account for aneuploidy observed after RB acute loss in HCT116 cells, as well as MAD2 depletion in human primary fibroblasts since MAD2 depleted cells showed a different chromosomal instability phenotype.

Role of the cannabinoids in the control of the mouse gastrointestinal mechanical activity

Amato A., Baldassano S., Mulè F.
Dipartimento di Biologia Cellulare e dello Sviluppo "A.Monroy", University of Palermo, Italy

Numerous investigations have recently demonstrated the important roles of the endocannabinoid system in the gastrointestinal tract under physiological and pathophysiological conditions. The endocannabinoid system consists of multiple endogenous ligands, like anandamide and 2-arachidonoylglycerol, biosynthetic enzymes, cannabinoid receptors and uptake and degradation systems. The endocannabinoids are lipids that are able to act on two different seven-transmembrane G-protein-coupled receptors called cannabinoid type 1 (CB1) and cannabinoid type 2 (CB2) receptors. CB1 receptor are highly expressed in the central and peripheral nervous system, but non-neuronal expression sites such as adipocytes and endothelial cells have been described. CB2 receptors are predominantly but not exclusively present in immune cells, suggesting that endocannabinoids have roles as immunomodulators. The cannabinoids are able to modulate several functions in the gastrointestinal tract, including gastric secretion, gastric emptying and intestinal motility. In particular, CB1 receptors are involved in the regulation of smooth muscle contractility and intestinal motility, through a mechanism mainly related to reduction of acetylcholine release from cholinergic nerve endings. The physiological role of CB2 receptors in gastrointestinal tract is not clear. Moreover, to date, the role of cannabinoid receptors in the control of spontaneous mechanical activity of gastrointestinal tract, *in vitro*, is unexplored yet. Therefore, the aims of the present study were: i) to verify whether cannabinoid receptor agonists were able to influence the spontaneous mechanical contractile activity in mouse stomach and ileum; ii) to analyze the mechanisms of action responsible for the observed effects; iii) to examine the receptor type involved.
For these purposes we used organ bath technique. The mechanical activity of stomach, mounted in an horizontal organ bath, was recorded as changes in endoluminal pressure, while the mechanical activity of longitudinally oriented ileum segments was recorded as change isometric tension.

Stomach: Neither the cannabinoid receptor agonists nor the cannabinoid receptor antagonist had any influence on the basal tone or on the spontaneous activity (amplitude of contractions, contractile frequency) of mouse gastric preparations. So we tested the cannabinoid drugs on the responses evoked by electrical field stimulation (EFS). EFS (0.5 ms duration, supramaximal voltage, in trains of 5 s, 2-16 Hz) caused a frequency-dependent contraction, which was abolished by the muscarine receptor blocker, atropine (1 μM), indicating its cholinergic origin. WIN 55,212-2 (1 nM – 1 μM), non-selective synthetic cannabinoid receptor agonist, and anandamide (1 -100 μM), non-selective endocannabinoid, caused a concentration-dependent reduction of the electrically-evoked contractile responses (Fig. 1A), which was antagonized significantly both by the cannabinoid CB1 antagonist, SR141716A (0.1 μM) and by the CB2 receptor antagonist, AM630 (0.1 μM). suggesting the involvement of both receptor subtypes in the action of the cannabinoids. To further confirm the subtype of CB receptors, we tested the evoked cholinergic contraction in the presence of the selective CB1 and CB2 receptor agonists. ACEA (0.01 – 10 μM), selective CB1 receptor agonist, produced a concentration-dependent reduction of cholinergic contractile responses to EFS, prevented only by SR14116A (0.1 μM). Also, JWH133 (0.1 – 10 μM), selective CB2 receptor agonist, caused a concentration-related inhibition of electrically-evoked contractions, prevented only by AM630 (0.1 μM). None of the cannabinoid drugs used had a significant influence on the carbachol (1 μM)-evoked smooth muscle contraction.

Ileum: Isolated segments of mouse ileum displayed spontaneous contractions with amplitude of about 300 mg and frequency of about 30 cpm. Anandamide (1 -100 μM) (Fig. 1B), ACEA (100 nM – 10 μM), but not JWH 133 (100 nM - 10 μM), reduced in a concentration-dependent manner the spontaneous mechanical activity. The inhibitory effect consisted in a decrease of the mean amplitude of longitudinal spontaneous contractions, without changes in the resting tone. The inhibitory effect induced by cannabinoids was significantly antagonized by the SR141716A (100 nM), but not by the AM630 (100 nM). None of the cannabinoid antagonists, at the concentration used, did affect the spontaneous mechanical activity. The ACEA-induced reduction of spontaneous contractions was almost abolished by tetrodotoxin, atropine or apamin and it was unaffected by hexamethonium or N_{ω}-nitro-L-arginine methyl ester (L-NAME), inhibitor of nitric oxide synthase. The myogenic contractions evoked by carbachol were not affected by ACEA.

In conclusion, the results obtained in mouse stomach suggest that the cannabinoids play a prejunctional modulatory role on the cholinergic excitatory neurotransmission and represent the first experimental evidence that activation of CB2 receptors is able to inhibit cholinergic neurotransmission. In the ileum the activation of cannabinoid CB1 receptors may play a role in the control of spontaneous mechanical contractile activity through inhibition of acetylcholine neural release. Small conductance Ca^{2+} -dependent K^+ channels appear to be involved in this mechanism.

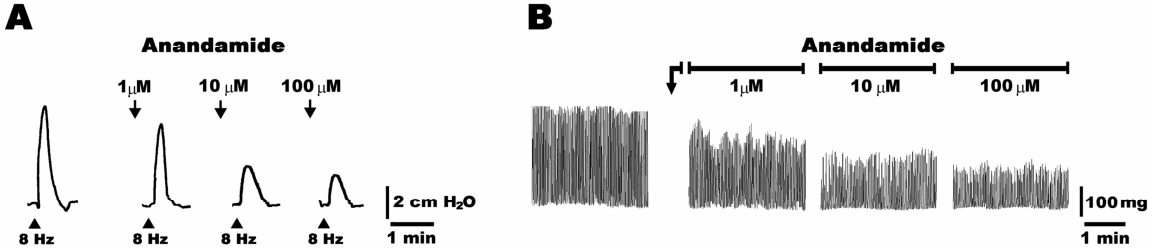


Fig.1: A. *Effects of anandamide on cholinergic contractile responses evoked by EFS in mouse stomach* ***B.*** *Effects of anandamide on the ileum spontaneous mechanical activity.*

Genetic polymorphism of the bitter taste TAS2R38 gene in central Sicily

Barbata G., Caradonna F., Catanzaro I., Mauro M., Saverini M., Sciandrello G.
Dipartimento di Biologia Cellulare e dello Sviluppo "A.Monroy", University of Palermo, Italy

The capability to perceive bitter compounds is regulated by genetic differences in some taste genes. In an evolutionary scenario this ability has played an important role to prevent ingestion of toxic substances.
The first study on the bitter taste perception was carried out in the 1930s when some individuals (nontaster) were found to show a decreased sensitivity to phenylthiocarbamide (PTC), a very bitter substance.
The bitter perception in human is due to a family of 25 genes named TAS2R that encode for G-protein coupled receptors (GPCRs); in particular TAS2R38 gene codes a 7-transmembrane 333 aminoacids-long protein that recognizes PTC as ligand. This gene, also called PTC gene, is located in the chromosome 7 and consists of a single exon 1002 bp. Today it is known that there are 5 SNPs in the TAS2R38 gene that induce aminoacid variations in 5 positions (49,80,262,274 and 296) of the PTC receptor resulting in 32 different haplotypes, those predominant being: PAV (corresponding to taster phenotype) and AVI (corresponding to nontaster phenotype).
The purpose of the present study is to determine PTC gene polymorphism and its correlation with phenylthiocarbamide perception in the central Sicily people. To assess individual threshold of bitter perception, we provided volunteers with paper samples soaked in different concentrations of PTC; moreover, to collect data concerning lifestyle and diet habits we administered a detailed questionnaire. In order to determine the two predominant haplotypes and then the three possible genotypes, we amplyfied a fragment of the PTC gene corresponding to position 49 of PTC. DNA extracted from saliva was amplified by Allelic Specific Olygonucleotides (ASO)-PCR; polymorphisms were evaluated by gel electrophoresis.
Until now, we examined 89 subjects and found that homozygous PAV/PAV genotype is more prevalent (79.78%) than homozygous AVI/AVI (3.37%) and heterozygous PAV/AVI (16.85%) genotypes. All genotypes correlate significantly with phenotypes; in fact the prevalent phenotype is "taster".
These data, pooled with those collected from other italian countries, will contribute to realize the genetic map of TAS2R38 alleles distribution and bitter perception in Italy.

.....

Mung Bean nuclease mapping of RNA 3' end

Bellavia D., Papadopoulos G., Sisino G., Cellura D. and Barbieri R.
Dipartimento di Biologia Cellulare e dello Sviluppo "A.Monroy", University of Palermo, Italy

S1 mapping and /or primer extension methods are used to map the 5'-end of an RNA. Although the mapping of the 3'-end of RNAs is of less importance with respect to the mapping of the 5'-ends, mainly because most of the RNA analysed are poly-A mRNAs, in some instances it could be necessary to map 3'-ends of these molecules (i.e., ribosomal RNAs and small RNAs) and, up to now, no standard procedure has been developed to map 3' ends of RNAs. Here we describe a simple, reliable method to map the 3'-ends of *non* poly-A RNAs.
This method is reminiscent of S1 mapping procedure, and was carried out using a labelled DNA probe complementary to the 3'-end of the RNA, which contains the supposed 3'-end of the RNA under analysis. After the probe was annealed to his target, Mung Bean nuclease was used to digest single strands of both DNA and RNA. Mung Bean nuclease is in fact a single-strand-specific nuclease which digests single strand DNA or RNA with an higher specificity than S1 Nuclease.
This simple procedure is described in Figure 1.

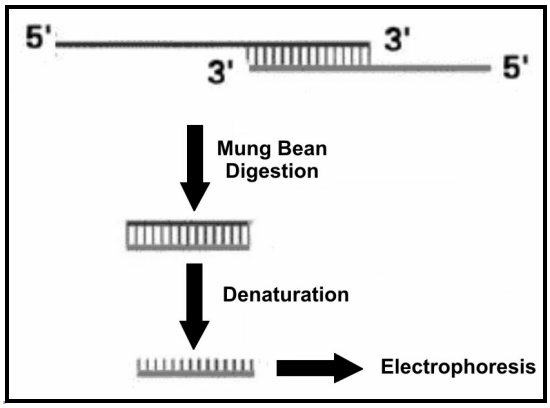


Figure 1

After denaturation, undigested labelled DNA fragments, whose length corresponds to the length of the "protected" DNA probe, is electrophoretically fractionated side by side with a sequence reaction of a known DNA. The last one is used as a DNA marker to measure the length of the undigested probe fragment.
In this abstract we report Mung Bean nuclease mapping of the 3'-ends of both 18S and 26S sea urchin rRNAs, performed using the above described protocol. We incubated 10µg of total RNA extracted from *Paracentrotus lividus* eggs and 100 ng of a labelled DNA probe in the presence of 10U of Mung Bean nuclease at 37°C for 10 minutes.
The result of this experiment is shown in Figure 2, in which an evident single band is visible indicating the length of "protected" probes corresponding to the 3'-ends of 18S and 26S rRNAs.

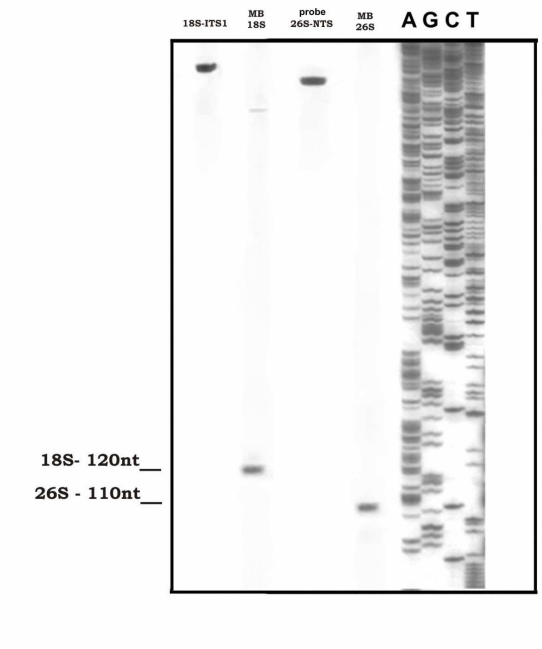


Figure 2

.....

Gene expression profiling of HT-29 cells in response to induction of oncogenic H and K-Ras

Bellavia M. (1), Eterno V. (2), Miranda F. (1), Blanda V. (1), La Farina M. (1), Albanese I. (1)
(1) Dept of Cellular and Developmental Biology , University of Palermo-ITALY
(2) Dept of Medicine, Pneumology, Physiology and Human Nutrition, University of Palermo-ITALY

Ras is often mutated in different types of human cancers and its specific mutations are related with some aspects of the phenotype shown by cancer cells. In more than 90% of tumors, mutations in the ras oncogene affect codon 12 and 13, more rarely codon 61. In all these cases the functional effect of the mutations is the impossibility to switch off the Ras signal in the cell.
In human cells there are three main isoforms of Ras (H, K, N-Ras) which differ in their carboxy-terminal domain which is target of specific post-translational changes. This probably confers to the different isoforms the capacity to localize in different sites within the cell membrane, triggering then alternative pathways of signal transduction. In particular, in different normal and cancer cell lines, it has been observed that induction of oncogenic H-Ras is responsible, in concert with TGF beta and/or ROS formation, for the acquisition of a new phenotype, referred to as epithelial-mesenchymal transition (EMT), which is related with metastasis.
In order to investigate the effects of expression of different oncogenic isoforms of Ras in colorectal carcinoma cells, we stably transfected HT-29 cells with cDNAs coding for H-Ras and K-Ras, mutated in codon 12 and in codon 12 and 13 respectively, under the control of an inducible promoter. The positive clones were called H4 (transfected with H-Ras mutated in codon 12), K1b (transfected with K-Ras mutated in codon 13) and Kr3 (transfected with K-ras mutated in codon 12). We then compared their behaviour in condition of induction of expression of oncogenic Ras (H or K-Ras) and not.
At a morphological level, 48 h after induction, the most interesting date was the acquisition, by the H4 cells, of a shape similar to that of mesenchymal cells. Besides this, 15 days after induction these cells acquired the capacity to form some multicellular bodies which could be described as "colonies". K1b and Kr3 cells also showed some morphological changes, but less evident.
We next performed a proliferation assay which showed for all three clones a growth rate, when cultured in conditions of induction, lower than that in the absence of it. Again the most interesting date was that H4 cells, 48 h after induction, showed a pronounced decrease in their rate of proliferation which didn't increase in the following 6 days. 9 days after

induction the proliferation rate began to increase remaining, however, lower than that in the absence of expression of oncogenic H-ras.
In order to identify genes involved in the acquisition of the changes observed in the examined clones, we performed a comparative analysis of mRNA extracted from HT-29, H4, K1b and Kr3 cells 48 h after induction, using the Differential Display RT-PCR approach. The results of this analysis showed that some cDNA seemed to be differentially expressed in the different clones. We chose and sequenced some of them. On the basis of the sequences we identified three genes in KIb cells (WD 34, RSRC2, RPL10) and three genes in H4 cells (H2AFJ, COX-2, HNRPDL) whose expression appeared increased upon induction of the oncogenic Ras. Northern blot analysis of H2AFJ mRNA levels in H4 cells under condition of induction , confirmed the differential expression of this gene. Using the same approach, we are now analysing the mRNA levels of the other identified genes, trying to confirm their differential expression.
Besides, we have isolated "colonies" formed by H4 cells and in the next future we will analyse their transcriptome to understand the possible molecular mechanisms responsible for the apparent "new behaviour" of these cells.
We believe that such and other studies could be crucial to understand the effects of activation of different oncogenic ras pathways during colon cancer progression.

