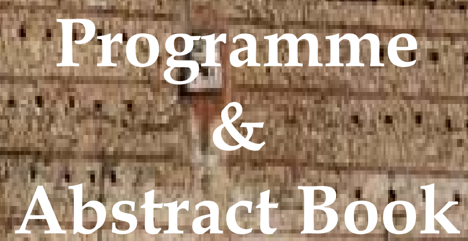

Programme
&
Abstract Book

Venetian Institute of Molecular Medicine
9th Annual Retreat

Marostica 12 - 13 November, 2010

Tel. 338 3261684

Manifestazione organizzata
con il patrocinio e il contributo della

Città di Marostica

Venetian Institute of Molecular Medicine

9th Annual Retreat

Marostica, 12 - 13 November 2010

Programme and Abstract Book

Welcome

Dear Friends,

As in the last annual meeting of the VIMM, three prizes will be awarded to recognize the work of three young Ph.D. students or postdocs working at our Institute.

The awards have been generously offered by Mrs Manzin, as a tribute to her father and her husband.

Welcome to Marostica to the 9th annual VIMM retreat.

Tullio Pozzan

VIMM Scientific Director

At the 9th annual meeting of the VIMM we have invited also the members of "*Centro Interdipartimentale di Ricerca per lo Studio dei Segnali Cellulari*".

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DI RICERCA PER LO STUDIO DEI SEGNALI CELLULARI Via Orus 2 -Padova

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Programme

November 12, Friday

8:00 Departure From Padua
09:30 Arrival in Marostica at Hotel Europa
09:30 – 09:50 Coffee Break at Hotel Europa

Congresso presso il Castello Inferiore

10:00 – 10:30 *Welcome Sindaco di Marostica, Prof. Muraro, Prof. Pagano*

10:30 – 11:00 **Paolo Lorenzon - gruppo Lodovichi**
Role of afferent activity in circuit formation in the olfactory system

11:00 – 11:30 **Cristina Cerqua - gruppo Scorrano**
Trichoplein/mitostatin regulates endoplasmic reticulum-mitochondria juxtaposition

11:30 – 12:00 **Valentina Giorgio - gruppo Bernardi**
The effects of Idebenone on mitochondrial and cellular bioenergetics

12:00 – 12:30 **Tania Zaglia - gruppo Mongillo**
Emerging roles of cardiac sympathetic nervous system in the modulation of cardiomyocyte trophism and microcirculation

12:30 - 14:30 Lunch at Hotel Europa
14:15 – 14:30 allestimento poster

14:30 – 15:00 **Barbara Molon - gruppo Bronte**
Improving cancer immunotherapy by preventing chemokine nitration

15:00 – 15:30 **Francesco Zonta - gruppo Mammano**
Connexin 26 structure and function investigated in silico by Molecular Dynamics simulations

15:30 – 16:30 **VIMM General Assembly**

16:30 – 17:15 **Prof. Angelo Avogaro**
Lecture: *The Diabetes Mellitus: from the metabolic to the epigenetic alterations.*

17:30 – 19:30 Poster Session at Castello Inferiore and Coffee Break
20:30 Dinner and Music at Ristorante Al Castello Superiore

November 13, Saturday

09:45 – 10:15 **Christian Borgo - gruppo Pinna**
Aberrant signalling by protein kinase CK2 in imatinib-resistant chronic myeloid leukemia cells

10:15 – 10:45 **Graziano Lolli - gruppo Battistutta**
Regulation by ordered aggregation of protein kinase CK2 as revealed by the crystallographic structure of an octameric form

10:45– 11:15 **Stefano Ciciliot - gruppo Schiaffino**
Role of the muscle regulatory factor Mrf4 in adult skeletal muscle

11:15 - 11:45 Coffee Break

11:45– 12:15 **Luisa Coletto - gruppo Sandri**
Role of autophagy in muscular dystrophies linked to collagen VI deficiency

12:15 – 13:00 **Prof. Francesco Di Virgilio**
Lecture: *In cauda venenum: the notorius P2X7 receptor*

13:00 - 15:00 Lunch at Hotel Europa

15:00 - 15:30 **Lorenza Sisinni - gruppo Zanotti**
Structural studies of proteins involved in different pathways of Helicobacter pylori

15:30 – 16:00 **Sebastian Martewicz - gruppo Elvassore**
Functional in vitro maturation of human embryonic stem cells-derived cardiomyocytes requires the cell-substrate interaction

16:00 – 16:30 **Gaia Codolo - gruppo de Bernard**
The Helicobacter pylori Neutrophil Activating Protein as an immune modulating agent: possible clinical application

16:30 – 16:45 Coffee Break

16:45 – 17:15 **Silvia Mirandola - gruppo Alberti**
Large scale survey of naturally occurring HBV polymerase mutations associated with anti-HBV drug resistance in untreated patients with chronic hepatitis B

17:15 – 17:45 **Elisa Ave - gruppo Semenzato**
The role of mesenchymal stromal cells in the pathogenesis of B cell chronic lymphocytic leukemia

17:45 – 18:15 **Marta Giacomello - gruppo Carafoli**
Defects of the plasma membrane Ca2+ pumps in hereditary deafness: analysis of mice and human mutants

18:30 Departure from Marostica

Lecture Abstracts

Invited Speakers

The Diabetes Mellitus: from the metabolic to the epigenetic alterations.

Angelo Avogaro
Venetian Institute of Molecular Medicine

The Diabetes mellitus (DM) is becoming increasingly prevalent in the general population and carries significant incremental morbidity and mortality. DM is characterized by a 2 to 4-fold increased risk of cardiovascular disease (CVD). This is generally attributed to the adverse effects of hyperglycemia and oxidative stress on vascular biology. It has been also shown that patients with prediabetic conditions, such as IFG and IGT, are at increased risk of CVD as well. DM is also associated with multi-organ involvement and increased all-cause mortality, resembling a precocious aging process. The mechanisms that account for this phenomenon are incompletely clear, but it is becoming recognized that longevity genes might be involved. Experiments with over-activation or disruption of key life-span determinant pathways, such as SIRT1, p66Shc and mTOR, lead to development of MS features in mice. These genes integrate longevity pathways and metabolic signals in a complex interplay in which life-span appears to be strictly dependent on substrate and energy bioavailability. Herein, we describe the roles and possible interconnections of selected lifespan-determinant molecular networks in the development of MS and its complications, describing initial available data in humans. A better understanding of these signals might help devise new therapeutic strategies to counter the metabolic syndrome epidemics.

In cauda venenum: the notorius P2X7 receptor.

Francesco Di Virgilio
Department of Experimental and Diagnostic Medicine, University of Ferrara

Adenosine triphosphate is the most fundamental intracellular energy currency, however not even Karl Lohmann would have anticipated that, a century after its discovery, this nucleotide was to enjoy a second life as an extracellular messenger and a mediator of inflammation. Over the last few years countless reports have proven beyond any reasonable doubt that this nucleotide is a strong inflammatory mediator acting at plasma membrane P2 receptors, among which P2X7 is most noteworthy. The P2X7 receptor is an intriguing plasma membrane molecule. Functionally, P2X7 is an ATP-gated, cation-selective channel. However, when overstimulated it undergoes a channel-to-pore transition that allows trans membrane fluxes to hydrophilic molecules of molecular mass up to 900 Da. Opening of such a pore is often, but by no means always, followed by cell death. P2X7 is made by the assembly of tri (but sometimes six) identical subunits, thus structurally is a homo-trimer (homo-hexamer). The ability to undergo the channel-to-pore transition is conferred by the extended carboxylterminal tail (about 200 aa long) that has no sequence homology to any mammalian protein so far known. Many polymorphisms are known, as well as splice-variants. The biological role of P2X7 is as yet unknown, but compelling evidence suggests that it has a central role in sensing release of damage-associated molecular patterns (DAMPs) and in the activation of the NLRP3-inflammasome. However, P2X7 might have a more complex function in pathophysiology as recent observations point to its involvement 1) in cell proliferation and 2) in host response to cancer. Although current opinion maintains that P2X7 is a cytotoxic receptor coupled to a number of cellular responses associated to cell death (caspase activation, mitochondrial swelling and uncoupling, plasma membrane blebbing), several groups have consistently shown that, while there is no doubt that pharmacological stimulation of P2X7 is detrimental, tonic stimulation due to locally released ATP has a trophic effect on cell metabolism and supports an increased rate of cell proliferation. We have fully dissected the chain of intracellular events responsible for the P2X7 trophic effect, demonstrating the role of cytoplasmic and intramitochondrial calcium changes and NFAT activation. In addition, recent observations by Zitvogel and coworkers indicate that a functional dendritic cell (DC) P2X7 receptor is necessary to allow efficient anticancer immunity. This effect seems to be due to the stimulation of this receptor by ATP released into the tumour microenvironment, and the ensuing local release of IL-1. These observations open and entirely novel perspective on the role of P2 receptors in the control of cell growth and on immunoregulation within the tumor microenvironment.

Oral Presentation Abstracts

(Underlined: speaker, **Bold:** Principal Investigator)

Role of afferent activity in circuit formation in the olfactory system.

Paolo Lorenzon[1], Nelly Redolfi[1], **Claudia Lodovichi**[1,2,3]

[1]Venetian Institute of Molecular Medicine (VIMM), Padua;
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[3]Armenise Harvard CDA

Electrical activity plays a critical role in circuit formation. Although largely investigated in other sensory modality such as the visual system, the role of activity in the development of the olfactory system (OS) remains largely obscure and controversial. In previous works, we found that homologous glomeruli in each olfactory bulb are linked through a specific and reciprocal inhibitory link, related to external tufted cells. These experiments were performed in adult animals. Now we are studying the development and the role of electrical activity in the formation of the intrabulbar link. To address these questions we analyzed the intrabulbar circuitry at different stages of development in normal animals and in mice in which the electrical activity is abolished by genetic manipulation (over expression of Kir 2.1 channel in OSN) and/or surgical methods (naris occlusion) and in controls. Using these approaches we plan to understand whether in these conditions: 1) the intrabulbar link is still precisely targeted between homologous glomeruli; 2) the extension of the projection is still confined to a single glomerulus. We also studied whether and how post synaptic elements, in particular mitral and granule cells, are affected by manipulation of OSN electrical activity. Preliminary data indicated that the absence of afferent activity, affect both the intrabulbar circuit formation and the development of post synaptic cells.