.....

Extracellular release of Hsp70 from A6 mouse stem cells

Candela ME., Turturici G., Aglieri L., Giacchino MC., Picozza M., Giudice G., Geraci F., Sconzo G.
Dipartimento di Biologia Cellulare e dello Sviluppo "A.Monroy", University of Palermo, Italy

Our previous studies had demonstrated that A6 stem cells synthesize the inducible form of Hsp70 without any stress. In order to study the Hsp70 localization we performed several experiments. We found that Hsp70 co-localizes with actin microfilaments by immunofluorescence assays and that there is a physical interaction between these two proteins such as shown by immunoprecipitation assays. No co-localization or physical interaction between Hsp70 and tubulin has been found. We also observed that some of the Hsp70 is released in the extracellular space. Moreover we found that A6 stem cells are able to release into the extracellular space membrane vesicles after culture medium replacement. These vesicles are produced either in the presence or in the absence of serum, and also in 8% O_2 atmosphere. The presence of Hsp70 inside the vesicles has been demonstrated both by immunoblot assays of isolated vesicles and by double fluorescence assays of cytocentrifugated vesicles. On the basis of Hsp70 intracellular localization we might hypothesize that actin cytoskeleton is involved in the HSP70 release through the vesicles. For this reason we have treated cells with cytochalasin A and nocodazole, two drugs that damage actin and tubulin cytoskeleton. The results showed that actin cytoskeleton, but not tubulin one influence the Hsp70 presence inside the vesicles, while microtubules damage affects vesicles formation.
Inside the vesicles we have also found caveolin-1 and GM1, that are known as two markers of detergent resistant microdomain (DRM). To demonstrate whether or not vesicles are released in the extracellular space involving these microdomains, we have treated cells with methyl-beta-cyclodestrin (MBC), a drug which destroys microdomains by cholesterol capture. We found that vesicle release decreases after MBC treatment, while Hsp70 inside the vesicles does not change. According to these results we are able to hypothesize that vesicle release is related to DRMs.
Poster presentation

.....

Possible mechanisms regulating the expression of nuclear and mitochondrial genes

Cannino G.(1), Ferruggia E. (1), Di Liegro C.M., Sala S. (1,2), Ortega A.(2)., Cuezva J.M. (2) and Rinaldi A.M. .(1)
(1)*Dipartimento di Biologia Cellulare e dello Sviluppo "A.Monroy", University of Palermo, Italy*
(2)*Universidad Autonoma de Madrid, Centro Biologia Molecular Severo Ochoa*

In higher eukaryotes, nucleus encodes all mitochondrial proteins with the exception of 13 subunits of the respiratory complexes. These subunits, together with 2 rRNAs and 22 tRNAs, specific for the mitochondrial translation machinery, are coded by mitochondrial genome (Fernandez-Silva et al., 2003). Cytochrome *c* oxidase (COX) complex and H^+ATP- synthase are built up with both nucleus and mitochondrion contribution. Biogenesis and assembly of the complexes thus requires fine interplay between the two compartments (Duchen, 2004; Cannino et al., 2007). In order to shed light on the regulation of nuclear-mitochondrial interactions, we studied the expression of COXIII (mitochondrion-encoded), COXIV (nucleus-encoded) and β-F1 (nucleus-encoded) subunits. We searched for RNA-binding proteins (RBPs) that could participate to the regulation of COXIII and COXIV in rat tissues and β-F1 subunit in human culture cells.
We previously demonstrated that the expression of COXIII and COXIV is different in diverse tissues analyzed and in the developing rat brain. Since the levels of COXIV protein and mRNA are not linearly related we hypotized post-

transcriptional model of regulation. We demonstrated, by T1 nuclease protection assay, that in developing rat brain the cytoplasmic and mitochondrial fractions, contain two specific COXIV RNA-binding factors (20 and 60 kDa). The 60 kDa mitochondrial factor decreases during development with inverse correlation with accumulation of mature COXIV in the organelles (Cannino et al. 2004). Then, we found that cytoplasmic fractions of heart, kidney and testis contain a factor binding the 3'-UTR of COXIV mRNA, and that mitochondrial extracts of brain and testis, but not of other tissues, contain a protein specifically binding COXIII mRNA. These proteins were not found in the cytoplasmic fractions of the same tissues. These results may suggest the occurrence of tissue-specific post-transcriptional regulation, or post-translational modifications of the involved proteins. Recently, we found that 60 kDa factor binds only the first 38 nucleotides of 3'UTR COXIV. The function of this factor is not yet known but it could have some role in the messenger RNA translation. The factor of 60 kDa binding 3'UTR COXIV has been isolated by streptavidin affinity chromatography and has been sequenced.
The expression of the catalytic subunit (β) of H^+ATP-synthase complex is also exerted at level of mRNA translation during development and cancer cells (Izquierdo JM 1997, Cuezva JM 2007).
Since the RNA binding proteins seem to regulate the expression of these mRNA, we used two methodologies to enriche and characterize the factors: the streptavidin affinity chromatography and a particular maltose-binding protein affinity chromatography (Zhou Z, 2002). We identified four proteins that have been sequenced and characterized as Ras-GTPase-activating protein-binding protein 1 (G3BP); Insulin-like growth factor 2 mRNA binding protein (IMP); B23 nucleophosmin (NPM) and HuR. In addition we verified the effect of these proteins in *in vitro* translation of β-F1 ATPase mRNA assay. In this system G3BP, NPM and IMP inhibit the translation of mRNAs for β- and ARF- mRNAs, while IMP specifically interferes only with the translation of β-F1 ATPase mRNA.

References:

Cannino G., Di Liegro C., Di Liegro I. and Rinaldi A.M. (2004) Neuroscience, 128:91-98.
Cannino G, Di Liegro CM, Rinaldi AM (2007) Nuclear-mitochondrial interaction. Mitochondrion 7(6):359-66
Duchen M.R. (2004) Molecular Aspects of Medicine, 25(4):365-451
Fernandez-Silva P., Enriquez J.A., Montoya J. (2003) Exp Physiol., 88:41-56.
Izquierdo JM, Cuezva JM (1997). Control of the translation efficiency of β-F_1ATPase mRNA depends on the regulation of a protein that binds the 3'untranslated region of the mRNA. Mol. Cell. Biol. 17: 5255-5268.
Cuezva JM, Sánchez-Aragó M, Sala S, Blanco-Rivero A, Ortega AD.(2007) A message emerging from development: the repression of mitochondrial beta-F1-ATPase expression in cancer. J Bioenerg Biomembr. 39(3):259-65
Zhou Z., Licklider L.J., Gygi SP, Reed R. (2002). Comprehensive proteomic analysis of the human spliceosome. Nature, 419: 182-185.

.....

Cytochemical and molecular analyses on mitochondria of immortalized and neoplastic epithelial cells from the human breast

Cannino G., Luparello C. and Rinaldi A.M.
Dipartimento di Biologia Cellulare e dello Sviluppo "A.Monroy", University of Palermo, Italy

We correlated the immortalized non-tumoral HB2 cells (courtesy of Cancer Research UK) and the ER^- MDA-MB231 neoplastic cells, both derived from human breast epithelium, for their mitochondrial mass, respiratory activity and expression of some genes encoding for mitochondrial proteins. In a first set of experiments, we inquired whether the distribution and number of mitochondria could differ between the two cell lines; to this purpose, the same number of cells was incubated with MitoTracker Green fluorochrome, which specifically and permanently binds the lipids of mitochondrial membranes, independently from potential, and therefore represents a suitable tool to measure the mass of the organelles. Mitochondrial respiratory activity was verified with MitoTracker Orange CM-H2 TMROS, the reduced form of the tetramethylrosamine, which becomes oxidized and fluorescent only when enters active cells, representing an optimal tool to measure mitochondrial oxidative activity. After analysis under a laser-scanning confocal microscope, to calculate the total mitochondrial mass we integrated the values of pixel intensities from 10 different focal plans of 50 different microscopic fields, thereby demonstrating that the mitochondrial mass of the two cell lines was comparable. Surprisingly, when the same microscopic fields and focal plans were evaluated for the pixel intensity of MitoTracker Orange we observed a significant increase of oxidative activity in the tumoral cell line. In a second set of experiments we analyzed the expression levels of some genes encoding for mitochondrial proteins, i.e. the nuclear gene for the subunit IV (COXIV) and the mitochondrial gene for the subunit III (COXIII) of the cytochrome oxidase complex (COX). We demonstrated by Northern blots that the amount of COXIV mRNA in the two cell lines was comparable, while COXIII mRNA was more abundant in-tumor cells. In addition, we studied by Western blots the accumulation of COXIII, COXIV and hsp60 proteins, demonstrating that COXIII and COXIV were more abundant in tumor cells, differently from hsp60 whose amount, surprisingly, was higher in immortalized non-tumoral breast cells.

Poster presentation

Astenozoospermy and mitochondrial DNA

Carra E., Salerno B., Gerardi E. and Rinaldi A.M.
Dipartimento di Biologia Cellulare e dello Sviluppo "A.Monroy", University of Palermo, Italy

Male infertility, inability to fecundate the oocyte, is due to alterations of sperm characteristics (low number, low motility, altered morphology), to the quantity of semen or to the presence of alterations in the male genital line.
75% of infertile men deal with untreatable sub-fertility. These subjects are either oligospermic producing less than the normal number (20 million sperm/ml) of sperms, or astenozoospermic with high percentage of immotile sperms. It is almost universally recognized that mitochondria play a crucial role in sperm health and fertility, in fact defective mitochondria have been associated with male infertility (Cummins et al., 1994; 1997; 1998) and it has been hypothesized that deletions or multiple rearrangements of mtDNA can cause defective sperm function (Etienne et al, 1997, 2001). St. John et coll. (2001) demonstrated that the multiple deletions of mtDNA are associated with poor quality of the seminal liquid. Ruiz-Pesini et al. (1998) identified a specific mtDNA aplogroup (T) more abundant in astenozoospermic populations and specific differences in the activity of some oxidative phosphorylation (OXPHOS) enzymes, suggesting that the lower ATP production capacity of T aplogroup might be responsible for the differences in sperm motility. Interestingly O'Connell et al. (2002 a, b) have observed a close relationship between mitochondrial DNA deletions and nuclear DNA fragmentation, probably because of the strict interaction between mitochondrial and nuclear genomes.
Motility, viability, concentration and mitochondrial respiratory activity "in vivo" of semen of 50 idiopathic astenozoospermic patients of age between 19 and the 48 and of 20 individuals with normal seminal parameters as control, were analyzed. DNAs purified form motile and immotile fractions of the same individuals were subjected to PCR with appropriate primers. Results showed that mtDNA and Y chromosome regions from the motile fractions of patients and controls gave amplifications, while, the non-motile fractions of the same seminal samples did not give amplifications of ND5, ND6 and regions inside the D-loop (Carra E. et al.2000, 2004). We recently submitted the DNA of patients to 10 different PCR assays using primers covering 25 mtDNA loci containing the majority of mitochondrial genes and giving rise to amplification of short and long DNA regions. The locus analyzed comprised the following genes:for ND6, ND5, ND4 and ND4L of the complex I; COI, COII and COIII of the complex IV; ATPase 6 and ATPase 8 of complex V. The results show that only for 14 patients exhibited the predicted PCR products. For the remaining 36 patients we found deletions in the genes coding for the subunits COI, COII, ND5 and ND6 and for the cytochrome b (CYTb). In addition, using primers for the region comprising the genes for ND6, tRNA Glu and the CYTb we found deletions in the mtDNA of 42 patients over 50, suggesting that the region analyzed was more prone to deletion incidence. We then related the deletions to sperm motility percentage, in order to contribute to a more precise and complete diagnosis of male infertility.

References

Carra E, Sangiorgi D., Montalto F., Amato C.,Gattuccio F., Rinaldi A.M. (2000) *Studio del DNA mitocondriale nell'infertilità maschile*, "G.I. di Ostetricia e Ginecologia", n. 3
Carra E., Sangiorgi D., Gattuccio F., Rinaldi A.M. (2004) *Male infertility and mitochondrial DNA*, "BBRC" 322: 333-339.
Cummins J.M. et al. (1998) *Semen levels of mitochondrial DNA deletions in men attending an infertility clinic do not correlate with phenotype;* "Int. J. of Andrology, vol.21, 47-52.
Ruiz-Pesini et al. (1998) *Correlation of sperm motility with mitochondrial enzymatic activities*; Clinical Chemistry, vol.44, n.8, 1616-1620.
St. John J.C., Roobin P. J., Barrat L.R.C.(2001), *Men with oligoasthenoteratozoospermia harbour higher numbers of multiple mitochondrial DNA deletions in their spermatozoa, but individual deletions are not indicative of overall aetiology*; "Molecular Human Reproduction, Vol. 7, No. 1, 103-111.

Poster presentation

.....

Ancient DNA analysis determines the taxonomic status of Orang-Utan (*Pongo pygmaeus*) museum specimen.

*[1] Catalano G., [2] Sineo L.& [1] Caramelli D.
[1]Dipartimento di Biologia Animale e Genetica ,Laboratorio di Antropologia, Universita` di Firenze, Italy
[2] Dipartimento di Biologia Animale "G. Reverberi" - Universita` di Palermo, Italy;

(∗author for correspondence, e-mail: giulio.catalano@unifi.it)

Keywords : Museum Collections, Ancient DNA, Conservation**.**

Using methods derived from the ancient DNA technologies, sequences of the mitochondrial ribosomal subunit 16S were obtained from a museum specimen of *Pongo pygmaeus* and analysed in order to discover its taxonomic status, and to ascribe it to one of the two still existing subspecies, the Bornean Orang-Utan (*Pongo pygmaeus pygmaeus*),

and the Sumatran Orang-Utan (*Pongo pygmaeus abelii*). The specimens, on the base of genetic analysis belong to the Bornean species. Our results demonstrate the efficacy of genetic testing in the identification of museum species.
In the near future the application of these methodologies would be of great help in the evaluation of the genetic structure and variability of extant species their representatively and availability is limited by issues in conservation.
Poster presentation

.....

Hbox12 homeodomain transcription repressor and the *Ecto-like* signaling molecule cooperatively direct the oral ectoderm network of the *Paracentrotus lividus* sea urchin embryo.

Cavalieri V.and Spinelli G.
Dipartimento di Biologia Cellulare e dello Sviluppo "A.Monroy", University of Palermo, Italy

Embryonic development is coordinated by networks of evolutionary conserved regulatory genes encoding transcription factors and components of cell signaling pathways (1, 2). In the sea urchin embryo, a number of genes encoding transcription factors display territorial restricted expression. Among these, the zygotic *Hbox12* homeobox gene is transiently transcribed in a limited number of cells of the animal-lateral half of the early *Paracentrotus lividus* embryo, whose descendants will constitute part of the ectoderm territory (3). To obtain insights on the regulation of *Hbox12* expression, we have explored the *cis*-regulatory apparatus of the gene. We show that the intergenic region of the tandem *Hbox12* repeats drives GFP expression in the presumptive aboral ectoderm and that a 234 bp fragment, defined aboral ectoderm (AE) module, accounts for the restricted expression of the *transgene*. Negative sequence elements that repress transcription in the vegetal lineages, and a stretch of sixteen *GA* repeats involved in the down-regulation of *Hbox12* at gastrula stage, are contained in the most upstream 60 bp region of the module. By contrast, loss of function assays, along with ChIP analysis at morula and gastrula stages, indicate that Otx, a regulator involved in the aboral ectoderm differentiation, binds to a canonical consensus site at the 3'-end of the AE module and acts as a direct positive driver input for *Hbox12* expression. As it has been reported, the Otx regulator function is absolutely required for aboral ectoderm specification. In agreement with these observations, we found that the ectopic expression of a dominant-negative Otx fusion had drastic defects in aboral ectoderm specification also in *P. lividus* embryos. Interestingly, embryos expressing such an obligate repressor appeared radialized and promoter activity of both *transgene* and endogenous *Hbox12* were severely reduced with almost identical kinetics. Moreover, functional experiments strongly suggest an early involvement of Hbox12 in the polarization of the oral-aboral axis, probably by repression of Nodal in the aboral side of embryos. Multiple gene copies of *Hbox12* exist in the *P. lividus* genome and most of them are tandemly arrayed. In such a genomic segment we identified two copies of a gene encoding for a RING-type E3 ubiquitin ligase, highly similar to the signaling molecule *Ectodermin*, that we called *PlEcto-like*. Interestingly, *Ectodermin* (*Ecto*) was discovered as a factor localized in the nuclei of the animal blastomeres of the *Xenopus* embryo (4). Functional experiments demonstrated that *Ecto* encodes a signaling molecule that antagonize the Nodal signaling pathway and is therefore essential for the specification of the ectoderm germ layer. The biological activity of Ecto primarily stems from the enzymatic activity of its RING-finger: Ecto induces Smad4 degradation via the ubiquitination-proteasome pathway. Similarly, we found that transcripts of *PlEcto-like* are distributed throughout the oral-aboral axis of the sea urchin embryo. Interestingly, *PlEcto-like* expression occurs early in the founder cells of the embryonic ciliary band and follows this lineage till later stages of development. Taken together, these results lead us to argue that restriction of the Nodal activity, in the sea urchin embryo, could result by a double effect: 1) the early inhibition of Nodal transcription in the aboral ectoderm via Hbox12 repression and 2) the restriction of Nodal activity probably by the *PlEcto-like* dependent degradation of the terminal transducer Smad4. Additional experiments are necessary to confirm this possibility.

References:

1. Angerer, L. M., and Angerer, R. C. (2003). Patterning the sea urchin embryo: gene regulatory networks, signaling pathways, and cellular interactions. *Curr Top Dev Biol* **53,** 159-98.
2. Davidson, E. H., Rast, J. P., Oliveri, P., Ransick, A., Calestani, C., Yuh, C. H., Minokawa, T., Amore, G., Hinman, V., Arenas-Mena, C., Otim, O., Brown, C. T., Livi, C. B., Lee, P. Y., Revilla, R., Rust, A. G., Pan, Z., Schilstra, M. J., Clarke, P. J., Arnone, M. I., Rowen, L., Cameron, R. A., McClay, D. R., Hood, L., and Bolouri, H. (2002a). A genomic regulatory network for development. *Science* **295,** 1669-78.
3. Di Bernardo, M., Russo, R., Oliveri, P., Melfi, R., and Spinelli, G. (1995). Homeobox-containing gene transiently expressed in a spatially restricted pattern in the early sea urchin embryo. *Proc Natl Acad Sci U S A* **92,** 8180-4.
4. Dupont S., Zacchigna L., Cordenonsi M., Soligo S., Adorno M., Rugge M., Piccolo S. (2005). Germ-layer specification and control of cell growth by Ectodermin, a Smad4 ubiquitin ligase. *Cell* 2005 Apr 8; **121**(1):87-99.

Poster presentation

A metallothionein family member interacts with the intracellular domain of the low density lipoprotein (LDL) receptor

Costa S., Nicosia A., Ragusa M.A., Pollaccia D., Valenti V., Cefalù A.B., Averna M.R. and Gianguzza F.
Dept. of Clinical Medicine and Emerging Diseases; Dept of Cellular and Development Biology –Palermo
E-mail: cefaluab@unipa.it; costas@unipa.it;

Background: Familial Hypercholesterolemia (FH) is an autosomal dominant disorder caused by mutations in the low-density lipoprotein receptor (LDLR) gene, characterized by elevated plasma LDL cholesterol (LDL-C) levels which results in premature coronary artery disease. LDL-C is mostly removed from the plasma through a specific binding between LDLR and apolipoprotein B (apoB), which is the main proteic component of LDL, and the complex LDL/LDLR is internalised in a clathrin-dependent manner. So far, several scaffold and adaptor proteins involved in the LDLR endocytosis have been described, such as SNX17, ARH, Dab-2, Numb, b arrestin 2, but little is still known about the protein-protein interaction and the cascade that lead to the internalisation and recycling of the LDLR.
Methods: In order to investigate novel proteins interacting with cytoplasmic domain of LDL receptor we screened a human liver cDNA library using a Two-Hybrid assay. LDLR transcript coding LDLR intracellular domain (aa residues 788-839) was retro-transcribed, amplified and cloned into pGBKT7 vector to be expressed in yeast as a fusion protein with the DNA binding domain of Gal4 and the c-myc epitope tag. 5.7 x 106 clones were screened, 700 colonies of interactors were identified and analyzed by sequencing; 31% encoded metallothionein (MT2 -accession number: M26637.1). Physical interaction between MT2 and the LDLR cytoplasmic domain was confirmed in a coimmunoprecipitation assay with the LDLR-cytosolic tail c-Myc epitope tagged and MT2 containing an HA epitope tag, both in vitro transcribed and translated in presence of 35S methionine and cysteine. The complex was precipitated using c-Myc monoclonal antibody or HA-Tag poyclonal antibody in two separate reactions.
In order to map the protein region responsible for the interaction, we generated three deletion mutants missing respectively 14, 39 and 73 aa residues at the C-terminal end. No one of the three clones was able to interact with the cytoplasmic domain of LDL receptor in a two hybrid assay, so restricting the position of the binding site at C-terminal end exactly in the last 14 aa of the protein.
Results: Our experiments suggest that MT2 could represent a new candidate protein involved in the internalisation of LDLR. Further experiments will be performed in order to confirm this preliminary results and to assign a possible functional role for MT2/LDLR interaction.

.....

Supporting a Molecular Phylogeny of Cebidae Family group by Reciprocal Chromosome painting.

Dumas F.[1], R. Stanyon[2], L. Sineo[1], G. Stone[3], F. Bigoni[2]
[1]*Dipartimento di Biologia animale (DBA) Università degli Studi di Palermo, via Archirafi 18. Palermo, Italy*
[2]*Dipartimento di Biologia Animale e Genetica, Laboratori di Antropologia, Via del Proconsolo 12, 50122 Firenze, Italy*
[3]*Comparative Molecular Cytogenetics Core, National Cancer Institute, Frederick Maryland*
key words: Primates, Platyrrhine, Chromosome Painting, Phylogeny.

A recent molecular phylogeny divides Platyrrhine into three families: Atelidae, Cebidae, and Pithecidae. The Cebidae group is the most controversial including species belonging to two different families if considered the traditional taxonomy (Cebidae, Callithricidae). Here we analyse this new hypothesis using a Reciprocal Chromosome Painting approach, after chromosome flow sorting of metaphases of several species included in the Cebidae family: *Callithrix argentata* (silvered-marmoset), *Cebuella pygmaea* (pygmy marmoset), *Callimico goeldii* (Goeldi's marmoset) and *Saimiri sciureus* (squirrel monkey). The reciprocal painting of monkey probes on human chromosomes revealed that 21 breakpoints are common to all four studied species. Here we demonstrate that the five chromosome associations previously hypothesized to phylogenetically link tamarins and marmosets are homologous and represent derived chromosome rearrangements, which occurred in a common ancestor. Four of these derived homologous associations tightly nest *Callimico goeldii* with marmosets. One derived association 2/15 may place squirrel monkeys within the Cebidae assemblage. Those reciprocal painting results, in general, support the molecular genomic assemblage of Cebidae.
Poster presentation

Caracterization of a 4.6 Kb upstream region necessary for the specific α2 neural tubulin gene expression in *P. lividus* embryos.

Emanuele M., Nicosia A., Longo V., Costa S., Ragusa M.A., Casano C., Gianguzza F.
Dipartimento di Biologia Cellulare e dello Sviluppo "A.Monroy", University of Palermo, Italy

P. lividus alfa-tubulin gene PlTα2 is expressed exclusively in the neurogenic territory of the embryo from blastula to pluteus stage. In order to characterize this gene, we isolated the PlTα2 genomic clone and we determined its genomic organization (Costa et al.2004). To test whether the 4.6Kb upstream region of PlTα2 drives gene expression correctly, we cloned the region in frame with GFP reporter gene.
Microinjection analysis showed that the complete construct (4.6kb-GFP) contains all the informations necessary for the correct temporal and tissue gene expression profile.
Functional analysis of deletion constructs suggest that the region from -4.6kb to -4.2 kb contains negative cis-regulatory elements that restrict the spatial expression of PlTα2 gene just in the neural structures of the embryo. Deletion of this region, in fact, cause GFP ectopic expression in all embryo tissues. Comparing fluorescence levels of 3.5Kb and 2.5Kb construction microinjected embryos we observed an abrupt decrease of fluorescence level so we hypotize the presence of an enhancer element in this region.
Furthermore analysis of deletion clones demonstrated that the region from -203 to +95 is sufficient for the basal gene expression and TATA-box remotion impairs the reporter gene expression.
EMSA and footprinting analysis allowed us to identify the potential binding sites for a homeobox family Nkx2 factor at -580 and -230bp from the starting point, in opposite orientations.
Specific DNA–protein interactions, GATA family and Sp1 binding sites, were also detected and identified using probes covering the region spanning from -4.6 Kb to -4.2 Kb.
Biochemical analysis of the presumptive enhancer region will be necessary for a complete characterization. In vivo competition assay using multimers of identified binding sites and knock down experiment using morfolino technology for putative transcription factors should be performed.
Poster presentation

.....

hisX*1 plays an essential role in the histidine biosynthetic pathway of S*treptomyces coelicolor

Fanale M.G., Calamia E., Girgenti S., Giordano G., Giunta A., Rizzuto B. and Puglia A.M.
Dipartimento di Biologia Cellulare e dello Sviluppo "A.Monroy", University of Palermo, Italy

The biosynthesis of histidine and its regulation have been extensively studied in enteric bacteria such as *Escherichia coli* and *Salmonella typhimurium* and in the Gram-positive *Bacillus* spp. where the genes involved in histidine biosynthesis are finely regulated at different levels including attenuation.
In the soil-dwelling Gram-positive bacterium *Streptomyces coelicolor,* little information is available on histidine biosynthetic genes regulation. Comparative analysis of RNA regulatory elements of amino acid metabolism genes in Actinomycetes does not reveal conserved regions upstream of histidine biosynthetic genes.
In *S. coelicolor* an unusual feature of a histidine operon is the occurrence of a small open reading frame, *his*X1, to which no function has been ascribed.
In order to elucidate the role of *his*X1 we constructed knock out mutants and strains in which *his*X1 is over expressed.
The *his*X1 of *Streptomyces coelicolor* has been disrupted using a PCR targeting based method called Redirect© technology. This method allows the replacement of a chromosomal sequence within a *S. coelicolor* by a selectable marker that has been generated by PCR using primers with 39 nt homology extensions.
Interestingly, the *S. coelicolor* Δ*his*X1 mutant does not grow on minimal medium unless supplemented with histidine.
Moreover, in silico analysis of aminoacid sequence of *his*X1 does not show conserved domains and similarity with proteins of different organism. On the other hand structure prediction analysis using Internet Service Robetta by de novo structure prediction method suggests that HISX1 could be involved in transcriptional regulation mechanisms.
A *hisX*1 over expressing mutant strain has been constructed, and his characterization is under investigation.
Our resulted suggest that orf1 plays an essential role in histidine biosynthetic pathway of S*treptomycin coelicolor*

References

Hodgson DA. *Adv Microb Physiol.* **42**,47-238 (2000).
Hu, D.S.J., Heisdstra R. & Hodgoson D.A. *Molecular Microbiology* **32(4),** 869-880 (1999).
Gust B. *et al.* "PCR-targeting system in *Streptomyces ceolicolor". John Innes Centre. (*2002)

Poster presentation

Cadmium insult and defence mechanisms in *Paracentrotus lividus* embryos and larvae

Filosto S., Agnello M., Gabriele G., Bonaventura R.*, Matranga V*, Luparello C., Roccheri M.C.
Dipartimento di Biologia Cellulare e dello Sviluppo "A. Monroy" Viale delle Scienze, Palermo
**Istituto di Biomedicina ed Immunologia Molecolare "A. Monroy" Consiglio Nazionale delle Ricerche, Palermo*

Marine invertebrate embryos represent a suitable model system where to investigate on the effects of many stressors, like heavy metals, on development and cell viability. Here we studied toxic effects and defense response by cadmium in *Paracentrotus lividus* sea urchin embryos and larvae, cultured with $CdCl_2$ since fertilization. We demonstrated that the metal is accumulated by the embryos depending both on concentration used and on time of exposure. In addiction we showed the possible competition between cadmium and calcium ions for using the same ionic channels. Our previous works described morphological abnormalities and apoptotic events induced by cadmium for both concentrations of 10^{-3} M and 10^{-4} M (Agnello et al, 2006; 2007). In this study we reduced noticeably the concentration of cadmium and prolonged the exposure time of *Paracentrotus lividus* zygotes until the feeding larval stages up to the metamorphosis, in order to test the toxic effects of low (10^{-12}M), medium (10^{-9}M) and high (10^{-6}M) cadmium chloride concentrations, mimicking environmental natural or polluted sea waters. We demonstrated that prolonged exposure to lower cadmium concentrations causes similar defects to those observed for short treatments with higher concentration of the metal. Moreover, we showed DNA fragmentation and activation of caspase-3, following $CdCl_2$ exposure. Thus, we can hypothesize that in sea urchin embryos/larvae apoptosis can be considered as part of a defence strategy in response to cadmium.
In addiction, we investigated the expression of caspase-3 protein and the transcription of relative mRNA during first stage o development of *P. lividus*. Immunocytochemical experiments demonstrated that caspase-3 protein is expressed in stage-dependent manner, during early development and it is quite represented since mesenchime blastula stage, 17h after fertilization. RT-PCR assays showed a modulation of transcription of mRNA of caspase-3 during first cellular divisions: caspase-3 mRNA goes to accumulation until mesenchime blastula stage; it is partially degradated during gastrulation and newly accumulated at pluteus stage. Other caspases seem to have a similar behaviour of transcription, suggesting that this could be a general synthesis strategy. In conclusion, our data suggest that sea urchin embryos would not be competent to activate defensive apoptotic mechanisms during stage preceding the mesenchime blastula stage.
Poster presentation

.....