Trichoplein/mitostatin regulates endoplasmic reticulum-mitochondria juxtaposition

Cristina Cerqua[1], Vassiliki Anesti[1], Aswin Pyakurel[2], Dan Liu[2], Deborah Naon[1], Gerhard Wiche[3], Raffaele Baffa[4], Kai Stefan Dimmer[1], **Luca Scorrano**[1,2]

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Trichoplein/mitostatin (TpMs) is a keratin-binding protein that partly colocalizes with mitochondria and is often downregulated in epithelial cancers but its function remains unclear. In this study, we report that TpMs regulates the tethering between mitochondria and endoplasmic reticulum (ER) in a Mitofusin 2 (Mfn2)-dependent manner. Subcellular fractionation and immunostaining show that TpMs is present at the interface between mitochondria and ER. The expression of TpMs leads to mitochondrial fragmentation and loosens tethering with ER, whereas its silencing has opposite effects. Functionally, the reduced tethering by TpMs inhibits apoptosis by Ca2+-dependent stimuli that require ER–mitochondria juxtaposition. Biochemical and genetic evidence support a model in which TpMs requires Mfn2 to modulate mitochondrial shape and tethering. Thus, TpMs is a new regulator of mitochondria–ER juxtaposition.

The effects of Idebenone on mitochondrial and cellular bioenergetics

Valentina Giorgio[1], Valeria Petronilli[1], Maurizio Prato[2], Anna Maria Ghelli[3], Michela Rugolo[3] and **Paolo Bernardi**[1]

[1]Consiglio Nazionale delle Ricerche Institute of Neuroscience and Department of Biomedical Sciences, University of Padova;
[2]Department of Pharmaceutical Sciences, University of Trieste;
[3]Department of Biology, University of Bologna, Bologna

Idebenone [2,3-dimethoxy-5-methyl-6(10-hydroxydecyl)-1,4-benzoquinone] is a synthetic short-chain analogue of coenzyme Q10 (CoQ10). A variety of quinones (including idebenone) have been shown to affect the mitochondrial permeability transition pore (PTP), a high-conductance inner membrane channel modulated by the proton electrochemical gradient and by many signaling molecules. The PTP links oxidative stress to cell death, and may be involved in the pathogenesis of Leber's hereditary optic neuropathy (LHON) and possibly to other conditions with complex I deficiency. Given these complex effects of idebenone, we have investigated its effects on bioenergetics and PTP modulation in intact cells. Our results indicate that: (i) idebenone promotes CsA-sensitive opening of the PTP and subsequent loss of pyridine nucleotides; (ii) dithiothreitol prevents PTP opening and its detrimental consequences; (iii) idebenol does not cause PTP opening, and stimulates electron transfer at complex III of the respiratory chain; and (iv) idebenol-stimulated respiration is coupled to ATP synthesis both in rotenone-treated normal cells and in RJ206 cells (harboring the 3460/ND1 LHON mutation) and XTC.UC1 thyroid oncocytoma cells (bearing a disruptive frameshift mutation in the MT-ND1 gene, which impairs complex I assembly). Thus, under proper experimental conditions idebenol can be a useful tool to bypass complex I defiencies.

Emerging roles of cardiac sympathetic nervous system in the modulation of cardiomyocyte trophism and microcirculation

Tania Zaglia [2], Giulia Milan [1], Giulia Carotta [2], Valentina Mazzariol [2], Francesca Da Broi [2], Anna Pia Plazzo [2], Mauro Franzoso [2], Maura Francolini [3], Tullio Pozzan [1,2], Marco Sandri [1,2], Stefano Schiaffino [1] and **Marco Mongillo** [1,2]

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[2], Department of Biomedical Sciences, University of Padova
[3], CNR Institute of Neuroscience, Department of Medical Pharmacology, University of Milano.

Scientific background. The sympathetic nervous system (SNS) mediates cardiomyocyte (CM) postnatal hypertrophic growth and modulates cardiac activity in stress conditions. Alterations in SNS patterning and activity occur spontaneously during aging and are associated to several cardiac diseases. However, little is known about the molecular pathways involved in SNS impairment and its causal relationship with cardiovascular pathologies. **Aims.** The aims of this study are to: *i)* characterize the cardiac SNS patterning and *ii)* investigate the role of SNS in the adult myocardium physiology in murine models. **Results.** Immunofluorescence analyses demonstrated that sympathetic terminals (STs) are mainly concentrated in the atria and the right ventricle and interact with coronaries, CM and capillaries. SNS ablation was accompanied by cardiac atrophy, as demonstrated by a 20% decrease in heart body/weight ratio and CM size. Quantitative PCR showed a 5-fold increase of the ubiquitine-ligase MuRF1. Vascular alterations, such as reduced capillary size and increased capillary density, were also observed. The decreased cardiac sympathetic innervation occurring during aging was accompanied to similar trophic and microvascular alterations. **Conclusions and perspectives.** i) SNS modulates CM trophism by regulating the balance between hypetrophic growth and protein degradation. SNS plays also a crucial role in the regulation of cardiac angiogenesis. Experiments are in progress to elucidate the molecular pathways involved in such processes. ii) Since SNS patterning is crucial for the maintenance of myocardial homeostasis, experiments are in progress to elucidate the molecular and functional organization of 'neuro-cardiomyocyte junctions'.

Improving cancer immunotherapy by preventing chemokine nitration.

Barbara Molon [1,2], Stefano Ugel [1,2], Federica Del Pozzo [2], Cristiana Soldani [3], Debora Avella [3], Antonella De Palma [4], Ana Monegal [5], Benedetta Savino [3], Alberto Gasco [6], Antonella Viola [3], **Vincenzo Bronte** [1,2].

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The goal of all immunotherapeutic approaches against solid tumors is the induction and expansion of cytotoxic T lymphocytes (CTLs) that invade tumor mass and kill transformed cells. Tumor-promoted constrains negatively affect CTL trafficking to tumor core. As a matter of fact even when able to reach the tumor, antigen-experienced T cells tend to remain at the periphery. Production of reactive nitrogen species (RNS) within the tumor microenvironment has been reported in mouse and human neoplasias. We observed a singular pattern of T lymphocyte distribution opposite to nitrotyrosine staining in many human tumors, with CTLs concentrating at the border of the neoplastic lesions and the bulk of tumor mass expressing substantial amount of nitrotyrosines. We provided evidence for a novel and previously unexplored mechanism of tumor immune escape based on RNS-dependent post-translational modification of chemokines. We found that the chemoattractants CXCL12, CCL21, and CCL2 lost their ability to recruit T lymphocytes when exposed to RNS that are generated within the tumor microenviroment. However, the modified CCL2 retains its capacity of recruiting myeloid cells thus favoring tumor immune escape. Based on our findings, drugs controlling the *in situ* production of RNS might be useful to aid immunotherapeutic approaches for the treatment of cancer, by creating a favorable tumor environment for lymphocyte recruitment and activation. Pre-conditioning of the tumor microenvironment with our novel drug, AT38 ([3-aminocarbonyl]furoxan-4-yl)methyl salicylate) that control RNS production by modulating ARG and NOS activity, facilitates infiltration of immune cells within the tumor, and improves the efficacy of adoptive cell therapy with tumor-specific CTLs. Our results unveil an unexpected mechanism of tumor evasion and open new avenues for cancer immunotherapy.

Connexin 26 structure and function investigated in silico by Molecular Dynamics simulations

Francesco Zonta[1], Giuseppe Zanotti[2,3], **Fabio Mammano**[1,3]

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About half of all cases of human deafness in countries surrounding the Mediterranean have been linked to mutations in the GJB2 gene, which encodes the gap junction protein connexin 26 (Cx26) (1). We chose a Molecular Dynamics approach aiming to construct a computational environment for the interpretation of experimental results and the prediction of effects not yet observed. Starting from the recently published crystal structure of the Cx26 gap junction channel (2), we built a fully atomistic model of the Cx26 connexon including plasma membrane phospholipids, water and ions (the whole system contains 206188 atoms) and followed its equilibrium dynamics for about 13 ns. This in silico approach enlivens the “frozen” crystallographic structure of Cx26 and permits us to study the protein in a realistically simulated environment. The analysis of temporal trajectories of each molecule provides critical insight into the interactions that shape the tertiary and quaternary structure of the protein complex. In addition, we can simulate equilibrium thermal fluctuation of the protein and study the dynamics of ions and secondary messenger transit through the pore, which provides invaluable mechanistic insight into channel function and dysfunction, including permeation and gating mechanisms.

Aberrant signalling by protein kinase CK2 in imatinib-resistant chronic myeloid leukemia cells

Christian Borgo[1,2], Luca Cesaro[1], Maria Ruzzene[1,2], Stefania Sarno[1,2], **Lorenzo A. Pinna**[1,2], Arianna Donella-Deana[1]

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Chronic myeloid leukemia (CML) is driven by the fusion protein Bcr/Abl, a constitutively active tyrosine kinase. Despite the great efficacy of the Bcr/Abl-specific inhibitor imatinib, resistance to this drug is a major problem in CML-patient treatment. Protein kinase CK2 is an ubiquitous and constitutively active Ser/Thr kinase, implicated in several pathways of cell life and frequently overexpressed in human cancer. The CK2 role in imatinib-resistance was studied by analyzing its potential cross-talk with Bcr/Abl in a CML cell line, imatinib-sensitive (LAMA84-S) or -resistant (LAMA84-R). We found that in LAMA84-R cells, characterized by Bcr/Abl overexpression, both CK2 protein-level and activity are higher than in sensitive cells. Co-immunoprecipitation and proteomic analyses showed that CK2 interacts with Bcr/Abl in imatinib-resistant cells, where a CK2α Tyr-phosphorylation is also detectable. Experiments performed with inhibitors specific for CK2 or Bcr/Abl suggest that Bcr/Abl is likely involved in CK2α Tyr-phosphorylation. The viability of LAMA84-S/R cells is affected by the CK2 inhibitor CX-4945, and CK2 inhibition makes LAMA84-R cells sensitive to a low imatinib-concentration. Our results suggest that, besides Bcr/Abl overexpression, CK2 abnormal level plays a role in the imatinib-resistance mechanisms of LAMA84-R cells and that the combined treatment with inhibitors directed to Bcr/Abl and CK2 might be a tool in counteracting the imatinib-resistance in CML pathology.