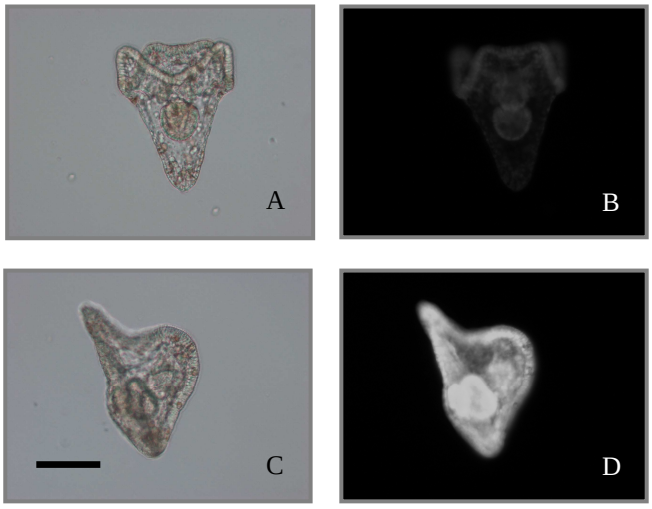
Interference of metals in sea urchin embryo development

Filosto S., Pinsino A., Agnello M., Balistreri R., Roccheri M.C.
Dipartimento di Biologia Cellulare e Dello Sviluppo "A.Monroy", Università degli Studi di Palermo

Continuous exposure to all organisms to environmental stressors leads to greater risk of harmful health outcomes. Among compounds metals are a major contaminants generated by human activities and represent an actual hazard for aquatic ecosystems. Marine organisms can take up metals from solution and diet, which may consist of particles in suspension or deposited in the sediment. A great number of factors may influence dose-effect and dose-response relationships between metals and organisms.
Life began in the sea, and its tolerance to and use of trace metals reflect seawater concentrations. Trace metals are of environmental interest both as limiting nutrients (Fe, Zn, Mn, Cu, Co, Mo and Ni), playing important roles in metal-requiring and metal-activated enzyme systems, as toxicants. Toxic metals include heavy metals as Cd, Hg, Ag, Pb, Sn and Cr, but several nutrients metals can act as toxicants at elevated concentrations. Responses to toxicants are developmental stage specific, and for many echinoids, pluteus and larva stages are more sensitive to toxicants than earlier developmental stages (Gopalakrishnan et al, 2007). The stressors can trigger biological responses at the organisms only after initiating biochemical and cellular events. In the present study we investigated the distinct interference of cadmium and manganese in sea urchin embryo development and the capacity of these to withstand to each single insult activating different mechanisms.
We studied the effects of cadmium on sea urchin embryos and larvae (*Paracentrotus. lividus*), cultured in the presence of $CdCl_2$ since fertilization. We demonstrated that cadmium is accumulated during treatment depending both on metal concentration and on exposure time (AAS analysis). The accumulation leads to developmental delay and morphological abnormalities. In addiction, we found a probably relationship between cadmium and calcium bivalent ions for using the same ionic channels. We showed that cadmium exposure triggers: decrese of pattern proteins, HSPs synthesis, and/or DNA fragmentation, which is, almost partially, caused by apoptotic events, linked by the activation of caspase-3. Moreover, production of reactive oxygen species (ROS) was explained following cadmium treatment (Fig. 1).
We also investigated on effects of manganese on sea urchin embryos continuously cultured in the presence of the $MnCl_2$ from fertilization. manganese showed inhibitory effects on embryo development, producing specific malformation in a time- and dose-dependent manner (Fig. 2). More then 80% of the embryos treated with the highest

manganese concentration exhibit major skeletal defects, that could be due to perturbation to PMC migration into the bastocoel. Manganese (Mn^{2+}) accumulation into the embryos tissues was determined by AAS analysis. We explored whether or not different concentrations of manganese exposure, would produce a cellular stress response in sea urchin embryos, by the up-regulation of synthesis of key stress proteins or by apoptosis. We found that manganese exposure caused an increase in HSC70 and HSC60 levels, but not in HSC90 levels. On the contrary to cadmium exposure, manganese not induce new synthesis of the inducible forms of HSPs and embryos show no increases in apoptosis in comparison to controls. Apoptosis was measured by the TUNEL assay.

FIGURE 1



ROS induction by cadmium in aberrant pluteus stage (fluorescence microscopy): A, B) Control Pluteus (42h), light and fluorescence microscopy. C, D) Pluteus treated for 42h with $CdCl_2$ 100 µM since fertilization, light and fluorescence microscopy. Bar = 70µm.

FIGURE 2

Sea urchin embryos (46h of development) continuously cultured in the presence of 61mg/l Mn^{2+} from fertilization: A) Control pluteus; B) Treated pluteus. Bar = 35µm.

.....

Proteomic analysis of *Amycolatopsis balhimycina* to reveal novel links between primary metabolism and antibiotic production

Gallo G., Li Greci L., Alduina V., Renzone G.[*], Scaloni A.[*] and Puglia A. M.
Dipartimento di Biologia Cellulare e dello Sviluppo, Università degli Studi di Palermo, viale delle Scienze, 90128 Palermo, Italy. Email: giumir@msn.com.
[*]*Proteomics and Mass Spectrometry laboratory, ISPAAM, National Research Council, via Argine 1085, 80147 Napoli, Italy.*

The glycopeptides, "drugs of the last resort" in treating life-threatening infections, are an important class of antibiotics with vancomycin and teicoplanin currently in clinical use. The actinomycete *Amycolatopsis balhimycina*, producing the glycopeptide antibiotic vancomycin-like balhimycin, has been used as model strain to study the genetic and the biochemistry of glicopeptide biosynthesis[1-5]. The *bal* gene cluster, responsible for balhimycin biosynthesis, was so far sequenced[1] and the whole *A. balhimycina* genome sequencing, started in the within of the European COMBIG-TOP project, is almost accomplished.
The aim of this work was to investigate the relationships between primary metabolism and antibiotic production by proteomic approach. In particular, a comparative study of *A. balhimycina* global protein expression profiles was performed on the wild-type (Wt) strain before and during balhimycin production as well as on two isogenic non-producing strains, SP1.5[1] and *ΔoxyD*[4], at late-growth stages by using the following procedures: 2-D gel electrophoresis for protein separation; *in silico* 2D-gel image comparative analysis for quantitative protein profiling; MS-MALDI-TOF analysis for protein identification.
Using three pH ranges for isoelectrofocusing ranging from pH 3 to 10, from 4 to 7 and from 4.5 to 5.5, respectively, three proteome reference 2D-maps were constructed comprising a total of 257 identified protein spots. Among these them, 139 are primary metabolism proteins differentially expressed before and during balhimycin production in the Wt strain as the rest show constant expression values. These proteins, that will be further described in detail, were clustered in functional groups according to BioCyc (http://www.biocyc.org/) and KEGG

(http://www.genome.ad.jp/kegg/kegg2.html) databases. Quantitative RT-PCR analysis of 14 genes, encoding differentially expressed products, revealed that in most cases the transcriptional and translational expression profiles coincided. Furthermore, comparative proteomic analysis showed that the 2D-protein profiles of the non producing strains during late growth stages are very similar to that one of the Wt strain before starting antibiotic production.
In conclusion, this study suggests novel links between balhimycin production and primary metabolism, and reveals putative target genes to construct a rationally engineered high yielding antibiotic producer strain.

References

[1]Pelzer S., Süßmuth R., Heckmann D., Recktenwald J., Huber P., Jung G., and Wohlleben W. (1999). *Antimicrobial Agents and Chemotherapy*, p. 1565-1573, Vol. 43, No. 7.
[2]Pfeifer V, Nicholson GJ, Ries J, Recktenwald J, Schefer AB, Shawky RM, Schroder J, Wohlleben W, Pelzer S. (2001). *J Biol Chem*. Oct 19;276(42):38370-7.
[3]Puk O., Huber P., Bischoff D., Recktenwald J., Jung G., Sussmuth R.D., van Pee K.H., Wohlleben W., Pelzer S. (2002). *Chem Biol.* 2002 Feb;9(2):225-35.
[4]Puk O., Bischoff D., Kittel C., Pelzer S., Weist S., Stegmann E., Sussmuth R.D., Wohlleben W. (2004). *J Bacteriol.*;186(18):6093-100.
[5]Stegmann E., Pelzer S., Bischoff D., Puk O., Stockert S., Butz D., Zerbe K., Robinson J., Sussmuth R.D., Wohlleben W. (2006). *J Biotechnol.* Aug 5;124(4):640-53.

Poster presentation

.....

Intracellular and extracellular Hsp70 in A6 mouse stem cells.

Geraci F., Turturici G., Candela ME., Giudice G., Sconzo G.
Dipartimento di Biologia Cellulare e Dello Sviluppo "A.Monroy", Università degli Studi di Palermo

Hsp70 was originally described as an intracellular protein with a chaperone role, induced by several kinds of stress such as environmental, pathological and physiological stress. Recently an additional role has been ascribed to it as danger signal molecule released in the extracellular space and as activator of the immune system. In our laboratory we have demonstrated that A6 mesoangioblast stem cells constitutively synthesize the inducible Hsp70. For this reason we carried out both in *vivo* and in *vitro* experiments to determine which factor are involved in this basal transcription. Super shift assays demonstrated that HSF1 is responsible for the stress response, but neither HSF1 nor HSF2 are involved in the basal HSE-protein complex. In order to verify if in the A6 stem cells the basal hsp70 transcription was due to CHBF/Ku, as it has been described in other cell lines, we carried out experiments both in *vitro* and in *vivo*. Supershift assays showed the ability of subunit Ku70 to bind HSE consensus in vitro, and chromatin immunoprecipitation assays, using anti-Ku70 antibodies, confirmed that in *vivo* Ku is bound on the proximal hsp70 promoter region, containing two HSE consensus. The importance of this consensus in constitutive Hsp70 expression is demonstrated by reporter gene assays. The expression of the β-gal reporter gene under the control of the prossimal promoter of hsp70.3 deleted in the HSE region, was highly reduced. A reduction in β-gal expression was also observed if the promoter was muted in the GC and in the GAGA region. All together these results demonstrated that the HSE consensus is involved in Hsp70 basal expression and Ku is responsible for this transcription.
To investigate the role of the constitutive Hsp70 expression in A6 stem cells we carried out a stable RNA interference of both hsp70.1 and hsp70.3, the two genes that in mouse transcribe for Hsp70. We have obtained the A6-NM3 clone with a 55% knockdown for Hsp70. Test of cytostaticity indicated that there are not any differences between A6 and A6-NM3 in cell distribution during the cell cycle phases. On the contrary, proliferation assays showed that A6-NM3 have a longer doubling time than A6 (23.4 vs h 16 h). These results indicated that in A6 stem cells the basally expressed Hsp70 might influence the cell proliferation.
We also observed that part of the Hsp70 is released in the extracellular space. In addition we found that A6 stem cells after culture medium replacement are able to release into the extracellular space membrane vesicles. These vesicles are produced either in the presence or in the absence of serum, and also in 8% O_2 atmosphere. These vesicles are about 1 μm in size and contain caveolin-1 and GM1, known to be markers of detergent resistant microdomains (DRMs). Moreover, immunoblot assays and double fluorescence revealed the presence of Hsp70 inside the vesicles released by A6 cells.
Moreover, we found that part of the intracellular co-localize with actin microfilaments, and that there is a physical interaction between these two proteins such as shown by immunoprecipitation assays. No co-localization or physical interaction between Hsp70 and tubulin has been found. On the basis of intracellular localization we studied whether the actin cytoskeleton is involved in the HSP70 release through the vesicles. For this reason we have treated cells with cytochalasin A and nocodazole, two drugs that damage actin and tubulin cytoskeleton. The results showed that actin cytoskeleton, but not tubulin one influences the Hsp70 presence inside the vesicles, while microtubules damage affects vesicles formation.
As vesicles contain DRM markers, to demonstrate whether or not they are released in the extracellular space with the involvement of these microdomains, we have treated cells with methyl-beta-cyclodestrin (MBC), a drug which

destroys microdomains by cholesterol capture. We found that vesicle release decreases after MBC treatment, while Hsp70 inside the vesicles does not change. According to these results we are able to hypothesize that vesicle release is related to DRMs.

.....

Role of proteolytic enzymes on ECM degeneration into two different mouse's pathological system

Lo Cascio L., Thumbi J., Bongiovanni A. (1) and Ghersi G.
Dipartimento di Biologia Cellulare e dello Sviluppo, Università di Palermo; (1) *Istituto di Biomedicina e di Immunologia Molecolare, C.N.R., Palermo.*

Aim of our work is to investigate the possible variations on the expression of proteolytic enzymes and adhesion molecules in pathologic conditions of the skeletal muscle. The contributes of the mesenchymal and satellite cells of mice are also investigated about the expression of these molecules. Our investigation is carried on primary cultures from wild type and pathological mice.
Proteases are a heterogeneous family of enzymes with degradative activity on protein substrates. Into specific our work focuses on two classes of proteases that work on the outer space of the cellular membrane. Membrane Metalloproteases (MMPs) are enzymes that need zinc ions in their catalytic site to work properly, and could be as transmembrane-type as soluble-type. MMPs are synthesized as pro-enzymes and successively activated. MMPs are classified on the basis of substrate specificity as ECM's component, other proteases or adhesion molecules. MMPs studied in our work were: MT1-MMP and MMP-2 / MMP-9 (gelatinase).
The other class of enzymes considered in our studied belong to serine proteolytic enzyme family and are characterized by amino acid serin in their catalytic site, also the members of this family are both transmembrane-type and soluble-type. Our study is focused to Seprase and Dipeptydil-Peptidase 4, Finally we have investigated adhesion molecules to extra-cellular matrix as β_1-integrin because its involvement in event as re-shaping of ECM during angiogenesis. It was described that β_1-integrin interacts with several α-integrin subunits, and plays roles in signaling and activation of degenerative pathways.
In our work we used two mice model, phenotypically linked to two different human diseases, both characterized by ECM degradation on skeletal muscle. Mdx mice have a mutation in the dystrophin's gene, mapped on X-chromosome. This mutation produces a non fuctional protein. Dystrophin is a member of a multiproteic complex that allows a physical link between actin cytoskeleton and members of ECM. Dystrophin have an actin-binding region and a dystroglican-binding region. Dystroglican is a trans-membrane-type protein capable to link the cell to the outside laminin. An aberrant complex produces a less strong resistance to mechanical stress but also alters permeability to ions in extracellular environment. In DMD patients dystrophin's mutation produces two different types of muscular dystrophy: Becker and Duchenne. Patients afflicted by these diseases have a progressive degeneration of muscle tissue, that is substituted by fibrotic tissue and adipocytes. At the end patients dies to asphyxia due to toracic muscles failure or for heart failure.
The second mouse model was generated in Dott. Alessandra d'Azzo's laboratory at St. Jude C.R.H, Memphis, TN. These mice have a mutation on Neur1's gene that codifies for a lysosomal enzyme. This aberrant enzyme is the cause of the lysosomal storage disease sialidase. Sialidase is a childhood disease due to systemic degeneration; recently it was observed that has strong effect also in skeletal muscle. Gene Neur1 codifies a glycosidase that plays a role into catabolism of protein with sialic acid. Also in this experimental model it was observed a wide degeneration of ECM.
In this work we applied several methods to comprehend different aspects. We made zymographies, using gelatin as substrate, which demonstrate an arisen activity of the pro-form of enzymes such as MMP-2 and MMP-9, in the pathological model. Protein extracts were not denatured nor reduced, to preserve their activity. Western Blotting assays confirmed an increased amount of protein's levels such as Seprase, DPPIV and MT1-MMP, but also β_1-integrin. Primary cultures from skeletal muscle allowed to identify and separate two different cell populations: satellite and mesenchymal cells. The latter, probably responsible of the tissue degradation, were separated on discontinuous density gradient in Percoll. Stained with falloidin, these cells show morphologic differences, to ipotize at least three sub-population; in part confirmed by rt-PCR analyses.
The obtained results demonstrate increased activity of several proteases in the ECM degeneration in pathological models. Degeneration is probably due to mesenchymal cells that are quite heterogeneous. Future studies will be focused on other member of physiological pathways of reshaping of ECM.