Regulation by ordered aggregation of protein kinase CK2 as revealed by the crystallographic structure of an octameric form

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CK2 is a protein kinase involved in many fundamental aspects of the normal cell life. It sustains cell survival but, under special circumstances, it also enhances tumour growth. Its most simple form is a hetero-tetramer composed of two catalytic subunits α and two regulatory subunits β. Differently from many other protein kinases, CK2α is not regulated through an on/off mechanism but its activity can be modulated (enhanced, reduced or preferentially directed to a subset of its many substrates) through association with CK2β, phosphorylation events and also via formation of ordered macromolecular assemblies. The C-terminal tail of human CK2α was found prone to degradation and all crystallographic studies were performed using a C-terminal truncated variant (54 amino acids missing). Therefore, the structural role of this C-terminal extension, which has evolved in vertebrates, has been neglected and its function is still unknown. Using a stabilized we were able to isolate a new octameric form of the full-length CK2α holoenzyme whose structure has been solved at 3.1 Å resolution. This corresponds to the "ring-like" structure of the holoenzyme observed by electron microscopy and associated with maximal activity. From crystallographic considerations we propose models also for the "filamentous" form of CK2, associated with very poor activity. A possible mechanism of regulation is that CK2 filaments, pool of inactive or sparsely active aggregates, can reversibly supply fully active CK2 when needed.

Role of the muscle regulatory factor Mrf4 in adult skeletal muscle

Stefano Ciciliot[1,2], Elisa Calabria[1,2,3], Irene Moretti[1,2], Marta Murgia[2], Anne Picard[2], Kenneth A. Dyar[1], Reimar Abraham[1,4], **Stefano Schiaffino[1,2]**

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Muscle regulatory factors are a family of four basic helix loop helix proteins playing a crucial role in myogenesis. Experiments of gene targeting have shown that MyoD, Myf5 and Mrf4 act as muscle determination factors during embryonic development while Myogenin acts as differentiation factor. The role of these factors in adult skeletal muscle fibers is not known. Myf5 is not expressed in adult fibers, MyoD and myogenin are expressed at low levels in fast and slow muscle fibers, respectively, whilst Mrf4 is the only factor expressed at high levels in adult skeletal muscle. Here we show that Mrf4 is expressed at similar levels in slow and fast rat muscles. To determine the role of Mrf4 we used in vivo transfection by electroporation to either overexpress or knockdown this transcription factor in adult slow and fast rat muscles. We find that Mrf4 overexpression impairs muscle growth in regenerating muscles, whereas Mrf4 RNAi induces muscle hypertrophy in both regenerating and adult muscles. This effect is rescued by co-transfection with siRNA-insensitive Mrf4 from mouse or human. The findings indicate that Mrf4 acts as a negative regulator of muscle growth. Microarray analysis from muscles transfected with RNAi constructs is being used to explore the mechanism of Mrf4 action.

Role of autophagy in muscular dystrophies linked to collagen VI deficiency

Luisa Coletto,[1]*Paolo Grumati,[2*] Patrizia Sabatelli,[3] Matilde Cescon,[2]Alessia Angelin,[4] Enrico Bertaggia,[1] Bert Blaauw,[5] Anna Urciuolo,[2] Tania Tiepolo,[2] Luciano Merlini,[6] Nadir M. Maraldi,[3,7] Paolo Bernardi,[4] **Marco Sandri**,[1,4] Paolo Bonaldo[2]

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4Department of Biomedical Sciences, University of Padova, 35121 Padova, Italy.
5 Department of Human Anatomy & Physiology, University of Padova, 35121 Padova, Italy.
6Department of Experimental and Diagnostic Medicine, University of Ferrara, Ferrara, Italy.
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Autophagy is an evolutionarily conserved process that is crucial in the turnover of cell components both in constitutive conditions and in response to starvation. Clearance of damaged cell components by the autophagic/lysosomal pathway is essential for tissue homeostasis. Defects of this degradative system play a role in various diseases, but little is known about autophagy in muscular dystrophies. We previously found that congenital muscular dystrophies linked to collagen VI deficiency are characterized by the presence of dysfunctional mitochondria and spontaneous apoptosis, which ultimately lead to myofiber degeneration. Here we show that persistence of abnormal organelles and apoptosis are caused by defective autophagy. Indeed, skeletal muscles of collagen VI knockout (Col6a1–/–) mice displayed an impairment of autophagic flux, which matched the lower induction of Beclin1 and Bnip3 and the lack of autophagosomes after starvation. Notably, forced activation of autophagy by genetic, dietary and pharmacological approaches restored myofiber survival and ameliorated the dystrophic phenotype of Col6a1–/– mice. Furthermore, muscle biopsies from patients affected by Bethlem myopathy (BM) and Ullrich congenital muscular dystrophy (UCMD) showed reduced levels of Beclin 1 and Bnip3. These findings indicate that defective activation of the autophagic machinery plays a pathogenic role in congenital muscular dystrophies

Structural studies of proteins involved in different pathways of Helicobacter pylori

Lorenza Sisinni[1,2], Laura Cendron[1,2], **Giuseppe Zanotti**[1,2]

[1] Dept. of Biological Chemistry, University of Padua, Padua, Italy
[2]Venetian Institute of Molecular Medicine (VIMM), Padua, Italy

Helicobacter pylori is a gram-negative, ureolytic organism which colonizes the stomach of about half of the human population. Its infection is associated with a spectrum of gastric pathologies, ranging from mild gastritis to peptic ulcers and gastric cancer. A strong predictor of a severe disease outcome is the infection with a bacterial strain harboring the cytotoxin associated gene pathogenicity island (cag PAI), a 40kb stretch of DNA that encodes homologues of several components of a type IV secretion system (1). This project is aimed at the structural and functional characterization of H. pylori proteins involved in pathogenicity, or relevant for colonization and persistence of the bacterial infection. In particular, studies on proteins of the following three systems are under way: CagL, a protein belonging to the cagPAI; HP0797, a flagellar sheath adhesin; HP1286, a YceI potential periplasmatic protein and it is induced by osmotic stress created by NaCl or by high pH. T4SS forms a pilus for the injection of virulence factors into host target cells such as the CagA oncoprotein. This is accomplished by a specialized adhesin of the pilus surface, the CagL protein, which binds to and activates host cell integrins for subsequent delivery of CagA across the host cell membrane. Injected CagA becomes tyrosine-phosphorylated by Src and Abl family kinases and mimics a host cell protein in binding and activation of multiple signalling factors.Here we present recent advances in the structural characterization of CagL using different techniques, like Small-angle X-ray scattering (SAXS), light scattering and mass spectrometry, to clarify the oligomerization state of this protein in solution and its tridimensional structure. REFERENCE: Backert S, Selbach M. Role of type IV secretion in Helicobacter pylori pathogenesis. Cell Microbiol.2008 10(8):1573-81

Functional **in vitro** *maturation of human embryonic stem cells-derived cardiomyocytes requires the cell-substrate interaction.*

Sebastian Martewicz[1], **Nicola Elvassore**[1,2]

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Human cardiac precursors or immature cardiomyocytes (hES-CM) derived from embryonic-like stem cells are potentially the best cellular model for *in vitro* and *in vivo* studies, being representative of the human physiology. The main limitation is their maturation stage: hES-CM phenotype and physiology are comparable to fetal/neonatal cardiomyocytes, rather than adult. The aim of this study is to investigate the effects of the hES-CM-substrate interaction into their functional differentiation through the calcium dynamics analysis. hES-CM were obtained from embryoid bodies, HES2 cell line, with a specific cardiac differentiation protocol (Yang et al, Nature 2008). The derivates of this differentiation protocol, once seeded in monolayer culture, express most of the cardiac markers and show spontaneous contractions and calcium handling machineries. The calcium transients were analyzed in hES-CM deriving from EB at 22, 32 and 52 days of differentiation and cultured in monolayer for additional 2, 4 and 6 days on glass. We observed a gradual shortening of the Ca^{2+} cycle with the increase of the culture time as monolayer on rigid support, while the phase of the differentiation protocol didn't alter the Ca^{2+} analysis. These experimental data were correlated with a mathematical model describing the calcium dynamics in adult human cardiomyocytes. SERCA pumps were identified as the most sensitive parameter on calcium SR uptake; experimental data were properly correlated with the inhibition of the SERCA pumps in the model (100% inhibition at T+2 time point, 80% at T+4 and <50% at T+6). Immunofluorescence analysis on SERCA (2a) confirmed the model derived-hypothesis. On the other hand, analysis on actin showed hES-CM morphological changes according to the time in culture as monolayer. The substrate elasticity-dependence of cell structural elements organization has been tested by seeding the cells on hydrogels of different elastic modulus. These findings confirm our hypothesis on the importance of the cell-substrate interaction in the maturation of hESC-CM.

The Helicobacter pylori Neutrophil Activating Protein as an immune modulating agent: possible clinical application

Gaia Codolo[1,2], Matteo Fassan[3], Fabio Munari[2,4], Piefrancesco Bassi[5], Massimo Rugge[3,6], Francesco Pagano[2], **Marina de Bernard**[1,2]

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Intravescical Bacillus Calmette-Guérin (BCG) is the gold standard treatment for intermediate and high-risk non muscle-invasive bladder cancer. BCG therapy is the most successful example of immunotherapy in cancer. Unfortunately, the treatment-related side effects are still relevant. Furthermore, non-responder patients are candidate to radical cystectomy in absence of valuable alternative options. Both mentioned findings have prompted the search of newer biological response modifiers (BRM) with a better benefit/side effects ratio. To this regard the Toll-like receptor (TLR) 2 ligand, *Helicobacter pylori* protein HP-NAP, has been shown to deserve a potential role as BRM. HP-NAP is capable of driving the differentiation of T helper (Th) 1 cells, both *in vitro* and *in vivo*, because of its ability to create an IL-12 enriched milieu. Herein we report that local administration of HP-NAP decreases tumor growth by triggering tumor necrosis in a mouse model of bladder cancer implant. The effect is accompanied by a significant accumulation of both $CD4^+$ and $CD8^+$ IFN-γ-secreting cells, within tumor and regional lymph nodes. Noteworthy, HP-NAP-treated tumors show also a reduced vascularisation due to the anti-angiogenetic activity of IFN-γ induced by HP-NAP. Our findings strongly indicate that HP-NAP, might become a novel therapeutic "bullet" for the cure of bladder tumours.

Large scale survey of naturally occuring HBV polymarese mutations associated with anti-HBV drug resistance in untreated patients with chronic hepatitis B

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Background: Drug resistance is a major limitation for the long-term efficacy of antiviral therapy with nucleos(t)ide analogues (NAs) in chronic hepatitis B (CHB). Antiviral resistance mutations may pre-exist in the overall viral population of untreated patients.We aimed to assess the prevalence of such HBV variants in a large cohort of NAs-naïve patients with CHB and to explore possible association with viral and host variables. Methods: Serum samples from 286 NAs-naïve consecutive patients with CHB were tested for serum HBV DNA and 255 of them having HBV-DNA> 1000 IU/mL were further analysed for drug resistance mutations by INNO-LiPA HBV DRv2/v3 and by direct sequencing. 39% of the patients had previous treatment with interferon alfa (or PEG-IFN) while the remaining 61% had never received any type of antiviral therapy. Results: NA-naïve patients analysed were mainly male (73%), Caucasians (85%), HBeAg-negative (79%) SD). Using± 13.4 years (±and genotype-D (69%), with a mean age of 43.2 the INNO-LiPA technique, HBV mutations associated with NAs drug resistance were detected in 13 (5%) patients : 3 had the rtM204V lamivudine resistance mutation always associated with the rtL180M compensatory mutation. Interestingly, all these 3 patients were infected with HBV genotype-C. Four patients had the rtI233V mutation that may reduce sensitivity to adefovir and 3 patients had the rtM250L/V mutation typical of entecavir resistance. Isolated lamivudine compensatory mutations rtL80V and rtV173L were seen in 2 and 1 patients, respectively. Several mutations with or without a defined clinical significance were detected by direct sequencing in 31% of the analysed patients. No relationship was seen between presence of resistant or compensatory mutations and HBV-DNA levels, HBeAg/anti-HBe status or previous IFN therapy. Conclusions : these results confirm that HBV mutations which confer resistance against currently available anti-HBV NUCs may already exist in patients who have never received the drug.

The role of mesenchymal stromal cells in the pathogenesis of B cell chronic lymphocytic leukemia

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B-cell chronic lymphocytic leukemia (B-CLL) is a clonal lymphoproliferative disease, characterized by the accumulation in peripheral blood, bone marrow and lymphoid tissues of small B lymphocytes expressing a typical phenotype (CD5+/CD19+/CD23+) and blocked in the G0/G1 phase of cellular cycle. Localization of CLL-B cells in bone marrow and/or lymphnodes is a crucial step for neoplastic clone survival: bidirectional interactions between malignant lymphocytes and by-stander cells may led to the establishment of an abnormal microenvironment which confers growth advantages and extended survival to the neoplastic clone. We focused our attention on mesenchymal stromal cells (MSCs), in order to clarify whether they play an active role on survival and accumulation of leukemic B cells. MSCs were isolated from the bone marrow of 33 B-CLL patients and characterized through flow cytometric analysis (CD73+, CD90+, CD105+; CD31-, CD34-, CD45-) and for their ability to differentiate into adipocytes and osteocytes. Co-colture experiments of CLL-MSCs and leukemic lymphocytes demonstrated that MSCs have an antiapoptotic effect on neoplastic B cells, with a significative increase of B-CLL cell survival after 7 days of co-colture (60%±17,3 in presence *vs* 14%±11,7 in absence of MSCs) but not of normal B lymphocytes (30,20%±15 with MSCs *vs* 6,20%±5 without MSCs). The analysis of neoplastic cell migration in the presence of conditioned *medium* derived from MSC cultures demonstrated the MSC ability to produce chemiotactic stimuli that drive the leukemic clone to the bone marrow microenvironment. We did not observed the same effect on normal cells. Recent studies indicated the role of the chemokine CCL5/RANTES in neoplastic cell homing and metastasis. We found that the co-colture with normal and leukemic B cells modulates CCL5 expression by MSCs and the expression of its receptors CCR1, CCR3, CCR5 on B cells. Taken together, these findings suggest that MSCs derived from patients with B-CLL, despite an apparent normal phenotype and differentiation ability, provide survival signals to neoplastic cells extending their lifespan and producing chemotattic factors favouring their accumulation in the bone marrow.

Defects of the plasma membrane Ca^{2+} pumps in hereditary deafness: analysis of mice and human mutants

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Mutations of isoform2 of the plasms membrane Ca^{2+} pump (PMCA2) produce deafness in mice and humans, frequently in association with mutations in the stereocilia protein cadherin23. PMCA2 is the resident isoform of the stereocilia of the outer hair cells of the inner ear. It has peculiarly high activity in the absence of the natural activator calmodulin, i.e. it ejects Ca^{2+} to the endolymph that bathes the stereocilia at an essentially constant rate. The properties allow the pump to control the uniquely low concentration of Ca^{2+} in the endolymph. The defects described so far, and analyzed in model cells overexpressing the pumps, concern the long term ejection of Ca^{2+} by the pump more than its short term ability to be activated by Ca^{2+} pulses. We analyzed the molecular defects of the pump in two deafness producing mutants: a human V586M and a mice T692K replacement. Whereas the mice mutant had the expected defect in the long term ejection of Ca^{2+}, the human mutant, which also had the cadherin mutation, was essentially similar to the wt. The emptying of the ER stores by IP_3 linked agonist activates the plasma membrane store activated Ca^{2+} influx (CCE). Preliminary results suggest the possibility that the pump mutation may differentially interfere with CCE. We are curently analyzing the possibility of a direct interaction of PMCA with components of the CCE system as well as the degree of emptying of the ER Ca^{2+} store in cells overexpressing the different PMCA mutants.

Poster Abstracts

(Underlined: speaker, **Bold:** Principal Investigator)

Translational suppressions of atrophic regulators by a microRNA integrate resistance against skeletal muscle atrophy

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Muscle atrophy occurs in many pathological states, which is caused by accelerated protein degradation. Recent research revealed that two muscle-specific ubiquitin ligases, atrogin-1/MAFbx and muscle RING-finger 1 (MuRF1), are prominently induced during muscle atrophy to mediate atrophy related protein degradation. In muscle protection against atrophy, the expression blockade of these two genes are essential. Here we report a single miRNA suppresses these key regulators to lead to muscle resistance against atrophy. We show that miR-23a suppresses the translation of both MAFbx/atrogin-1 and MuRF1 in their 3′ UTR dependent manner. Ectopic expression of miR-23a is sufficient to protect muscle from atrophy in vitro and in vivo.Furthermore, miR-23a transgenic mice are significantly resistant against skeletal muscle atrophy. These data suggests that multiple suppression of regulators by a single miRNA can lead significant phenotypes in adult tissues.

Defective recruitment, survival and proliferation of endothelial progenitor in diabetic wounds

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Bone marrow (BM)-derived endothelial progenitor cells (EPCs) are involved in endothelial homeostasis and tissue healing. Diabetic patients showed altered EPCs biology, which might contribute to end-organ complications. We tested the hypothesis that diabetes impairs the contribution of BM-derived EPCs at sites of wound healing. After 4 weeks of streptozotocin-induced diabetes, hind limbs skin wounds were created and monitored while granulation tissue was analysed. Diabetes delayed wound healing, reduced granulation tissue thickness, vascularity and increased apoptosis. Circulating $CD34^+$/Flk-1^+ EPCs, quantified by flow cytometry before on day 1 and day 4 after wounding, are not modified by diabetes. In separate experiments, we transplanted GFP+ BM into myeloablated wild-type mice 4 weeks before induction of diabetes. We quantified proliferation (phospho-histone H3), apoptosis (in situ TUNEL) and endothelial differentiation (vWF expression) of recruited GFP^+ cells. GFP^+/VWF^+ cells within the granulation tissue, as well as the percentage of GFP^+ cells expressing VWF were significantly reduced in diabetic as compared to control mice. GFP^+ cells showed increased apoptosis and decreased proliferation in diabetic versus non diabetic wound tissues. Net recruitment, estimated by subtracting cell count before wounding and the contribution of apoptosis and proliferation to the local tissue pool of BM-derived GFP^+ cells, was reduced on day 1 after wounding, in diabetic mice compared to non diabetic mice. Further investigations will be addressed to unveil molecular mechanisms, and potential pharmacological targets, which restore EPCs recruitment.

High Abundance Plasma Proteins Depletion vs Low Abundance Proteins Enrichment: Comparison of Methods

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To date, the complexity of the plasma proteome exceeds the analytical capacity of conventional approaches to isolate lower abundant proteins that may prove to be informative biomarkers. Only complex multistep separations strategies have been able to detect a substantial number of low abundant proteins (<100 ng/ml). The first step of these protocols is generally the depletion of high abundant proteins by the use of immunoaffinity column or the low abundant protein enrichment by the use of solid phase hexapeptides ligand libraries. Here we present a direct comparison of these two approaches. After the immunodepletion of high abundant proteins or the enrichment of low abundant proteins, the plasma sample was further fractionated by SCX chromatography and analyzed by RP-LC-MS/MS with a Q-TOF mass spectrometer. The depletion of the 20 higher abundant plasma proteins allowed for the identification of a larger number of proteins (about 60% more) with respect to that obtainable after low abundant proteins enrichment. However, from a technical point of view, the enrichment approach seems to be more suitable as first stage of a complex multi-step fractionation protocol. Moreover, the 2 datasets are only partially overlapping, showing that the 2 methods are somehow complementary. Advantages and disadvantages of both methods are discussed.

Role of the TpF1, a protein of **Treponema pallidum**, *in inducing the production of T reg cells and in driving inflammation via the activation of inflammasome*

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Syphilis, which is caused by *Treponema pallidum*, is a chronic disease and if is not treated can result in severe cardiovascular and neurological symptoms. With an estimated 12 million new cases annually, syphilis is a global health problem. The local and systemic immune responses elicited by the bacterium have not been well studied in humans. TpF1 is a protein produced by *Treponema* and is homologous to HP-NAP of *Helicobacter pylori* and to NapA of *Borrelia burgdoferi*, both proteins with immunomodulatory properties. We tested whether TpF1 might contribute to the chronicity of the disease. We found that TpF1 triggers the release of IL-1β, IL-6, TGF- β and IL-10 from monocytes. This cytokine milieu is expected to trigger the differentiation of T helper cells towards the T reg phenotype. Treg cells suppress the effects of macrophages and this strategy has been suggested as a possible mechanism for *Treponema* to escape elimination by the immune system. Accordingly, we found that a significant proportion of T cells isolated from syphilis patients are T reg (CD25+ FoxP3+ TGF-β+ cells) and an high percentage of these are specific for TpF1. Furthermore, we found that TpF1 promotes the secretion of IL-1β via the inflammasome, a multi-proteins complex that, through the activation of caspase-1, leads to the processing and secretion of pro-inflammatory cytokines, which exacerbate the inflammation.