Identification of the pathway-specific regulatory genes of the erythromycin biosynthesis

Lo Piccolo L., Echenique - Rivera H., Tarantino S., Di Cesare E., Puglia A. M. and Alduina R.
Dipartimento di Biologia Cellulare e dello Sviluppo, Università di Palermo
valduina@unipa.it

Saccharopolyspora erythraea, a mycelium-forming actinomycete, is the major producer of the clinically important macrolide antibiotic erythromycin. Extensive genetic studies have provided some insight into the genes involved in erythromycin biosynthesis. The erythromycin biosynthetic genes are clustered on the *S. erythraea* chromosome similarly to other secondary metabolic pathway genes. The erythromycin gene cluster contains 20 genes involved in the biosynthesis of erythromycin A. The genes involved in the biosynthesis of the polyketide ring, the biosynthesis and attachment of mycarose to the macrolide ring, and the biosynthesis and attachment of desosamine to the macrolide ring have been designated *eryA, eryB, and eryC* genes, respectively. Additionally, there are three genes encoding modifying enzymes, designated *eryF, eryG, and eryK*, as well as *ermE*, encoding the rRNA methylase conferring erythromycin resistance on the host organism. Finally, two open reading frames (ORFs), *eryBI*, which is not essential for erythromycin biosynthesis, and *orf5*, encoding a putative type II thioesterase, are also located in the *ery* gene cluster. The putative regulatory gene has not been identified in *ery* gene cluster. Erythromycin is a clinically important macrolide antibiotic; it is also the parent molecule for several commercially successful semisynthetic macrolide and ketolide derivatives. It would be of both great biological and commercial interest to understand the regulatory mechanisms that control the level of production of this important antibiotic.
To perform a platform for rapid isolation of DNA-binding proteins, gel shift analysis of *ery* promoters was carried out in presence of *S. erythraea* native proteins extracted from biomass samples collected before and during antibiotic production. This analysis revealed that *eryBI* and *eryBIII* promoters are bound by two different putative regulators before production. In parallel, the transcription profile of key *ery* genes was analysed by quantitative RT-PCR revealing that the expression of *eryBI* and *eryBIII* is down-regulated before production. All together these results strongly suggest that *eryBI* and *eryBIII* are negatively controlled by two different regulators. Different fractionation procedures are ongoing to purify from the crude extracts the specific DNA-binding proteins.
Our analysis suggested that different negative regulators are involved in *ery* gene expression revealing that the regulation of erythromycin biosynthesis is more complex than previously believed.

Poster presentation

……

Relation of primary metabolism on antibiotic production in *Streptomyces coelicolor*.

Marineo S., Giardina A., Mandalà S., Bravatà V., Canepa A., Ledda M., Di Carlo E., Curcurù L. and Puglia A.M.
Dipartimento di Biologia Cellulare e dello Sviluppo "A. Monroy" Viale delle Scienze, Palermo

Streptomycetes are Gram-positive bacteria producing a multitude of complex secondary metabolites. While much attention has been paid to the pathways and regulation of secondary metabolism, less has been paid to the pathways and the regulation of primary metabolism, which supplies the precursors (Hodgson et al., 2000). In *Streptomyces coelicolor* both L- and D-tryptophan are incorporated into the calcium dependent antibiotic (CDA). In order to obtain higher amounts of CDA, we have adopted two different strategies aimed at increasing aminoacid concentration. We constructed (1) mutants in which tryptophan biosynthetic genes are overexpressed and (2) several mutant strains impaired in aromatic aminoacid catabolism.
As little information is available on tryptophan biosynthetic gene expression, the expression of anabolic genes has been investigated by qRT-PCR at different growth stages in *Streptomyces coelicolor* grown in different media. *trp* gene expression appears to be regulated by growth phase but not by feedback repression. There are only a few cases of end product repression.
We created knock-out mutants in almost all *trp* genes and their phenotype analysis revealed that not all the genes are essential for the aminoacid biosynthesis, except for *trpA* and *trpB* (codifying both subunits of tryptophan syntase) and trpX (a putative regulator) (Hu *et al.*,1999).
Interestingly, a *trp*B and *trpA* overexpressing mutants produce early CDA compared to the wild type strain.
Several *S. coelicolor* mutant strains, affected in the catabolic pathway of aromatic aminoacids, have been constructed using transposon mutagenesis (Bishop et al., 2004). Microbiological assays showed that some of these mutant strains produce a higher yield of CDA than the wild type strain.
Microarray analysis of RNA extracted from these mutant strains have been carried out in order to study global gene expression changes with a particular interest for the CDA biosynthetic genes.

References

Hodgson DA. *Adv Microb Physiol.* **42**,47-238 (2000).
Hu, D.S.J., Heisdstra R. & Hodgoson D.A. *Molecular Microbiology* **32(4),** 869-880 (1999).
Bishop A., Fielding S., Dyson P., Herron P. (2004) Systematic insertional mutagenesis of a streptomycete genome: a link between osmoadaptation and antibiotic production. *Genome Res*. **14:**893-900.
Keywords: Calcium Dependent Antibiotic, aminoacid metabolism.

Poster presentation

Mapping the locus specific HSA7q11.23 "William-Beuren" probe on primates

Picone B. (1*), Dumas F. (1), Stanyon R. (2), Lannino A. (1), Vitturi R.(1), Privitera O. (3), Sineo L. (1).
*(1) Dipartimento di Biologia animale "G. Reverberi" – Università degli Studi di Palermo – b.picone@unipa.it / * Department of Botany and Zoology-Evolutionary Genomics Group - University of Stellenbosch, Cape Province - South Africa / (2) Dipartimento di Biologia animale e Genetica – Università degli Studi di Firenze / (3) Laboratorio di Citogenetica – Unità Ospedaliera Legnano - Milano*

The mapping of small locus specific probe (800-450 Kb) gives a considerable value to the study of chromosomal rearrangements used in evolutionary analysis. The locus Williams-Beuren, maps in the q arm of chromosome 7. The human synteny 7 has a very complex origin (Murphy et al., 2001; Müller et al., 2004). During the eutherian evolution the synteny 7 presents divided into two distinct segments; the little fragment is frequently associated with other human syntenies such as HSA 16 ("ancestral Eutherian" and Strepsirrhini) and HSA 5 (Platyrrhini). Fluorescence *in situ* hybridization has been performed on metaphases of: *H.sapiens, P.pygmaeus, P.troglodytes, G.gorilla, C.aethiops, C.neglectus, M.fascicularis, T.cristata, C.argentata (M.argentatus), C.cupreus, A.caraja, S.oedipus, C.goeldii, S.sciureus, A.paniscus, L.lagothrica, C.pygmaea*. The research (partly published: Sineo and Romagno 2006; Sineo et al., 2007) have showed that the locus HSA7q11.23 in the different Anthropoidea species is included in the region of the synteny HSA 7: 7p22-qter - 7q 22.22, with one exception (*C.cupreus*). These data permitted to assess the evolution of the human synteny 7 and to debate on phylogenetic relationships between species.

References:

Müller S et al, . 2004. Genomics 84: 458-467.
Murphy WJ, et al., S. 2001. Genomic Biology 2 (6: Rev. 0005.1-.8)
Sineo L and Romagno D. 2006. In: Sineo L, Stanyon R (eds) Primate Cytogenetics and Comparative Genomics. Firenze Firenze University Press, pp. 57-66.
Sineo L, et al., . 2007. William Beuren mapping in C. argentata, C. cupreus and Alouatta caraja indicates different patterns of chromosomal rearrangements in neotropical primates. J Zool Syst Evol Res Published article online: 14-May-2007

Poster presentation

.....

Characterization of a nuclear factor associated to the chromatin of sea urchin histone genes

Melfi R., Lo Cicero A., Scuderi T., e Spinelli G.
Dipartimento di Biologia Cellulare e dello Sviluppo "A. Monroy" Viale delle Scienze, Palermo

The sea urchin early histone gene cluster, containing the five genes H2B, H3, H2A, H1 and H4, is strictly regulated during embryonic development. Gene expression starts at morula stage and is downregulated by the early blastula stage to be then permanently silenced.
The early histone gene H2A is under the control of several cis-acting sequences, including the M30 enhancer upstream the promoter, and a sequence of 462 bp (sns 5) responsible for the correct temporal pattern of expression, which lies immediately downstream the coding region (1-3). We have shown that sns5 contains a fragment of 265 bp, sns, that behaves as an insulator in enhancer blocking assays (2). Furthermore in the 5' region, upstream the enhancer, we have identified another sequence, which contains four GAGA tandem repeats, needed for the silencing of the α-H2A gene at gastrula stage .
Some recent experimental evidence strongly suggest that some epigenetic events are also involved in the downregulation of the α-H2A gene at the gastrula stage.
On the promoter and the 3'regulatory region, at morula stage, it as been shown that there is a strong correspondace between gene transcription, the absence of an histone deacetylase (HDAC-1) and the high level of H3K9ac toghether with a poor H3K9me2. While, at gastrula stage, HDAC-1 is associated to the same DNA fragments which also show, as expected, under-acetylated H3K9 and dimethylated H3K9 (4).
But the question arises how the enzimes responsible for histone tails modifications are recruited to the regulatory regions of H2A gene.
By two hybrid screening, we have identified a cDNA fragment coding for a nuclear factor, called BBF, which specifically binds the BoxB, a direct repeat of 20 bps, which is one of the four negative cis regulatory nuclear factor binding sites identified in the sns5 fragment, shown to be essential for both downregulation and insulating function.
Complete cDNA sequence of BBF has been found by data base search among the clones of a *Paracentrotus lividus* library generated within NoEMG (Network of Excellence Marine Genomics). BBF contains an HTH motif immediately followed by an homeodomain, probably both implicated in DNA binding.
To characterize BBF function and to confirm its in vivo binding to sns5, we performed Chromatin immunoprecipitation (ChIP) experiments. In order to make an antibody against the protein, necessary to perform this kind of assay, we expressed the BBF gene in *E.Coli*.

Many attempts have been done to obtain a soluble fusion protein to inject into rabbit for polyconal antibody production. Finally we succeeded in expressing a soluble fusion protein (BBFRI/RV-GST) by cloning a fragment of BBF cDNA in frame with GST gene in a pGEX vector
The antibody was purified from the total serum of a rabbit injected with BBFRI/RV-GST protein and assayed against nuclear extracts and purified BBF fusion proteins.
The purified antibody was then used in ChIP experiments which have confirmed the in vivo binding of BBF to sns5 sequence at gastrula stage.
It is remarkable that the BoxB lies in the insulator sequence. Thus it will be interesting to investigate the role of BBF in recruiting other chromatin proteins and eventually their involment in the mechanism of downregulation, but we also expect that BBF could be a key factor in the insulating activity of sns.

References

1) Spinelli, G. & Birnstiel, M. L. (2002). The modulator is a constitutive enhancer of a developmentally regulated sea urchin histone H2A gene. Bioessays, 24, 850–857.
2) Melfi R., Palla F:, Di Simone P., Alessandro C., Calì L., Anello L. and Spinelli G. J. Mol. Biol. (2000) 304, 753–763
3) Di Caro D., Melfi R., Alessandro C., Serio G., Di Caro V., Cavalieri V., Palla F. and Spinelli G. J. Mol. Biol. (2004) 342, 1367–1377
4) Di Caro V., Cavalieri ., Melfi R. and Spinelli G.. J. Mol. Biol. (2007) 365, 1285–1297

Poster presentation

.....

Manganese effects on sea urchin embryos

Pinsino A.[1], Scudiero R.[2], Matranga V.[3], Bosco L.[1], Roccheri M.C.[1]
Dipartimento di Biologia Cellulare e Dello Sviluppo "A.Monroy", Università degli Studi di Palermo[1]
Dipartimento delle Scienze Biologiche-Sezione di Biologia Evolutiva e Comparata, Università Federico II di Napoli[2]
Consiglio Nazionale delle Ricerche, Istituto di Biomedicina ed Immunologia Molecolare "A. Monroy"[3]

Manganese (Mn) is a naturally abundant metal in nature. It exists in a number of physical and chemical forms in the water, in the earth's crust and in the atmosphere's particles. It is a trace metal essential for maintaining good health, involved in the normal functioning of various enzymes of metabolic processes. Manganese-dependent enzymes are found within different locations in the cell, including the Golgi, mitochondria and cytoplasm. On the other hand, this essential element is potentially toxic when in excess, and, consequently, represents a new important factor in environmental contamination. In the marine environment increased concentrations of bio-available manganese often result from anthropogenic activity, in fact, emission occurs from metallurgic and chemical industries including municipal wastewater discharges, sewage sludge, mining and mineral processing, productions of alloy, steel, and iron (World Health Organization 2004). The reduced and solubilised manganese (Mn^{2+}) released from the sediment surface result in an exponential increase of its concentration in the overlying water. In this preliminary study, we investigated on effects of Mn^{2+} on sea urchin (*Paracentrotus lividus*) embryos continuously cultured in the presence of the $MnCl_2$ from fertilization. Manganese exposure caused inhibitory effects on embryos development, producing specific malformation in a time- and dose-dependent manner. More then 80% of the embryos treated with the highest manganese concentration tested exhibit major skeletal defects. Mn^{2+} accumulation was detected by atomic absorption spectrometry (AAS) analysis. We found that Mn^{2+} is accumulated into the embryos since the beginning of development, in a dose-dependent manner. We explored whether or not different concentrations of Manganese exposure, would produce a cellular stress response in sea urchin embryos, by the up-regulation of synthesis of key stress proteins or by apoptosis. A direct correlation has been observed between malformations, accumulation of Mn^{2+} and cellular response of embryos exposed to Manganese. We found a moderate dose-dependent increase in HSC70 levels at pluteus stage, evaluated by Western blotting using an anti-HSP/HSC70 antibody but not the induction of the inducible forms of HSPs assess by methionine ^{35}S labelling. Apoptosis was measured by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labeling (TUNEL) assay. Gastrula and Pluteus embryos show no increases in cells displaying DNA fragmentation in comparison to controls.

Poster presentation

Triiodothyronine effect on the brain specific proteins PIPPin and LPI/PEP19

Proia P.*, Compagno V., Schiera G.**, Donatelli M.,
and Di Liegro I.**
*Dipartimento Biomedico di Medicina Interna e Specialistiche, *Dipartimento Universitario di Neuroscienze Cliniche, and **Dipartimento di Scienze Biochimiche,
University of Palermo, Palermo, Italy;*
E-mail: diliegro@unipa.it

In the intrauterine life, thyroid hormones are produced by the mother and even moderate and transient maternal hypothyroidism can alter brain organization of the fetuses. Although this dependence has been known for a long time, the molecular mechanisms through which these hormones act on developing brain are not completely understood. WE previously observed that T3 can induce in rat cortical neurons, cultured in a chemically defined medium (Maat medium, MM), the overall reorganization of chromatin that characterizes terminal differentiation of cortical neurons *in vivo*. More recently, we described a CSD-containing protein that seemed able to bind mRNAs encoding histone variants and is present both in the nucleus and in the cytoplasm of brain cells. As other CSD-containing proteins have the ability to interact both with RNA and chromatin, we investigated the possibility that PIPPin binds to chromatin. We also investigated for effects of T3 on PIPPin expression by comparing newborn euthyroid rats with newborns delivered by rats treated with 6-propyl-2-thiouracyl (PTU) since the last week of pregnancy. We analyzed in parallel rat cortical neurons, purified from brain cortices and cultured on laminin, in MM, with or without T3. The most significant difference among euthyroid- and hypothyroid- newborn rats concerns sumoylation of nuclear PIPPin, that is abolished by hypothyroidism. Moreover, we found that about one half of unmodified as well as all the sumoylated form of PIPPin could be extracted from nuclei with HCl, together with histones and is tightly bound to chromatin.
We recently reported identification of a cDNA that encodes a putative protein of 94 amino acids and expected molecular weight of 10.7 kDa, the C-terminal half of which is identical to that of PEP19, a small, brain-specific protein involved in Ca^{++}/calmodulin signaling. Preliminary experiments indicate that the novel rat-specific protein, tentatively named Long PEP19 Isoform (LPI), is an RNA-binding protein, possibly involved in the regulation of histone messenger expression. We therefore looked for effects of T3 on both LPI and the cognate peptide PEP-19. Here we report that a significant dicrease of the concentration of both proteins was noticed in hypothyroid rats, after two weeks of postnatal life.