Anion binding properties of the STAS domain of the motor protein prestin

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Prestin is a member of the Solute Linked Carrier 26 (SLC26) anion exchangers, a family of membrane proteins with important roles in human physiology, capable of transporting a wide variety of monovalent and divalent anions. Prestin is the motor protein responsible for the somatic electromotility of outer hair cells of the mammalian cochlea and is essential for normal hearing sensitivity and frequency selectivity. Although the molecular mechanism underlying electromotility has not been completely clarified, the binding of chloride ions to an intracellular portion of prestin, not identified yet, seems to have a fundamental role in the regulation of the protein function. Recently, we have determined the crystal structure at 1.57 Å resolution of prestin STAS domain. We have also characterized this domain in solution by heteronuclear, multidimensional NMR spectroscopy. A structure-function analysis suggests that this model can be a general template for most SLC26 anion transporters and supports the notion that STAS domains are involved in functionally important intra- and intermolecular interactions. In order to investigate a possible interaction between the STAS domain and chloride ions, we performed further X-ray diffraction and NMR experiments. Very recently, we have determined the crystal structure of the STAS domain in complex with iodide and bromide ions; the two halogens bind in the same site, on the surface that we predicted is facing the membrane. Preliminary NMR results confirm these data, showing that also chloride ions bind to the STAS domain. On the basis of these data, 8 mutations have been identified that should interfere with the halogen binding, and the corresponding mutants will be tested in transport and localization studies in mammalian cell lines, in collaboration with Prof. Dominik Oliver. This will allow to shed light on the functional role of chloride ions and other halogens on the modulation of prestin activity.

Autophagy in muscle-specific mTOR knockout mice

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Muscle growth is controlled by the IGF-1/Akt pathway through its downstream effectors FoxO and mTOR. Protein synthesis is controlled by mTOR, whereas protein degradation is induced by FoxO. Both mTOR and Foxo3, even if to a different extent, have been shown to control autophagy in skeletal muscle. Here we want to study the role of mTOR in autophagy regulation by using a genetic approach. The group of Gangloff has recently developed a muscle-specific mTOR knockout. These mice showed a myopathic phenotype with metabolic impairment. We used these animals and we monitored the level of activation of atrophy-related genes in fed and fasted mice by quantitative real-time PCR. Simultaneously, we checked the autophagy system and the AKT pathway. Our data suggest that autophagy can be induced by fasting even in the absence of mTOR, suggesting that other pathways play a major role in autophagy regulation. Moreover mTOR deficiency triggers p62 accumulation even in presence of LC3 lipidation suggesting that the presence of mTOR is required for the correct delivery of autophagosomes to lysosomes. The full characterization of these animals will dissect the role of mTOR in autophagy regulation in vivo.

Connexin expression and functional analyses of the novel Cx26 conditional null mouse model Cx26Sox10Cre

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Mutations in the GJB2 and GJB6 genes, respectively encoding for connexin 26 (Cx26) and connexin 30 (Cx30), are the major cause of prelingual non-syndromic hereditary deafness in several human populations. Here, we have analyzed inner ear connexin expression and function in Cx26Sox10Cre conditional null mice with targeted ablation of Cx26 in the sensory epithelium of the cochlea. Previous work indicated that Cx26 and Cx30, which share 77% amino acid identity, are essential for hearing and appear to be coordinately regulated. Adult Cx26Sox10Cre mice presented with a considerable hearing loss, assessed by auditory brainstem responses (ABR), and a reduced endocochlear potential. Cx30 expression was similar in adult Cx26Sox10Cre mice and in aged matched controls. In contrast, Cx30 was barely detectable in cochlear organotypic cultures obtained from Cx26Sox10Cre mice at P5 and, accordingly, non-sensory cells in these cultures presented with impaired transfer of the fluorescent tracer calcein through gap junction channels. Our findings support the notion that reduced biochemical coupling can cause defective hearing in mice and man. A key role might be played, at the developmental level, by spontaneous Ca2+ signalling activity, which was significantly decreased in cochlear cultures from P5 Cx26Sox10Cre mice compared to wild type controls.

Monitoring cAMP dynamics in the mitochondrial matrix

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In eukaryotic cells, energy production is functionally coupled to metabolic demands. Mitochondria constantly respond to changes in substrate availability and energy utilization to maintain cellular ATP supplies, and reversible phosphorylation of mitochondrial proteins has been proposed to play a fundamental role in metabolic homeostasis, but very little is known about the signaling pathways involved. cAMP is known to play several different roles in distinct cellular compartments, but its impact on mitochondrial functions is still emerging. In particular, increasing evidence indicates the cAMP/PKA signaling pathway on the cytosolic surface of mitochondria as essential in modulating the apoptotic response and the mitochondrial metabolic activity. In contrast, data on a possible role for cAMP in the matrix are still scattered and contradictory. Recently, it has been supported the existence of a soluble adenylyl cyclase (sAC)-cAMP-PKA signaling cascade wholly contained within mitochondria, which would serve as a metabolic sensor modulating ATP generation and ROS production. To directly investigate the cAMP dynamics within the living mitochondria, we targeted genetically encoded cAMP sensors to the matrix and we generated stable cell lines. Our results indicate that mitochondrial cAMP increases are detectable in different cell lines as well as in primary cardiomyocites in response to bicarbonate and calcium, consistently with the existence of a mitochondrial form of sAC.

Activity-dependent and –independent control of circadian rhythms in mammalian skeletal muscle

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Physical inactivity and dysregulation of circadian rhythms are both well-established risk factors for metabolic diseases, such as type 2 diabetes. Previous studies have suggested that physical activity may participate in regulating the skeletal muscle circadian transcriptome, including the muscle core clock, yet the role activity plays directing skeletal muscle circadian gene expression and its potential mediators remain largely unexplored. We asked whether reduced activity can alter the skeletal muscle circadian transcriptome, including the core clock genes, by performing comparative circadian expression profiling in mouse skeletal muscles with graded activity levels: frequently active slow soleus (SOL), sporadically active fast tibialis anterior (TA) and completely inactive denervated muscle. In contrast to relatively minor changes to core oscillator gene expression observed after denervation, including modest yet significant changes in the expression profiles of many core clock genes, we noted that activity plays major roles in regulating circadian gene expression in both fast and slow muscle. Furthermore, we identified the Ca2+-dependent Calcineurin-NFAT pathway as a mediator of activity-dependent circadian regulation in skeletal muscle, as circadian rhythms of in vivo nucleocytoplasmic shuttling and transcriptional activity of NFATs and their target genes, such as Rcan1, peak during the active phase, are abrogated by denervation, yet remain essentially unaffected by a restricted day feeding schedule. We conclude that both activity and feeding rhythms coordinately control circadian gene expression in skeletal muscle.

C57BL/6 YFP-MTAEQ: A mouse model for the monitoring of mitochondrial calcium

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Monitoring Ca^{2+} dynamics in primary cell cultures is important to study the complexity of Ca^{2+} signalling pathways. To bypass the esperimental limitations linked to low efficiency of plasmids transfection, and to the problems connected with viral infections and the consequent laborious steps, we have decided to generate a transgenic mouse which expresses constitutively the Ca^{2+} sensitive protein aequorin. We have chosen mitochondrial aequorin (mtAEQ fused to the yellow fluorescent protein, YFP) since mitochondrial Ca^{2+} homeostasis dysfunctions are prominent in numerous disease conditions, including the neurodegenerative disorders which are topics of interest to our Laboratory. The monitoring of mitochondrial Ca^{2+} would be made much easier by crossing animal models with neurodegenerative features with the mouse expressing YFP-mtAEQ. The work to obtain the transgenic model carrying YFP-mtAEQ has continued for six generations of mice derived by crossing of transgenic animals with the littermate to select the animal with the lowest number of stable copies of the gene and the highest level of aequorin activity in selected tissues. We are presently at the stage when we have produced YFP-mtAEQ mice with 2 functional copies.

A widespread increase in myeloid calcifying cells contributes to ectopic vascular calcification in type 2 diabetes

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Acquisition of a pro-calcific phenotype by resident or circulating cells is important for calcification of atherosclerotic plaques, which is common in diabetic patients. Herein, we demonstrate for the first time that a distinct subpopulation of circulating myeloid cells expressing osteocalcin and bone alkaline phosphatase (OC+BAP+) has pro-calcific activity in vitro and in vivo. Myeloid calcifying OC+BAP+ cells (MCCs) could be differentiated from peripheral blood mononuclear cells, and generation of MCCs was associated with expression of the osteogenic transcription factor Runx2. In gender-mismatched bone marrow transplanted humans, circulating MCCs had a much longer half-life compared to OC-BAP- cells, suggesting they belong to a stable cell repertoire. Interestingly, the percentage of MCCs was higher in peripheral blood and bone marrow of patients with type 2 diabetes compared to non diabetic patients, but was lowered toward normal levels by optimization of glycemic control. Furthermore, compared to non diabetic, diabetic carotid endoarterectomy specimen showed higher degree of calcification and amounts of cells expressing OC and BAP in the- SMA- areas surrounding calcified nodules, where CD68+ macrophages co-localize. Collectively, these data identify a novel source of blood-derived pro-calcific cells potentially involved in atherosclerotic calcification of diabetic patients.

Interplay among cGMP, cAMP and Ca^{2+} *in living olfactory sensory neurons,* **in vitro** ***and*** **in vivo.**

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The mechanism of cGMP production in olfactory sensory neurons (OSN) is poorly understood, although this messenger takes part in several key processes such as adaptation, neuronal development and long term cellular responses to odorant stimulation. Many aspects of the regulation of cGMP in OSN are still unknown or highly controversial, such as its subcellular heterogeneity, mechanism of coupling to odorant receptors (OR) and downstream targets. Here we have investigated the dynamics and the intracellular distribution of cGMP in living rat OSNs in culture transfected with a genetically encoded sensor for cGMP. We demonstrate that OSN treated with pharmacological stimuli able to activate membrane or soluble guanylyl cyclases (mGC and sGC) presented an increase in cGMP in the entire neuron, from cilia-dendrite to the axon termini-growth cone. Upon odorant stimulation, a rise in cGMP was again found in the entire neuron, including the axon termini, where it is locally synthesized. The odorant-dependent rise in cGMP is due to sGC activation by NO and requires an increase of cAMP. The link between cAMP and NO synthase appears to be the rise in cytosolic Ca2+ concentration elicited by either plasma membrane Ca2+ channel activation and Ca2+ mobilization from stores via the guanine nucleotide exchange factor Epac. Finally we show that a cGMP rise can elicit both in vitro and in vivo the phosphorylation of nuclear CREB.