References

Bono E. et al. (2007). Endocrinology 148: 252-257
Sala A et al. (2007) Int J Mol Med 19: 501-509
Derrigo M. et al. (2000) Int J Mol Med. 5: 111-123.
Nastasi T. et al (2000) NEUROREPORT 11: 2233-2236
Nastasi T. et al. (1999) J Biol Chem 274: 24087-24093
Scaturro M et al. (1998) J Biol Chem 273: 22788-22791
Castiglia D et al. (1996) Biochem Biophys Res Commun 218: 390-394

Poster presentation

.....

Isolation of five Gram-positive bacterial strains that degrade long-chain *n*-alkanes

Quatrini P[1]., Catania V[1]., De Pasquale C[2]., and Puglia A.M[1].
1Dipartimento di Biologia Cellulare e dello Sviluppo "A. Monroy", Università di Palermo. Viale delle Scienze 90128- Palermo [2] Dipartimento I.T.A.F. Università di Palermo

Petroleum is a complex mixture of HC and other organic compounds, and the microbial catabolic pathways for their biodegradation have been widely investigated (van Hamme et al. 2003). Nevertheless oil and oil-derived compounds are still the most threatening contaminants in the environment.
Bioremediation, based on the microbiological decontamination of hydrocarbon polluted sites, is claimed to be an efficient, economic and versatile alternative to physicochemical treatments. Bioremediation involves the use of indigenous or introduced microrganisms to degrade enviromental contaminants, i.e. hydrocarbons and other recalcitrant molecules.
HC degrading microrganisms can be isolated from contaminated soils.
For aerobic alkane metabolism, the most extensively characterized alkane-degradation pathways are encoded by the OCT plasmids, carried by *Pseudomonas* and other Gram-negative bacteria (vanBeilen and Funhoff, 2007). Here, a membrane-bound mono-oxygenase (encoded by *alkB*), and soluble rubredoxin and rubredoxin reductase, convert the alkane into the corresponding alcohol that is further oxidized to aldehyde and acid, prior to proceeding into the βoxidation pathway.
Distantly related *alk*B homologues have then been found in GC-rich Gram-positive *Rhodococcus* spp. and *Amycolatopsis rugosa* where multiple *alk*B sequences have been found. Most of these bacteria are able to degrade

dodecane and hexadecane but the identity of enzymes and genes involved in degradation of *n*-alkanes longer than *n*-hexadecane (C_{16}) is still an open question. In this work we report of five Gram-positive GC rich *n*-alkane degraders that can degrade up to hexatriacontane (C_{36}).
The bacteria were isolated from a long-term contaminated beach in Sicily. Serial dilutions of soil and water samples of the contaminated site were inoculated on Bushnell-Haas mineral medium plates with a fuel mixture (green petrol and diesel oil, 1:1) as the sole C source. After incubation at 30° for 7 days, colonies were repeatedly streaked on the same medium several times. Five isolates, that that were able to use the fuel HC as the sole C and energy source, were analysed by PCR-amplification and sequencing of the 16S rRNA gene and identified as one *Nocardia*, two *Rhodococcus* and two *Gordonia* strains (Quatrini et al., in press). When single HC model molecules were used as C source results showed that all the isolates were able to grow on medium and long chain *n*-alkanes from *n*-octane (C_8) to *n*-hexatriacontane (C_{36}) and none used aromatics. GC-MS analysis confirmed that these strains can degrade long chain *n*-alkanes up to *n*-hexatriacontane (Quatrini et al., in press). The degrading capacity of *Nocardia* and *Gordonia* strain SoCp were also assessed in microcosms on standard soils with different physico-chemical characteristics, spiked with medium and long chain *n*-alkanes. This is the first report showing the ability of *Gordonia* and *Nocardia* isolates to degrade long-chain *n*-alkanes longer than C_{18}.
Diverging alkane-hydroxylase encoding gene sequences (*alkB*) were also detected by PCR using degenerated primers in all the strains isolated in this work; multiple sequences were only obtained from the *Nocardia* strain. The majority of the *alkB* sequences were related to *R. erythropolis alkB2* (Quatrini et al., in press).
Work is in progress to clone the *alk*B flanking regions and to assess the role of AlkB in degradation of long-chain alkanes.
Moreover, these specific *n*-alkane degraders actinobacteria, should prove to be interesting subjects for microbial remediation under dry, resource-limited conditions such as those found in southern Mediterranean shoreliness.

References:
Quatrini, P. Scaglione, G. De Pasquale, C.; Riela, S. and Puglia, A. M. (in press) Isolation of Gram positive n-alkane degraders from a hydrocarbon contaminated Mediterranean shoreline. J. Appl. Microbiol.. 2007 doi:10.1111/j.1365-2672.2007.03544.x
Van Beilen JB, Funhoff EG.(2007) Alkane hydroxylases involved in microbial alkane degradation. Appl Microbiol. Biotechnol. 13-21.
Van Hamme, J.D., Singh, A. and Ward, O.P. (2003) Recent advances in petroleum microbiology. Microbiol Mol Biol Rev 67, 503–549.
Poster presentation

.....

Expression and localization of AEG-1 in cadmium-treated MDA-MB231 breast cancer cells

Ragusano R., Sirchia R., Luparello C.
Dipartimento di Biologia Cellulare e Dello Sviluppo "A.Monroy", Università degli Studi di Palermo

It is know that cadmium (Cd) is able to exert various biological effects, both cytotoxic, which promote the production of specific stress response proteins that, in turn, in some cases determine the switching-on of apoptotic mechanisms, and carcinogenetic, which are responsible of neoplastic transformation of cells. Previously, we demonstrated that treatment of estrogen receptor-negative (ER^-) MDA-MB231 breast cancer cells with 5 μM Cd for 96h resulted in an about 50% reduction of cell number coupled with the varying modulation of genes encoding for stress response proteins and apoptosis factors (Luparello et al., 2007). In particular, semiquantitative-multiplex (SM)-PCR experiments indicated that the following genes were up-regulated: *hsf1* (+7.5folds), *hsp27* (+8.7folds), *metallothionein (MT) IA* (+2.34folds), *MTIF* (+3.65folds), *Waf-1* (+10.4 folds), *caspase-2* (+3folds), *-7* (+15folds), *-8* (+9.25folds), *-9* (+4.7folds), and, more prominently, *Dapk* (+55folds), and *caspase-1* (+106folds) and *-6* (+31.3folds). On the other hand the expression level of the following genes was decreased: *MTIG* (-18.8folds), *hsc70* (-4.9folds), *hsp75* (-9.5folds), *hsp90β* (-2.57folds) and, massively, *grp78* (-54.2 folds) and *Bcl-2* (-53folds). When we submitted RNA samples of control and Cd-treated tumoral MDA-MB231 cells to "differential-display"-PCR experiments, *AEG-1* (astrocyte elevated gene-1) gene, a.k.a. 3d3/lyric and metadherin (Britt et al., 2004; Brown and Ruoslahti, 2004; Sutherland et al., 2004; Kang et al., 2005; Emdad et al., 2006), was shown to be down-regulated after treatment with 5 uM Cd for 96h. From literature data, it appears that *AEG-1* gene is ubiquitously expressed, that its transcript is likely to undergo altermative "splicing" and that its product is a single-pass transmembrane protein of predicted molecular mass of 64 kDa and pI 9.3 probably localized in the endoplasmic reticulum and perinuclear region, although some authors found it localized to tight junctions of polarized epithelial cells, or in the plasmalemma of malignant cells. It seems plausible that intracellular distribution of AEG-1 is cytospecific. Recent data by Emdad et al. (2006) demonstrated also that in HeLa cells engineered for *AEG-1* up-regulation, this over-expression activates NF-κB pathway (Pahl, 1999) through IκBα degradation and direct interaction with p65 followed by nuclear import of the latter, resulting in the activation of different genes, such as *c-fos* and *c-jun*. To extend the knowledge in our model system, we submitted control and treated MDA-MB231 cells to analysis under the confocal fluorescence microscope after AEG-1

immunolocalization and propidium iodide counterstain: the results obtained indicated that the amount of intracellular AEG-1 decreased after Cd treatment, as well as the number of fluorescence pixel specifically-present in the nucleoplasm, thereby supporting the previous data about general *AEG-1* down-regulation and also suggesting a block of its nuclear import in treated cells. We have investigated the amount of p50 and p65 subunits of NF-κB in nuclear protein extracts from control and treated cells by an ELISA technique, but, at least under the experimental conditions used, we found no statistically-significant difference between them. On the other hand, we submitted cDNA preparations from control and treated cells to SM-PCR to evaluate the expression levels of *c-fos* and *c-jun* and the results indicated a down-regulation of both genes to about -3folds with respect to control. We have also checked whether such down-regulations were to be considered as a specific event of Cd-treated MDA-MB231 tumoral cells, by submitting non-tumoral immortalized HB2 epithelial breast cells (courtesy of Cancer Research UK) to the same treatment and then evaluating *AEG-1*, *c-fos* and *c-jun* expression levels. The results obtained indicate that in Cd-treted HB2 cells only *c-fos* appeared to be down-regulated to a lesser extent than in tumoral cells (about -2folds), thus confirmung that the described Cd-mediated effect on *AEG-1*, *c-fos* and *c-jun* expression levels is specific for the neoplastic cytotype.

.....

nCDase and SphK-1 localization in vesicles shed by tumour cells and their biological roles.

Rigogliuso S., Lo Baido G., Taverna S., Salamone M. and Vittorelli M.L.
Dipartimento di Biologia Cellulare e Dello Sviluppo "A.Monroy", Università degli Studi di Palermo

Sphingolipid metabolism is a dynamic process resulting in the formation of a number of bioactive metabolites including ceramide, ceramide-1-phosphate, sphingosine e sphingosine-1-phosphate (S1P). (Pyne and Pyne; Biochem. J. 2000; 349:385-402).

Following sphingomyelinase activation, sphingomyelin is hydrolyzed to ceramide, which is considered to be an inducer of cell growth arrest, differentiation and apoptosis. (Hannun et. al 1996; Science: 274:1855-1859). Ceramidase catalyzes the deacylation of ceramide to produce a free fatty acid and sphingosine. The enzyme sphingosine kinase (SphK) catalyzes the formation of S1P from sphingosine and ATP (Olivera et al. J.Biol.Chem. 1998; 273:12576-12583).

Two distinct SphK isoforms, SphK1 and SphK2, have been cloned and characterized. (Liu et.al. J.Biol.Chem. 2000; 275: 19513-19520) and recently, alternatively spliced variants of human SphK1 and SphK2, differing in their amino terminal portions, have also been described (Billich et. al J.Biol.Chem. 2003; 278; 47408-47415). SphK1 and SphK2 differ in their relative tissue distribution, sub cellular localization and biochemical activities, consistent with distinct biologic functions for these two enzymes (Saba et al. Circ. Res. 2004; 94:724-734). SphK2 presents a nuclear localization signal sequence and is localized in the cell nucleus (Igarashi et. al. J.Biol.Chem 2003; 278: 46832-46839). SphK1 is primarily localized in the cytosol. PMA and TNFα induce the phosphorylation of SphK1 Ser 225, through the activation of MAPK and ERK1/2. Phosphorylation of SphK1results in its plasma membrane localization and in its activation. (Pitson et al. Embo J. 2003; 22: 5491-550). SphK1 is a cell surface-active kinase and an extracellular protein.

As several secreted proteins, like for instance FGF-1 and FGF-2, SphK1 molecule lacks a conventional leader secretion signal sequence. The mechanism of its release from the cell occurs *via* a non classical pathway independent of the endoplasmic reticulum/Golgi system but requiring functional actin dynamics (Ancellin et al J.Biol.Chem. 2002; 277: 6667-6675). SphK1 activity, and therefore production of S1P at the cell periphery and/or in the extracellular medium, was shown to regulate a wide variety of cellular processes, including promotion of cell proliferation, survival and motility (Olivera et al. J.Biol.Chem. 2003; 278: 46452-46460). S1P is an important proangiogenic factor and its ability to promote capillary morphogenesis in endothelial cell is significantly enhanced when S1P is associated with FGF-2 (Harvey et al. J Lab. Clin. Med 2002; 140: 188-198).

Since we already reported that FGF-2 release occurs by vesicle shedding (Taverna et. al. J.Biol.Chem. 2003; 278: 51911-51919), we hypothesized and tried to demonstrate the possibility that S1P is produced in shed vesicles and that it exerts a synergic role with vesicle associated FGF-2, in the induction of endothelial cell differentiation. We also considered the hypothesis that enzymes involved in sphingolipid degradation could play a role in vesicle shedding.

Our experimental dates indicate:

- nCDase and SphK1 are both present in shed vesicles in biologically active forms, together with their lipidic substrates.
- The enzymes of sphingolipid metabolism are not involved in the process of vesicle shedding.
- In SK Hep-1 hepatocarcinoma cells, which we used in most of our experiments, FGF-2 and both nCDase and SphK1 are simultaneously released in shed vesicles.
- Shed vesicles exert chemiotactic effects on endothelial cells and have the ability to promote their morphogenesis in capillary-like structures.
- Since these effects are typical of both FGF-2 and S1P, to neutralize effects of vesicle-associated FGF-2, we denatured the protein components of vesicles by a 10 minutes treatment at 100 C°. This treatment is known to

denature FGF-2 (Vemuri et al. 1994; J Pharm Pharmacol. 46: 481-486) while S1P is reported to be stable at temperatures below 120 C° (Harvey et al. J Lab. Clin. Med 2002; 140: 188-198). Heat treatment substantially decreased the chemiotactic effect of vesicles but only had a small effect on their capability to induce formation of capillary-like structures.

- Promotion of endothelial cell morphogenesis was increased in vesicles shed by cells overexpressing SphK1 and it was decreased in cells expressing a dominant negative SphK1 isoform.

In summary our dates show that shed vesicles are vehicles for both FGF-2 and SphK1 secretion, and that FGF-2 and S1P, produced in the vesicle membrane by the joined catalytic action of nCDase and SphK1, exert a synergic action on endothelial cells inducing angiogenesis.

.....

Analyses of podosomes and invadopodia in invading Endothelial and Hepatocarcinoma Cells.

Salamone M., Taverna S., Ghersi G. and Vittorelli M.L.
Dipartimento di Biologia Cellulare e Dello Sviluppo "A.Monroy", Università degli Studi di Palermo

Cell invasive movements are associated with the acquired capability of cells to partially degrade extracellular matrix components. The cell capability to degrade matrix components was shown to be greatly increased at the level of specific membrane protrusions (Chen et al. 2003; *Current Topics in Development Biology*, 54: 207-27) and of shed membrane vesicles (Vittorelli 2003; *Current Topics in Development Biology*, 54: 417-27). Colocalization of several proteolytic enzymes in specific areas of the cell membrane, which are also specifically enriched in integrins, permits activation cascades and concentrate the proteases within the vicinity of their target; their release as shed membrane vesicles induces proteolysis in discrete pericellular regions.
For what proteases involved in cell invasion are concerned an emerging role appears to be played by proteinases which are stably associated to the plasma membrane. This group of proteolytic enzymes includes Mt1-MMPs, matrix metalloproteinases which are located at the cell surface through membrane anchors or transmembrane stretches; and type II transmembrane serine proteases (TTBPs): Seprase and its homolog DPP4. These proteolytic enzymes are generally found in specific domains of the cell plasma membrane. MT1-MMP, for instance, was detected at the level of melanoma cell invadopodia (Nakahara et al. 1997; *P.N.A.S.*, 94: 7959–64). While DPP4 is ubiquitously expressed in epithelial and endothelial cells (Chen, and Kelly, 2003; *Cancer Metast. Rev*, 22: 259–269), Seprase is not expressed in normal cells but only activated endothelial and fibroblast cells and is expressed in melanoma and breast carcinoma cells; Seprase is also clustered in invadopodia (Muller et al. 1999; *J Biol Chem*, 274: 24947-52).
Other involved proteolytic enzymes, while being secreted, are frequently found to be stably associated with specific domains of the cell membrane. Binding of uPA to the cell membrane is mediated by its receptor (Ellis 2003; Current Topics in Development Biology, 54: 263-312); MT1-MMP binds and activate MMP-2 (Sato et al. 1994; *Nature*, 370**:** 61–65); MMP-9 is bound to the cell membrane *via* CD44, the hyaluronic acid receptor (Yu and Stamenkovic 1999; *Genes Dev.* 13: 35–48). These proteinases are also clustered in specific domains of the cell membrane. For instance MMP-9, was recently, found in invadopodia of leukemia cells (Redondo-Mun˜ oz, et al. 2006; *Blood* 108: 3143–51); MT1-MMP and MMP-2 have been located in podosomes of endothelial cells (Tatin et al. 2006. *J. Cell Sci.* 119: 769–781). These same enzymes are also found in association with shed membrane vesicles (Salamone et al. submitted)
Cells migration, however, not only requires partial degradation of extracellular matrix components, but an increased cell motility driven by contacts with ECM and by remodelling of cytoskeleton components. Podosoma and invadopodia are two types of actin-rich adhesions structures, which both establish contact with substratum and are involved in matrix degradation (Linder 2007 *Trends Cell Biol.* 17: 107-17); they are frequently found in invasive cell types. Podosomes are formed in monocytic cells, such as macrophages, dendritic cells or osteoclasts; invadopodia, on the contrary, are typically found in carcinoma cells (Baldassarre et al. 2003 *Mol. Biol. Cell.* 14: 1074–84).
In this report we compared the migratory capability of resting (non stimulated) and EGF stimulated endothelial ECV-304 cells and of hepatocarcinoma Sk-Hep1 cells. In some experiments the cell morphology and attitude to degrade the rodaminated-FN film on which cells were cultured was analyzed. We observed that resting ECV-304 endothelial cells have a lower capability to degrade the substrate in the pericellular area compared to invasive SK-Hep1 hepatocarcinoma cells. Hepatocarcinoma Sk-Hep1 cells actively degraded Rh-FN and the digested FN was endocytosed. On the other hand, when treated with EGF, ECV-304 cells acquired degradation capabilities similar to those of Sk-Hep1 hepatocarcinoma cells. When cultured on a FN-film the two cell lines appeared morphologically similar; endothelial cells however presented ventral protrusions, structurally comparable to podosomes, which were not detected in Sk-Hep1 cells.
In other experiments the cell capability to invade three dimensional type-I collagen gels was analyzed. When, the two cell types (SK-Hep1 and EGF treated ECV 304 cells) were cultured in 3 D type-I collagen fibril gels, they showed a morphology completely different from that observed when the same cells were cultured in 2D systems. The structure of actin microfilaments was highly modified, forming, in spite of stress fibres, a network at level of the cortical zone. In 3D systems, endothelial cells formed numerous philpodia, while this kind of cell protrusion was not observed in SK-Hep1 cells.

Both cell lines shed membrane vesicles, but while ECV-304 cells released them randomly from all areas of the plasma membrane surface, Sk-Hep1 cells vesicles were shed mostly from membrane protrusions. When grown in 3D gels, the two cell lines showed the same pattern of secreted gelatinolitic enzymes, (including pro and activated MMP-2 and MMP-9) with the exception for the presence, in ECV-304 cell extracts of one high m.w. lytic band, which probably corresponds to seprase; this band was not detected in SK-Hep1 cells.

Poster presentation

.....

P38 MAPK pathway is involved in cadmium response by MDA-MB231breast cancer cells

Savona R., Sirchia R., Maira L. L., Casano C.,and Luparello C.

Dipartimento di Biologia Cellulare e Dello Sviluppo "A.Monroy", Università degli Studi di Palermo

Cadmium (Cd) is an underground mineral extracted as part of zinc deposits, which is widely used in the steel industry, in plastics, in batteries, mining and smelting, and in soluble form in many colour pigments used for painting, electroplating and galvanizing. It is an industrial and environmental pollutant released as air contaminant from fertilizers and, more prominently, in the form of wastewater, in fact it is found in high concentrations in coastal, especially in polluted estuary zones.

Cd is classified in group I of the International Agency of Research on Cancer categories of carcinogens and studies reported in the literature indicate that it may play a role in both the initiation of cancer, and in the progression of cancer. A number of data demonstrates that Cd not only promotes apoptosis in several experimental model systems, but also induces tumor suppression when administered at not overtly-toxic doses to tumor-cell bearing immuno-depleted mice.

The p38 MAPK pathway is crucially involved in cell response to the metal, in fact several results indicate that p38 activation is an early and specific regulatory event for Cd-induced apoptosis, at least in some cellular types. Four genes encode the known members of the p38 family, *p38α*, *p38β*, *p38γ*, *p38δ*. The kinases *p38α* and *p38β* seem to be expressed ubiquitously, while others *p38γ* and *p38δ* are differentially expressed in various tissues. Only *p38α* and *p38β* are inhibited by SB203580, whereas *p38γ* and *p38δ* are unaffected by the drug.

Because the p38 MAPK pathway is crucially involved in cell response to Cd, in this study we investigated the expression level of p38 genes in control, Cd-treated, SB203580-treated, and Cd/SB203580 co-treated cells.

cDNA preparations from control and treated cells were submitted to conventional qualitative PCR in the presence of the specific primers for *p38 α, β, γ, δ*. The results obtained indicated that the transcripts of all isoforms of p38 MAPK were present in the preparations from control and treated cells.

When the same cDNA preparations were submitted to semi-quantitative multiplex (SM)-PCR assays we observed that Cd appeared to promote the down-regulation of *p38α* (-8,3 folds) and *p38β* (-4,2 folds), the up-regulation of *p38γ* (+7,2 folds), whereas expression of *p38δ* was unaffected. Moreover, Cd/SB203580 co-treatment promoted the down-regulation of *p38α* (-2 folds), and *p38β* (-3,6 folds), the up-regulation of *p38δ* (+2,7 folds), whereas expression of *p38γ* was unaffected. On the other hand, single SB203580 treatment promoted the down-regulation of *p38β* (-16 folds) and *p38γ*(-2,5 folds), whereas expression of *p38α and p38δ* was unaffected.

To study the activation pattern of p38 MAPK in untreated and Cd-treated MDA-MB231 cells, 2D-electrophoretic analyses and Western blot assays with specific monoclonal antibodies that recognize the active (bi-phosphorylated) and inactive (unphosphorylated or mono-phosphorylated) form of the p38 MAPK were performed.

The anti-inactive p38 MAPK antibody revealed the presence of one isotype that increased up to 10% in Cd-treated cells *versus* control. On the other hand, when the anti-active p38 MAPK antibody was used, two isotypes (1 and 2) were revealed. Moreover, the extent of activation of the isotype 2 decreased down to 40% in Cd-treated cells *versus* control.

Poster presentation

Extracellular vesicles shed by astrocytes and neurons contain angiogenic factors.

Schiera G.*, Proia P.**, Savettieri G.** and Di Liegro I. *
**Dipartimento di Scienze Biochimiche, and **Dipartimento Universitario di Neuroscienze Cliniche, University of Palermo, Palermo, Italy;*
E-mail: diliegro@unipa.it

Brain capillary endothelial cells (RBE4.B), cultured on collagen IV, synthesize occludin and correctly localize it at the cell periphery only when cocultured with neurons for at least one week (Savettieri et al., 2000; Cestelli et al., 2001). Moreover, by using a three-cell type-culture system, we found that, in the presence of astrocytes, the neuron-induced synthesis and localization of occludin is precocious as compared to cells cocultured with neurons only (Schiera et al., 2003; 2005). In the three-cell type system of culture, neurons, astrocytes and endothelial cells are not in physical contact,so we hypothesized that the effects discovered were due, at least in part, to the diffusible factors. It was recently shown that transformed glial cells (oligodendroglioma cells) are able to shed extracellular vesicles (D'Agostino et al., 2006). We supposed that shedding could be also the way through which neurons and/or astrocytes address inductive signals to endothelial cells. We then looked, by immunofluorescence and western analysis, for factors, produced by neurons/astrocytes, with the potential to influence growth and differentiation of endothelial cells. Here we report that neurons and astrocytes produce both vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF-2), two well known angiogenic factors. More interestingly, we gained evidences that both factors are released by brain cells, at least in part, by shedding extracellular vesicles, that contain beta1-integrin, a membrane protein already known to be part of extracellular vesicles released by tumor cells. Shedding of extracellular vesicles by neurons and astrocytes was also confirmed by scanner electron microscopy. On the other hand, we demonstrated, by immunofluorescence analyses, that the vesicles released into the medium are not apoptotic bodies.
We are now analyzing the events involved in the synthesis and release of VEGF and FGF-2 and the mechanisms that enable endothelial cells to respond to these factors. We are also trying to understand whether brain cells release a single class of structures or a mixed population of vesicles that differ for size and constituents. Finally, we wonder which is the fate of vesicles after shedding into the extracellular environment.

References:
Cestelli A. et al., 2001, J. Controll. Rel. 76: 139-147.
D'Agostino S. et al., 2006, Int J Oncol, 29: 1075-1085.
Savettieri G. et al., 2000, NeuroReport 11: 1081-1084.
Schiera G. et al, 2003, J. Cell. Mol. Med. 7: 165-170.
Schiera G. et al., 2005, J. Cell. Mol. Med. 9: 373-379.

.....

Comparison of pacing activity in duodenal muscle cells from control and *mdx* (dystrophic) mice

Serio R., Zizzo MG, Mulè F.
Dipartimento di Biologia Cellulare e dello Sviluppo – Laboratorio di Fisiologia generale, Università di Palermo – Viale delle Scienze, 90128 Palermo, Italia
rserio@unipa.it

Our laboratories are currently interesting in studying differences in gastrointestinal motor function between normal and *mdx* mice, mutant animals for the gene encoding dystrophin, a cytoskeletal protein localised at the inner face of the plasma membrane in cells, including the intestinal muscle cells, the enteric neurones and the interstitial cells of Cajal (ICCs). Interstitial cells of Cajal (ICCs) are reported to pace slow waves and contraction of the intestinal muscle and changes in the ICC ultrastructure have been shown in *mdx* mice accompanied by changes in the functionality of intestinal segments (1). Since our previous studies have shown duodenal contractility disturbances in *mdx* mice (6,7), the aim was to compare the intracellular electrical activity of duodenal circular muscle cells between normal and *mdx* mice, in order to clarify the mechanisms underlying the observed alterations. Standard microelectrode technique was used to study intracellular activity in normal and *mdx* colon. In *mdx* mice, membrane potential (RPM) of duodenal circular smooth muscle cells was significantly depolarized compared to the control (about -45mV *vs* -65 mV). Spontaneously occurring slow waves were larger in amplitude (16mV *vs* 10 mV), with a less evident waxing

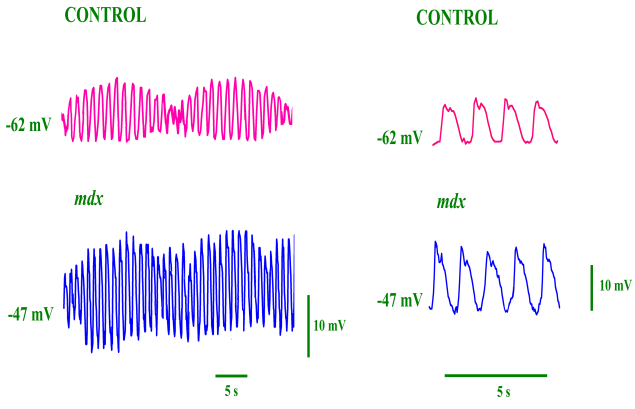


Fig. 1. Slow waves activity recorded from the circular muscle cells of duodenum in control and in in mdx mice.

and waning of the amplitude, and higher in frequency (53 *vs* 40 waves min^{-1}) (Fig 1).
Slow wave activity was not affected by L-type calcium channel blockers and was less sensitive than the control to the removal of extracellular calcium or to the block of the PLC/IP3 pathways. Moreover, slow wave activity was independent of nerve function: atropine, guanethidine, tetrodotoxin and nitric oxide synthase blocker had no influence on RMP, slow wave rhythmicity or amplitude. In conclusion *mdx* duodenal circular muscle cells display differences in the rhythmicity of the spontaneous electrical activity that can be related to a primary dysfunction in non-neural cells, likely in the ICCs. This alteration would match the observed differences in the motor pattern, i.e. sustained mechanical tone and motor hyperactivity.
Supported by Telethon (grant n. GGP030250).

References

1. Vannucchi M.G., Zizzo M.G., Zardo C., Pieri L., Serio R., Mulè F. and Faussone-Pellegrini M.S.
Ultrastructural changes in the interstitial cells of Cajal and gastric dysrythmias in mice lacking full-length dystrophin (*mdx* mice).
2. Zizzo M.G., F. Mulè and Serio R.
Duodenal contractile activity in dystrophic (mdx) mice: reduction of nitric oxide influence.
Neurogastroenterol. Mot, 2003,15, 559-565.
3. Zizzo M.G., Mulè F., Serio R.
Tachykinergic neurotransmission is enhanced in duodenum from dystrophic (*mdx*) mice.
Br. J. Pharmacol. 2005, 145:334-341.

Poster presentation

.....