Identification of 'neuronal-cardiomyocyte junction' : **in vivo** *and* **in vitro** *models*

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Introduction: Several cardiovascular pathologies associate with abnormal activity of the sympathetic regulation of cardiac activity. Such alterations are consistent with an impairment of the 'SN-CM' cell coupling. In addition, it is well accepted that sympathetic neuron survival requires target-derived neurotrophic factors (e.g. NGF). It has been proposed that specialized SN-CM interaction sites exist (neuro-cardiac junction, NCJ), similar to the well-characterized NMJ. However, the molecular determinants, and the functional role of the NCJ in SN-CM signaling and in retrograde CM-SN signaling are undetermined. **Aims**: To characterize the molecular organization and function of sympathetic NCJ in both *in vitro* and *in vivo* models. **Results**: CM from adult mice present dystrophin enrichment close to sympathetic terminals. Hearts from mdx mice show a 70% decrease in sympathetic innervation. In addition, our preliminary electron microscopy (EM) analyses strongly suggest that specialized NCJ might indeed be found in the myocardium. Co-cultures between SN and CM from neonatal rats were established as a versatile tool to characterize the molecular and functional aspects of NCJ. Enrichment in β-catenin and cadherins immuoreactivity was detected in the post-synaptic membrane close to the neuronal releasing active sites. In addition, interference with the main cell-to-cell adhesion molecules, as achieved by incubating co-cultures with a neutralizing anti-VCAM1 antibody, ablated the SN-CM contact and induced SN degeneration. **Conclusion**: Our results suggest that specific interaction sites between cardiac SN and CM (NCJ) exist and that the establishment and maintenance of NCJ is crucial for SN viability.

Expression and Purification of Ca^{2+}-ATPases for structural studies

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Ca-ATPases are among the major regulators of intracellular calcium levels. Plasma membrane calcium pump (PMCA) actively transports calcium from the cytosol to the extracellular space, while the sarcoplasmic reticulum (SERCA) pump controls the concentration of cytoplasmic Ca2+ in muscle cells, where its ability to remove Ca2+ from the cytosol induces relaxation. The 3D structure of calcium ATP-ases can help elucidate important aspects of the pumps activity and regulation. Two human diseases have been associated with SERCA pump gene defects, Brody's and Darier's disease. The former is a rare recessive muscular condition characterized by impaired relaxation, painless cramps and stiffness. Furthermore, SERCA is functionally decreased in nearly all models of heart failure and mutations of the SERCA2a protein may predispose to it. The crystal structure of the SERCA pump from rabbit skeletal muscle in different states is known. No information is yet available on the mutants. In order to study the effects of point mutations on the protein conformation and behavior and to understand the molecular aspects of the pathology, structural and functional study on the bovine and human SERCA proteins and of their mutants have been undertaken. Recently, cattle showing symptoms very similar to those of the Brody syndrome have been found to carry a mutation (R164) on the gene coding for the SERCA pump. Crystals of bovine wild-type SERCA have been already obtained in our laboratory, some crystallization trials on the mutant bovine SERCA have also been performed, so far without success. Data of the bovine E1 form have been measured at the European Synchrotron Radiation Facility (ESRF) of Grenoble (France) and the structure will be described. Human recombinant calcium pump as well as human recombinant SERCA-2a have been expressed in S.Cerevisiae.

MMP-9 and MMP-2 and their tissue inhibitors are involved in inflammatory and fibrotic phases in a mouse model of BLM-induced fibrosis and their expression is modulated by the kinase GSK-3.

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Background: Idiopathic pulmonary fibrosis (IPF) is a lung disease characterized by fibroblast proliferation and deposition of extracellular matrix (ECM). Its pathogenesis includes chronic injury to the alveolar epithelium, chronic inflammatory response and an imbalanced ECM turnover sustained by the matrix-metalloproteinases (MMPs) and their tissue inhibitors (TIMPs). Glycogen Synthase Kinase 3 (GSK-3) is a crucial mediator of inflammation homeostasis because of its role in the regulation of expression of some inflammatory cytokines. Recently, we demonstrated that SB216763, a specific GSK-3 inhibitor, shows therapeutic properties in a mouse model of bleomycin (BLM)-induced pulmonary fibrosis reducing T-cell recruitment, neutrophils activation and expression of inflammatory cytokines. **Aim of the study:** We investigated whether metalloproteinases MMP-9 and -2 and their inhibitors, TIMP-1 and -2, are involved in the inflammatory and fibrotic phases of BLM-induced lung fibrosis and whether in vivo inhibition of GSK-3 could modulate their activity. Moreover, in order to understand the mechanism through which GSK-3 modulates MMPs activity, in vitro experiments were performed using wild type and GSK-3β knock-out mouse embrional fibroblasts (MEFs). **Results:** The activity and gene expression of MMP-9 and -2, which are elevated in the BALF of mice with BLM-induced alveolitis, can be reduced by the in vivo administration of SB216763 which was also able to augment TNF-α-induced MMP-9 expression on primary skin fibroblasts and in MEFs knock-out for GSK-3β. Finally, when we tested the effect of GSK-3 blockade on MMPs expression upon cell exposure to TGF-β, TGF-β negatively regulates MMP-9 expression: this effect was mitigated in GSK-3β knock-out mouse fibroblasts. Concerning MMP-2, it was constitutively expressed in MEFs and GSK-3 knock-out showed a tendency toward an increased expression. **Conclusions:** Our data suggest a role for MMPs and theirs inhibitors in inflammatory and fibrotic phases in the mouse model of BLM-induced fibrosis. We also suggest a role for GSK-3 in MMPs trascriptional control under some signaling pathways such as TNF-α and/or TGF-β pathway.

Assembly of a Canine Adenoviral genome carrying the Plasma Membrane Ca^{2+}-ATPase coding sequence.

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Hearing dysfunction, and ultimately deafness, may be related to the impairment in Ca^{2+} ions fluxes, across the plasma membrane of outer hair cells (OHC), the sensory cells of the inner ear. Proper handling of Ca^{2+} by the OHC stereocilia requires the integrity of the Plasma Membrane Ca^{2+}-ATPase (PMCA pump), notably the *w/a* splicing variant of its isoform 2 (PMCA2*w/a*), whose mutation or deletion has been shown to cause deafness in mice models and human patients. One potential tool to repair such defects of Ca^{2+} pumping by the stereocilia might be a gene-delivering therapy, based on infections by specific viral particles, able to restore the expression of the wild type PMCA2*w/a* in the OHC stereocilia, without undesidered side-effects, as toxicity or unspecific infectivity. In preliminary result we have recently observed that CAV-derived (Canine Adenoviral) constructs are able to express the fluorescent reporter RFP in the OHC stereocilia with high efficiency, upon infection of organotypic utricle cultures, indicating that this system is very promising. Here, we show how we are constructing the 36 kbp Canine Adenoviral genome, carrying the 3.7 kbp coding sequence of the PMCA2*w/a* isoform, fused with the red fluorescent protein Kate. We also show that the PMCA2*w/a* pump, transiently expressed by these vectors in HeLa cells, is properly targeted to the plasma membrane and is fully functional in the ejection of Ca^{2+}. The basic properties of the construct pave the way for possible future applications in gene therapy. The final production of virus particles will be performed in collaboration with the group of Eric Kremer in Montpellier (France).

Human connexin 30 T5M causes hearing loss and reduces biochemical coupling among cochlear non-sensory cells in mice

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Mutations in the GJB2 and GJB6 genes, respectively coding for connexin26 (Cx26) and 30 (Cx30) proteins, are the most common cause for prelingual non-syndromic deafness in humans. In the inner ear, Cx26 and Cx30 are expressed in different non-sensory cell types, where they largely co-localize and may form heteromeric gap junction channels. Here, we describe the generation and characterization of a mouse model for human bilateral middle/high-frequency hearing loss based on the substitution of an evolutionarily conserved threonine by a methionine residue at position 5 near the N-terminus of Cx30 (Cx30T5M). The mutation was inserted in the mouse genome by homologous recombination in mouse embryonic stem cells. When probed by auditory brainstem recordings, Cx30 T5M mice exhibited a mild, but significant increase in their hearing thresholds of about 15 dB at all frequencies. Immunolabelling with antibodies to Cx26 or Cx30 suggested normal location of these proteins in the adult inner ear, but Western blot analysis showed significantly down-regulated expression levels of Cx26 and Cx30. In the developing cochlea, electrical coupling, probed by dual patch-clamp recordings, was normal. However, transfer of the fluorescent tracer calcein between cochlear non-sensory cells was reduced, as was intercellular Ca^{2+} signalling due to spontaneous ATP release from connexin hemichannels. Our findings link hearing loss to decreased biochemical coupling due to the point-mutated Cx30 in mice.

Tumour-associated macrophages as major source of APRIL in gastric MALT lymphoma

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Mucosa associated lymphoid tissue (MALT) represents a pre-neoplastic condition associated to *Helicobacter pylori* (HP) infection, which may evolve to a B cell lymphoma. While it is well established that the initial neoplastic proliferation of B cells is antigen-driven and dependent on the helper activity of HP-specific T cells, it needs to be elucidated which cytokine or soluble factor(s) are able to promote B cell activation and lymphomagenesis. Herein, we originally report that gastric MALT lymphoma express high levels of a proliferation inducing ligand (APRIL), a novel cytokine known to be crucial in sustaining B cell proliferation. By immunohistochemistry, we demonstrate that APRIL is produced almost exclusively by gastric lymphoma-infiltrating macrophages located in close proximity to neoplastic B-cells. We also show that macrophages produce APRIL upon direct stimulation with both HP and HP-specific T cells. Collectively, our results represent the first evidence for an involvement of APRIL in gastric MALT lymphoma development in HP-infected patients.

Hyperforin against Multiple Myeloma Plasma Cells

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Hyperforin (Hyp) is an active compound contained in the extract of Hypericum perforatum, well known for its antidepressant activity. It has been found to possess several other biological properties, including inhibitory effects on tumor invasion, angiogenesis, and to promote apoptosis of leukemic B cells. Here we studied the role of Hyp on survival of multiple myeloma (MM) plasma cells (PCs) and their secretion of matrix metalloproteinase-9 (MMP-9), a protease shown to be instrumental in invasion and neo-angiogenesis. Flow cytometry analysis of five out six MM cell lines and highly purified malignant PCs from ten patients with MM showed that Hyp induced apoptosis, as shown by dose-dependent stimulation of phosphatidylserine externalization. The averaged values of the five MM lines were 1.33 ± 0.05, 1.65 ± 0.09 and 2.24 ± 0.06 at the 0.5, 1.5 and 4.5 µM doses of Hyp, with a value of 1 attributed to untreated cells ($p < 0.01$); MM PCs from patients were divided into three groups (high, intermediate and low) on the basis of sensitivity to 0.5 µM Hyp and the mean value, at the three doses of Hyp, were 1.71 ± 0.2, 2.40 ± 0.19, 2.44 ± 0.62, with $p < 0.01$ for the high; 1.3 ± 0.04, $p < 0.05$, 1.48 ± 0.03, 1.87 ± 0.12 $p < 0.01$ for the intermediate; and 1.08 ± 0.02, 1.20 ± 0.05, $p < 0.05$, 1.68 ± 0.32, $p < 0.01$ for the low group, respectively. Disruption of the mitochondrial transmembrane potential and cleavage of the caspase substrate PARP-1 confirmed the apoptotic action of Hyp on MM cells. Treatment of MM cells with Hyp also resulted in a marked inhibition of their capacity to secrete the gelatinase MMP-9. These effects were dose-dependent and in the µM range measured in the blood of patients under Hypericum extract treatment.These capacities qualify Hyp as a lead structure for the development of new approaches for the treatment of a number of diseases, including some haematological malignant tumors such as MM.

Myotonic dystrophy protein kinase localizes to mitochondria and protects from several death stimuli

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Myotonic dystrophy1 (DM1) is a multi-system, autosomal dominant disorder which results from a CTG repeat expansion located in the 3′ untranslated region of DMPK gene coding for a Ser/Thr kinase. Mouse models have demonstrated that decreased levels of DMPK, Six5 (the DMPK-5′ flanking gene) and the expression of expanded CUG repeats independently contribute to the development of DM1 pathology. The DMPK protein is ubiquitously expressed and its biological functions are poorly understood. Here we investigate the effects of the expression of human DMPK isoform A in the SAOS-2 osteosarcoma cell model lacking the endogenous kinase. Our results indicate that DMPK-A, which localizes to the outer mitochondrial membrane, has a protective effect against death stimuli such as detachment of hexokinase II from mitochondria, nutrient depletion and oxidative stress. DMPK expression protects cells from death stimuli by inhibiting superoxide anion production by mitochondria, implying a possible alteration in respiratory chain activity or assembly. Ongoing work is directed towards characterization of the activity and the amount of respiratory chain complexes, as well as towards evaluation of possible alterations in mitochondrial biogenesis and elimination.

Chemotherapy disrupts tolerogenic niche in the spleen.

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Chemotherapy causes immunosuppression but also favours activation of immune effectors by either triggering immunogenic cancer cell death or removing immunosuppressive constraints established in the host by growing tumors. We found that chemotherapeutic drugs with different molecular targets can amplify the therapeutic efficacy of adoptively transferred, tumor-specific CD8+ T cells. This activity was long lasting, did not require a direct action on cancer cells, and entirely depended on the perturbation of a biological niche shared by central memory CD8+ T cells and a population of tumor-induced, and actively proliferating, immunosuppressive myeloid cells. This niche resides in the spleen and splenectomy completely abrogated tumor-induced tolerance. Increased circulating levels of the immunosuppressive myeloid cells correlated with worse prognosis and radiographic progression in breast and colorectal cancer patients, respectively. These results unveil an unanticipated tolerogenic niche and lay the basis for a rational use of chemotherapy as immune adjuvant.

Development of optogenetic tools to investigate ER/SR Ca2+ leak in cardiac cells

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Calcium leak from intracellular stores has been proposed as the major underlying mechanism of inherited arrhythmias such as cathecholaminergic polymorphic ventricular tachycardia (CPVT). Whether Ca2+ leak from the sarcoplasmic reticulum *per se* is sufficient to trigger arrhythmias remains unclear. We here aim to develop a molecular tool based on channelrhodopsin (ChR) to optically control Ca2+ release from the endoplasmic/sarcoplasmic reticulum (ER/SR) with high temporal resolution. Native ChRs are plasma membrane localized ion channels mostly permeable to sodium upon light stimulation. We thus aim to: i) engineer a ChR2 channel with high Ca2+ permeability and ii) target ChR2 to the ER/SR membrane. Bioinformatic analysis of ChR2 structure allowed us to identify candidate mutations to increase Ca2+ permeability. We generated a number of mutants and assessed Ca2+ permeability using patch-clamp recording and Ca2+ imaging.In parallel, we are engineering ER-retained ChR2 proteins by fusion with the first two transmembrane domains of the IP3 receptor and/or adding ER retention signals. This approach will result in development of a tool to non-invasively modulate ER/SR Ca2+ leak. In addition, light activation of ChR2 expressed in cultured neonatal myocytes allowed us to control membrane depolarization with millisecond resolution, mimic early (EAD) or delayed after depolarizations (DAD) and trigger an action potential activating Ca2+-induced Ca2+ release.

Mitotic phosphorylation of CK2a C-terminal tail: which regulation?

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CK2 is a protein kinase involved in many aspects of the cell life. As one of its roles, it sustains cell survival but, under special circumstances, it enhances tumour growth. CK2α C-terminal tail, which has evolved in vertebrates, contains four sites that were found phosphorylated at mitosis. This event is required for correct cell cycle C-terminal tail is however prone to degradation and progression. CK2α all structural studies were conducted on a truncated version of the enzyme. We were able to produce a homogeneous and stable full-length human CK2α and its phosphomimetic counterpart (the four phosphorylated residues were changed to glutamate) in E.coli by implementing a fast purification protocol, with high yields as required for structural analysis. Fast purification was needed in order to reduce the amount of C-terminally degraded CK2α that could be removed with a subsequent dedicated purification step. The isolated CK2α with complete C-terminus is stable over time and no more susceptible to degradation. Both wild-type and phosphomimetic full-length CK2α were crystallized. In both instances the C-terminal tail is disordered. Using the phosphomimetic CK2α we have verified that a new site of autophosphorylation is C-terminal tail was generated at the C-terminus. Phosphorylated CK2α proposed as docking site for mitosis-specific protein-protein interactions. We are currently verifying whether or not CK2α C-terminus phosphorylation is involved in substrate selection and attempting reconstruction of putative mitotic complexes.

Involvement of miRNAs in regulating Skeletal Muscle Atrophy

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Loss of muscle proteins and the consequent weakness has important clinical consequences. Thus, understanding the molecular mechanisms to counteract muscle wasting is important to develop new therapeutic approaches. Muscle atrophy involves a common pattern of transcriptional changes that control a small subset of genes named atrophy-related genes or atrogenes. Whether microRNAs play a role in the atrophy program and muscle loss is unknown. To understand the involvement of miRNAs in atrophy we performed miRNA expression profiling of mouse muscles under wasting conditions. We found that the miRNA signature is peculiar of each catabolic condition. We focus our attention on denervation and we revealed that the changes of miRNA expression is delayed compared to the transcriptional control of the atrophy-related genes. However, the induction of these microRNAs is not sufficient to induce muscle loss. We have characterized their pattern of expression, defined their targets and their role in muscle homeostasis. Thus miRNAs expression is important for fine balancing of the atrophy program and their modulation can be a novel potential therapeutic approach to counteract muscle loss and weakness in catabolic conditions.

Parkinson's disease: involvement of protein kinase CK2

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Parkinson's Disease is a neurodegenerative disease characterized by the development of cytoplasmic inclusions (insoluble fibrillar portion) called Lewy bodies, the major pathological hallmark of Parkinson's disease (PD). α-Synuclein is the major constituent of Lewy bodies. α-Synuclein is expressed throughout the brain at high levels, but there is little information on its normal function. The protein is intrinsically disordered or "natively unfolded". The acidic C-terminal domain has a strong negative charge. This region contains several phosphorylation sites: Tyr125, Ser129, Tyr133 and Tyr136. Phosphorylation appears to play an important role in the fibrillogenesis, Lewy bodies formation and neurotoxicity of α-synuclein in vivo. Protein kinase CK2 is one of several kinases, which phosphorylates α-synuclein at Ser129. More than 90% of phosphorylated α-synuclein in dementia with Lewy bodies occurs on Ser129, and several data suggest that α-synuclein Ser129 phosphorylation facilitates its aggregation and accumulation in Lewy bodies. We have observed that α-synuclein Ser129 phosphorylation by CK2 is positively modulated by tyrosine phosphorylation. Protein tyrosine kinase p72Syk, Syk, phosphorylates all the three tyrosyl residues (Y125, Y133 and Y136) located in the C-terminal domain. Syk pre-phosphorylated α-synuclein is more prone to interact with βCK2 and showed a reduced Km for CK2 when compared with α-synuclein wild type. This is the first example of tyrosine phosphorylation that can act as positive recognition determinant for CK2. Modulation of Ser129 phopshorylation of α-synuclein can be important in PD.

The mitochondrial fusion protein OPA1 is oxidized during ischemia/reperfusion of the heart

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The intense metabolical activity of mitochondria in the heart is accompanied by side effects, like the generation of reactive oxygen species (ROS), that has been implicated in several heart diseases. At least in a cellular model, mitochondrial ROS generation seems to depend on morphological changes of the organelle, regulated by a growing family of mitochondria-shaping proteins. In particular, the inner membrane mitochondria-shaping protein Opa1 regulates fusion of the organelle and cristae remodeling during apoptosis. Maintenance of the cristae structure is accomplished by an Opa1 containing oligomer that is disrupted early during cell death. Whether formation of this Opa1 oligomer and Opa1 itself are target of ROS is unknown. Here we show that Opa1 can be oxidized at specific cysteine residues and that this causes the formation of Opa1 aggregates during pro-oxidant and ischemia reperfusion (I/R) induced cell death. In whole hearts undergoing I/R, Opa1 was retrieved in high molecular weight aggregates that could be reverted by reducing treatments. This oxidative oligomerization was phenocopied in cardiomyoblasts and fibroblasts exposed to the pro-oxidant hydrogen peroxide. An homology modeling approach showed that 4 Cys residues, exposed on a surface region of Opa1, could homo-interact, mediating the oxidation-induced Opa1 aggregation. Mutants of these residues uncoupled Opa1 mediated mitochondrial elongation from the aggregation of Opa1 induced by oxidation, suggesting that different residues play different roles in the pathophysiology of Opa1. Our data show that Opa1 is a target for oxidative modification during I/R injury and point to a role of conserved Cys residues in this process.