Involvement of PKCη in type V collagen-induced apoptosis on 8701-BC breast cancer cells

Trigili C., Sirchia R., Luparello C.
Dipartimento di Biologia Cellulare e Dello Sviluppo "A.Monroy", Università degli Studi di Palermo

We have previously reported (Luparello and Sirchia, 2005) that type V collagen, a "minor" component of normal human breast stroma subjected to over-deposition in cases of ductal infiltrating carcinoma (d.i.c.), when used as a substrate for d.i.c. cells of the 8701-BC line promotes apoptosis-related events. As one of the various examples, by submitting RNA preparations from 8710-BC cells cultured onto either type IV- or type V collagen substrates to semiquantitative-multiplex (SM) PCR, we observed the up-regulation of the apoptosis-activating genes *Bcl-xS* (about +3folds), *Bad* (about +2folds), *Dap kinase* (about +2folds), *caspase-5* (about +10folds), and *-8* (about +17folds), whilst *Bcl-2*, an anti-apoptotic gene, appeared to be down-regulated (about -2.5folds) vs. cells plated onto control type IV collagen substrate. When we submitted RNA samples of cells cultured on either collagen to "differential-display"-PCR experiments, *PKCη* (protein kinase C η) gene, encoding for one of the Ca^{++}-independent isoforms of the enzyme, was shown to be down-regulated (about -6.5folds) when the culture substrate was type V collagen. It is known that this gene expression is increased during the post-natal differentiation of rat mammary gland, whereas its expression levels decrease drastically during the physiologic involution of the gland, an event that can be related to the onset of apoptotic phenomena typical of the involution process (Masso-Welch et al., 1998). In light of our and literature data, we wanted to check in our model system whether *PKCη* is involved in some way in the modulation of cell lethal phenotype induced by type V collagen substrates. To this purpose, we have seeded cells onto type IV collagen substrate, which represents the physiological support for cells of epithelial nature, and trasfected them with either an antisense oligonucleotide (asODN) targeting a 20-mer sequence from nt 1,762 of PKCη mRNA, or a scrambled asODN as a control, according to Brenner et al. (2003); anti-PKCη- and scrambled-asODN transfected cells seeded onto type IV collagen substrate were comparatively examined vs. untransfected cells seeded onto either type IV or type V collagen substrates for their morphological appearance, growth behaviour and gene expression pattern with reference to the above-mentioned type V collagen-modulated genes. The data obtained demonstrate that: i) under the phase-contrast microscope, scrambled asODN-transfected cells displayed the same spreaded, fibroblastoid and well-attached aspect as untransfected cells grown onto type IV collagen, whereas anti-PKCη asODN-transfected cells were less elongated and more rounded, as cells seeded onto type V collagen were, that is a typical morphology of loosely-attached suffering cells; ii) cell number at 48h, obtained after cell counting in a haemocytometer, diminished drastically when cells where transfected with anti-PKCη asODN (or untransfected but seeded onto type V collagen substrate) with respect to the other two experimental situations, i.e. untransfected cells and scrambled asODN-transfected cells both cultured onto type IV collagen; iii) the gene expression pattern of anti-PKCη asODN cells was similar to that of untransfected cells seeded onto type V collagen substrate. In fact, after SM-PCR assays, we observed the up-regulation or down-regulation of the same above-mentioned genes encoding for apoptotic factors, to the following extent: *Bcl-xS* (about +2folds), *Bad* (about +3folds), *Dap kinase* (about +2.5folds), and *Bcl-2* (about -2folds). Concerning *caspases*, expression of *caspase*-5 and *-8* was switched-on in anti-PKCη-asODN transfected cells, whilst no or weak positivity was found for cDNA preparations from untransfected or scrambled-asODN transfected controls. These cumulative results suggest that, apart from the physiologic involution of normal mammary gland, type V collagen-triggered *PKCη* down-regulation may be conceivably involved in the onset of apoptotic phenomena also in tumoral cytotypes like the d.i.c.

cell line under study. It is known that the abnormal deposition of type V collagen in d.i.c. stroma is to be ascribed to the fibroblasts of the host tissue and, in light of our results, this specific over-accumulation of a collagen species could be interpreted as an aspect of the tissue defensive reaction against neoplastic ingrowth. Further analyses will be necessary to identify the molecular mechanisms underlying type V collagen-promoted signal transduction, involving the switching-off of *PKCη* gene expression.

.....

Regulation of Hsp70 and its role and in A6 mesoangioblast stem cells

Turturici G., Candela ME., Aglieri L., Giacchino MC., Picozza M., Giudice G., Geraci F., Sconzo G.
Dipartimento di Biologia Cellulare e Dello Sviluppo "A.Monroy", Università degli Studi di Palermo

Previous studies demonstrated that A6 mesoangioblast stem cells constitutively synthesize the inducible Hsp70. Biochemical analysis indicated that there are two classes of HSE-binding factors: one is constitutive, while the other one is induced by stresses. These two HSE-protein complexes have different DNA binding stability depending on temperature. Supershift assays demonstrated that HSF1 is responsible for the stress response, but neither HSF1 nor HSF2 are involved in the basal HSE-protein complex. In other cell lines it has been demonstrated that a complex named constitutive HSE-binding factor (CHBF), binds constitutively HSE and was the same of autoantigen Ku. In order to verify if in the A6 stem cells the basal hsp70 transcription was due to CHBF/Ku, we carried out experiments both in *vitro* and in *vivo*. Supershift assays indicated the ability of protein Ku to bind HSE consensus in vitro, in addition chromatin immunoprecipitation assays, using anti-Ku antibodies, confirmed that in *vivo* Ku is bound on the proximal hsp70 promoter region, in which there are two HSE consensus. The importance of the HSE consensus in basal Hsp70 transcription is demonstrated by reporter gene assays. By using the β-gal reporter gene under the control of hsp70.3 promoter deleted in the HSE consensus, we observed a reduction in the β-gal activity. Moreover, to investigate the role of the constitutive Hsp70 expression in A6 stem cells, we have carried out a stable RNA interference to obtain the silencing of both hsp70.1/3. We have obtained A6-NM3 clone with a 55% knockdown for Hsp70. Test of cytostaticity indicated that there are not any differences between A6 and A6-NM3 in cell distribution during the cell cycle phases. On the contrary, proliferation assays indicated that A6-NM3 cells grow slower than A6 control cells and their doubling time is 23.4 hours versus 16 hours of the A6 cells. These results indicated that in A6 stem cells the basally expressed Hsp70 might influence the cell proliferation.

Poster presentation

.....

P38 MAPK expression during development and in stress response in sea urchin embryo

Valenti F., Savona R., Lo Medico I., Ragusa M.A., Gianguzza F. and. Casano C
Dipartimento di Biologia Cellulare e Dello Sviluppo "A.Monroy", Università degli Studi di Palermo

We have previously founded, by a search of EST *Paracentrotus lividus* (Pl) with *Lytechinus variegatus* (Lv), one sequence corresponding to p38 MAPK. By comparative analysis between Lv and Pl EST p38 nucleotide sequences we designed two primers, which were used to reverse transcribe a fragment 340 bp long. This fragment was used as probe in Southern blot experiments, showing the presence, in *Paracentrotus lividus* genome, of a single–copy gene, coding for α (or β) p38 subtype.
p38 belongs to the family of MAPK, that has a central role in many cellular processes, like stress responses, apoptosis, development, spiculogenesis and many others.
So we start to investigate both the post-translational mechanism of activation through phosphorylation, and the regulation of expression of p38 mRNA.
We performed semi-quantitative "multiplex" RT-PCR experiments, using specific p38 primers and specific 28S rRNA (Pl), to normalize p38 levels. This experiment were performed using total RNA from normal developing embryos and from differently stressed embryos.
The stress used are: deciliation, in which embryos are submitted to iper-osmotic condition, heat shock, in which embryos are submitted to 31°C temperature, and two stress with heavy metal, zinc and cadmium.
We used this stress because induces a different kind of cellular response, with activation of different sets of HSPs protein.
This experiments showed that p38 mRNA levels are differently modulated during developmental stage and after induction of different stress.
Moreover to study the p38 activity, we performed two-dimensional electrophoresis analysis using p38 monoclonal antibodies. We investigated p38 active and inactive forms in different developmental stage and after four different stress condition.
We shown the presence of isoforms of p38 MAPK, differently present and differently activated (or inactivated) during development and in different stress response.

Poster presentation

Intracellular mechanisms activated by ATP and ADPbetaS via P2Y receptors in mouse distal colon: evidence for a "Functional selectivity"

Zizzo M. G., Serio R.
Dipartimento di Biologia Cellulare e dello Sviluppo "A.Monroy" Laboratorio di Fisiologia Generale

Adenosine 5'-triphospate (ATP) released from nerves is involved in the non-adrenergic, non-cholinergic (NANC) enteric transmission *via* interaction with cell surface receptors, the P2 purinergic receptors. P2 receptors are further subdivided into two classes, the ligand-gated channels or P2X receptors and the G protein-coupled P2Y receptors. Numerous P2X and P2Y-receptor subtypes have been cloned and functionally defined on the basis of coupled signalling pathway. The diversity of functions within the purinergic receptor family is responsible of the tissue-specificity of nucleotide signalling. The presence of P2 purinergic receptors have been demonstrated in mouse gastrointestinal tract, but there have been no enough functional studies designed to look for their role in the control of gastrointestinal activity.

The paucity of reports regarding the influence of purines on the longitudinal muscle of mouse distal colon, prompted us to examine *in vitro* the involvement, of the purinergic system in the enteric neurotransmission, the subtypes of P2 purinergic receptors responsible of ATP-induced response and the transduction mechanism underlying the activation of the purinoreceptors.

Mouse colonic segments suspended in an organ bath containing oxygenated Krebs solution, exhibited spontaneous mechanical activity, consisting in rhythmic changes in isometric tension. Nerve stimulation elicited a transient nitrergic relaxation, followed by contraction. Contractile responses was reduced by PPADS, suramin, P2Y purinoceptor desensitisation, but not by P2X purinoceptor desensitisation. Exogenously applied ATP induced a concentration-dependent muscular contraction; adenosine 5'-O-2-thiodiphosphate (ADPbetaS), a preferential P2Y agonist, induced muscular contraction, with the same pharmacological profile as the ATP-induced response, instead the P2X-purinergic agonist, alpha,beta-methylene ATP (alpha,beta-meATP), was ineffective.

Cross desensitization experiments showed that the ATP induced contraction is more sensitive to desensitization, that is the response to ADPbetaS. The block of P2Y(1), P2Y(11), P2Y(12) or P2Y(13) receptors by pre-treatment with the selective antagonists MRS2179, NF 157, MRS 2211 or MRS 2395 respectively did not reduce the contractions induced by purinergic agonists.

The response to both ATP and ADPbetaS were not significantly changed in Ca^{2+}-free medium, but were abolished after the depletion of intracellular calcium stores. The blockers of PLC\IP3 pathway, neomycin, PLC inhibitor, or 2APB, IP3 receptor antagonist, reduced significantly ATP induced contraction; whilst ADPbetaS induced effects were unaffected by pre-treatment with neomycin or 2APB, but decreased by block of calcium release from ryanodine sensitive stores.

In conclusion our results indicate that in the longitudinal muscle of mouse distal colon, ATP and ADPbetaS contribute to the excitatory neurotransmission via a not yet determined P2Y receptors. The two purinergic agonists ATP and ADPbetaS activate different signal transduction pathways. In fact, ATP, but not ADPbetaS, stimulates IP_3 formation, via PLC activation, leading to a rise in cytosolic Ca^{2+} from IP3 sensitive stores. Instead ADPbetaS induces muscular contraction *via* ryanodine-sensitive Ca^{2+} release.

The differential activation of signal transduction pathways may be explained by the phenomenon known as "functional selectivity"

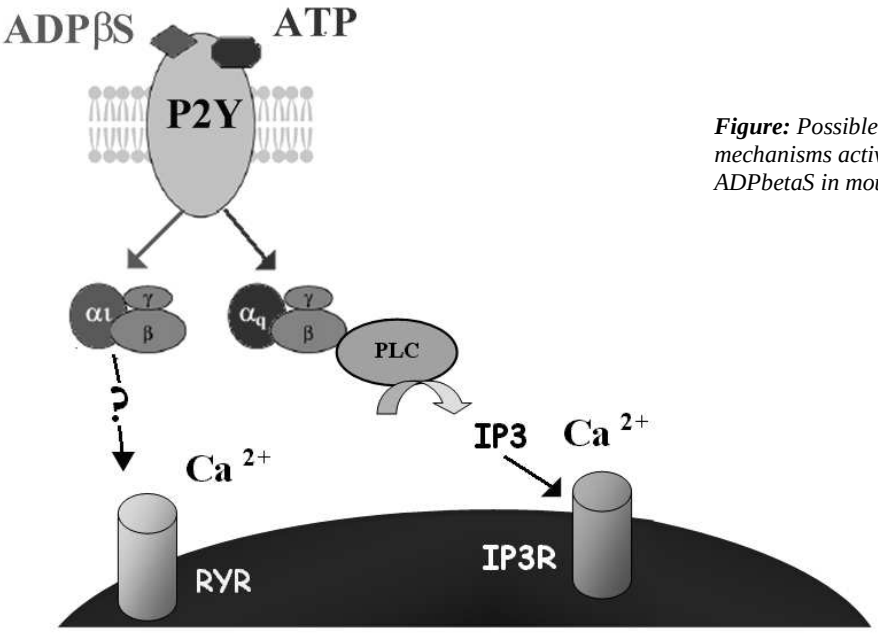


Figure: *Possible intracellular mechanisms activated by ATP and ADPbetaS in mouse distal colon*

Streptomyces coelicolor SCO3645 encodes a novel kynureninase

Zummo F.P. Marineo S., Bernardini F., and Puglia A.M.
Dipartimento di Biologia Cellulare e Dello Sviluppo "A.Monroy", Università degli Studi di Palermo

Tryptophan catabolism consists of a complex network of reactions which leads to different metabolites. Kynureninase or L-kynurenine hydrolase is an enzyme involved in tryptophan degradation and in the pathway for the conversion of tryptophan in quinolate. It's well-known that in eukaryotes, tryptophan and its derivate quinolate are NAD precursors. Instead, it was a common idea that in prokaryotes quinolate is synthesized only from aspartate.
Enzymology of the kynurenine pathway has been extensively studied in eukaryotes, from yeast to human, and all human genes have been characterized. In fact, in the central nervous system, many metabolites of the kynurenine pathway are neuroactive and they are believed to play an important role in the inflammatory processes and neuronal pathologies. For example alterations of this pathway are responsible for variations in brain serotonin levels. Moreover, it has been recently demonstrated that an enzyme involved in this pathway induces apoptosis of neurons prepared from rat striatum and may play a role in cataract formation in the mammalian eye. Precursors and quinolate concentrations are elevated in patients with AIDS-related dementia, Huntington's disease, and hepatic encephalopathy. Furthermore, quinolate acts on NMDA receptors in the brain causing neuronal injury and death.
Recent studies demonstrate that this important pathway belongs also to bacteria: enzymes have been found in *Ralstonia metallidurans*, *Escherichia coli*, *Pseudomonas fluorescens* and *P. aureofaciens*, *Bacillus cereus* and in several strains of the genus *Streptomyces*. This discovery could allow easier and faster identification of small molecule inhibitors that could be used for the treatment of human diseases.
In *Streptomyces coelicolor*, a $Gram^+$ soil bacterium well known for production of many bioactive compounds, only the first enzyme implicated in this pathway encoded by *SCO3646* has been identified. *In silico* analyses of aminoacidic sequence of SCO3645, using BLASTp (http://www.ncbi.nlm.nih.gov/BLAST/), indicate the presence of a conserved kynureninase domain and a high similarity with the same enzyme of different organisms. Moreover, the same analysis strongly suggests that *SCO3644* codifies for a second enzyme of the kynureninase pathway. This hypothesis is supported by MGDB (http://mbgd.genome.ad.jp/) that allowed us to find other genes possibly involved in the same pathway in different microorganisms. Genes homologous to *SCO3645* and *SCO3644* often show sinteny.
In order to demonstrate the activity of SCO3645 as a kynureninase we cloned and overexpressed the gene in *E. coli* and purified the protein. The purified protein has been used in kynureninase assay. This analysis revealed kynurenine disappearance.
Our results strongly suggest that SCO3645 is the first kynureninase described in Streptomyces coelicolor.
Further experiment could help us to elucidate the role of *SCO3645* and adjacent genes in tryptophan catabolism.