Structural and Functional Characterization of Proteins Related to Colonization and Pathogenesis from Helicobacter pylori

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H. pylori is a Gram-negative bacterium which establishes a life-long chronic infection in more than half of the human population. Infections are often asymptomatic, but, in an important minority, H. pylori causes a range of pathologies, including gastritis, peptic ulcers, adenocarcinomas and MALT lymphomas (Clyne et al., 2007). New factors important for colonization and persistance have been proposed (Baldwin et al., 2007). A pull of important H. pylori proteins was cloned, expressed in E. coli and purified for structural and functional studies. In particular HP0310, a secreted enzyme with peptidoglycan deacetylase activity, which catalyze the hydrolysis of acetyl groups from N-acetylglucosamine (Psylinakis et al., 2005), was purified in high yield. Peptidoglycan deacetylation, is a highly efficient mechanism used by Helicobacter to evade innate host defenses. In vitro fully N-deacetylated PG from Helicobacter pylori completely lost its ability to be sensed by both Nod1 and Nod2 (Boneca et al., 2007). Crystals of HP0310 were obtained and three datasets collected at a maximun resolution of 2.5 Å. The structure refining is now ongoing. HP1564, an outer membrane lipoprotein with function as substrate binding unit of ABC transporter for iron transport, was cloned, purified and crystallized. Crystals diffract to a maximum resolution of 1.67 Å. The three dimensional structure was solved and the refinement is in progress. Other H. pylori targets under investigation are HP1012, and HP0231. HP1012, a putative secreted zinc protease, was predicted to belong to the M16 family of peptidase. This protein was reported to be secreted outside the cell, being found in the extracellular proteome (Smith et al., 2007). HP0231, a secreted disulfide isomerase, is reported as one of the novel antigen with low similarity with other organisms and a candidate for potential H. pylori vaccine development (Sabarth et al., 2002). Both of them were cloned and purified and their functional characterization is in progress.

Defining microRNA role in differentiation and function of myeloid-derived suppressor cells.

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MicroRNAs (miRs) are short noncoding RNA molecules, between 18 to 25 nucleotides in length, which have been found to regulate a wide variety of cellular processes. Recently the alteration of miRNA expression has been associated with human tumor development and metastasis. We focused on the role played by specific miRs in myeloid suppressor cell (MDSC) differentiation from bone marrow precursors and in the regulation of their biological functions. MDSCs are expanded under pathological conditions, such as cancer development, and in mice are broadly defined by Gr-1 and CD11b surface markers. The comparison between the miR profiles of CD11b+ cells isolated from the spleen of healthy mice and from either spleen and tumor of tumor-bearing mice in 5 different tumor models unveiled a common miR signature shared by all the examined tumor histotypes (40 down-regulated and 25 up-regulated miRs). We identified a subset of miRs (cluster 17-92, miR 142-3p, 146a, 106a, and 223) constantly down-regulated in tumor-associated MDSCs and previously shown to be involved in haematopoietic commitment. MiR 142-3p over-expression in CD11b+ cells induced an almost complete abrogation of the immunosuppressive activity of *in vitro* generated MDSCs. We investigated the molecular pathway involved in this process and found that miR 142-3p can target IL6 receptor and the transcription factor cEBP-, both crucial for full MDSC maturation and activation. We are currently generating bone marrow (BM) chimeras by infecting BM progenitor cells with a miR 142-3p-expressing lentivirus, to exploit the possibility to target miR 142-3p *in vivo* in order to affect tumor growth and survival of tumor-bearing mice following adoptive immunotherapy with tumor-specific CD8+ T lymphocytes.

Opa1, mutated in dominant optic atrophy, forms high molecular weight with mitofilin to control viability

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Maintenance of the mitochondrial ultrastructure is key for the bioenergetic efficiency of the organelle: density of the cristae correlates with the energy requirement of the tissue. In addition, most of the cytochrome c that is released in the cytosol to activate caspases during cell death is contained in the cristal space. This requires the so called "cristae remodelling", which was discovered in our lab. Mitochondrial shape and ultrastructure is controlled by a growing family of shaping proteins that include Optic atrophy 1 (OPA1), the only inner membrane mitochondria shaping protein discovered so far. Complexes of high molecular weight and unknown composition that comprise Opa1 are responsible for the maintenance of cristae shape and are early targets during apoptosis. To identify the molecular components of the OPA1 complexes, and to understand the molecular mechanism that regulate the maintenance and disruption of mitochondrial cristae during apoptosis we are following a Three Dimensional Blue-Native, Blue-Native, SDS-PAGE approach. A good candidate to be part of the OPA1 complexes is Mitofilin, an inner mitochondrial membrane protein that protrudes to the intermembrane space. The two isoforms of mitofilin localize mainly close to the cristae junctions .Size exclusion chromatography shows that OPA1 and mitofilin co-fractionate. Three Dimensional BN-BN-SDS PAGE indicate that the two proteins are part of the same complex. Upon apoptotic stimulation, mitofilin leaves the Opa1 complex to take part in a complex with the BH3-only protein cBID. In addition, early during apoptosis mitofilin is cleaved independently of OPA1 and a crosslinkable complex between OPA1 and mitofilin is disrupted. Silencing of mitofilin induces mitochondrial fragmentation, and ablation of mitofilin sensitizes Opa1-/- cells to death, suggesting that the two proteins work in the same genetic pathway of apoptosis control. In conclusion, our data suggest that mitofilin and OPA1 participate in the same pathway that regulates cristae and mitochondrial morphology, and our approach promises to identify novel players involved in the regulation of cristae morphology during cell life and death.

A Ca^{2+}-regulated mitochondrial (permeability transition) pore in **Drosophila melanogaster**

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The mitochondrial permeability transition (PT) describes a process of Ca^{2+}-dependent, tightly regulated increase in the permeability of the inner mitochondrial membrane that has been discovered and studied mostly in mammals. The PT, which is critically involved in cell death, is due to the opening of the mitochondrial permeability transition pore (PTP), a high-conductance inner membrane channel with unknown structure. Although data are available also in yeast and plants, it is not clear whether the permeability changes observed in these organisms can be ascribed to the same molecular events involved in the mPT of mammals. We have studied the properties of the PT in mitochondria from the fruit fly *Drosophila melanogaster*. Experiments were carried out in permeabilized *Drosophila* embryonic S_2R^+ cells, where we demonstrate the occurrence of ruthenium red-sensitive Ca^{2+} uptake as well as of a ruthenium red-insensitive Ca^{2+} release following matrix Ca^{2+} overload (which in mammals is caused by opening of the PTP). Ca^{2+} release was insensitive to CsA, Ub0 and ADP, well-known inhibitors of the mammalian PTP; but was inhibited by Mg^{2+} (as is the PTP of all species) and Pi (as is the "pore" of yeast). Ca^{2+}-induced Ca^{2+} release could be triggered by the thiol reactive compound N-ethylmaleimide and by the dithiol oxidant diamide, indicating the existence of regulatory redox-sensitive sites, as is known for the mammalian PTP. Our results suggest that *Drosophila* mitochondria may possess a Ca^{2+}-regulated permeability pathway with features intermediate between the "pore" of yeast and the PTP of mammals.

The odorant receptor at the axon termini-growth cone in olfactory sensory neurons: mechanisms of activation and function

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In olfactory sensory neurons (OSN) the odorant receptor (OR) is not only involved in detection of odors but also in OSN axonal convergence, although the molecular mechanism underpinning the latter function remained largely unknown. In a previous study (Maritan et al.,2009) we demonstrated, for the first time, that the OR at the growth cone is capable of binding odors and coupled to local increases of cAMP and Ca2+. To assess whether molecules present in the olfactory bulb (OB) could be responsible for OR activation, we treated axon termini of OSN loaded with fura-2 or transfected with the sensor for cAMP with bulb extract (lysate, dialysate, dialysate processed through gel filtration chromatography or ion exchange chromatography or both). To evaluate whether gradients of cAMP and Ca2+ can affect the elongation and turning behaviour of the OSN growth cone, we are performing real time imaging on OSN axon termini-growth cone in response of pharmacological (i.e. forskolin) and physiological (odors) agents able to modulate Ca2+ and or cAMP level at the OSN growth cone.

Engineering an* in vitro *model of human muscle dystrophy for pre-clinical trials of cell therapy approaches

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Duchenne Muscular Dystrophy (DMD) is the most common, lethal, inherited disease of skeletal and cardiac muscles for which an effective therapy has not been developed yet. The most promising experimental strategies developed so far for the treatment of DMD patients are addressed to restore dystrophin expression in skeletal myofibers. The aim of this work is to obtain a patient-specific *in vitro* model of human dystrophic skeletal muscle, made of oriented and functional myofibers, suitable for testing the efficiency of different therapeutic strategies in restoring dystrophin expression. Through the proper design of the *in vitro* 2D cell culture microenvironment, in terms of both mechanical properties (substrate elastic modulus E≈12±4kPa) and topological organization, we guided the differentiation of human dystrophic myoblasts into myotubes exhibiting an highly defined sarcomeric organization of myosin heavy chain and α-actinin together with a remarkable dystrophin expression at membrane-level. We realized also a new *in vitro* 3D cell culture system made by micrometric channels (200μm in diameter and 1cm long) inside a polyacrylammide hydrogel scaffold, in order to improve functional maturation of the engineered skeletal myofibers. The developed *in vitro* models were then used to evaluate, through co-colture experiments, the capability of different donor cell types, in particular human primary myoblasts, satellite cells and mesoangioblasts, to restore dystrophin expression in dystrophic myotubes, both by immunofluorescence and western blot analyses. These results elucidate the possibility to use the realized *in vitro* models as a complementary tool in patient-specific pre-clinical trials, bridging the gap between conventional cell culture, animal models and patients.

Three-dimensional human adipose organ culture in microfluidic system for multi-parametric patient-specific screening

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Insulin Resistance (IR) plays a key role in Type 2 Diabetes Mellitus (T2DM), by affecting adipose tissue glucose uptake. It's known that this complex disease could require the treatment with different classes of drugs. Our aim is to develop a microfluidic platform able to perform *in vitro* tests on patient-derived three-dimensional human adipose tissue, controlling the temporal evolution of culture conditions and performing multi-parametric analyses. Microfluidic based technology uses nanoliter volumes giving a high ratio between culture and medium volumes. This intrinsic property enhances the insulin dependent-response of glucose uptake in organ culture leading to a high sensitivity of cell response. A microfluidic platform was fabricated using soft-lithographic techniques and the design and development were assisted by mathematical modeling. Biopsies of subcutaneous and visceral adipose tissues obtained from both T2DM and healthy patients were processed after surgery into 10-20mg samples. Samples were treated in a 24well plate or within microfluidic system for up to 4 days. Viability and histological analyses were performed at the end of the cultures and insulin dependent-glucose uptake was investigated. In order to confirm the activation of insulin pathway in organ culture, western blot analyses for IRS-1, Akt and GLUT4 were performed. MTT showed high tissue viability and no significant differences with controls in 24well plates. We observed in organ culture an enhancement of glucose uptake for increasing insulin concentration in healthy patients. Moreover differences in glucose uptake between insulin-sensitive and T2DM patients were reported. This system may open important perspectives towards the realization of *in vitro* high-throughput dynamic screening of anti-diabetic drugs on patient-derived three-dimensional human adipose tissue.

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