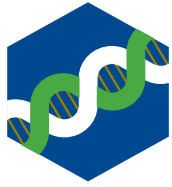


*Venetian Institute of Molecular Medicine
8th Annual Retreat*



MAROSTICA, October 23-24, 2009





FONDAZIONE PER LA RICERCA BIOMEDICA AVANZATA - ONLUS
ISTITUTO VENETO DI MEDICINA MOLECOLARE (VIMM)

Venetian Institute of Molecular Medicine

8th Annual Retreat

Marostica, October 23-24 2009

Programme and Book of Abstracts

INDEX

| | |
|--------------------------------|--------|
| INDEX ----- | - 3 - |
| WELCOME ----- | - 5 - |
| PROGRAMME ----- | - 7 - |
| LECTURE ABSTRACTS ----- | - 11 - |
| ORAL PRESENTATION ----- | - 15 - |
| ABSTRACTS ----- | - 15 - |
| POSTER ABSTRACTS ----- | - 23 - |
| LIST OF AUTHORS ----- | - 41 - |

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 8th annual VIMM retreat.

Tullio Pozzan

VIMM Scientific Director

At the 8th annual meeting of the VIMM we have invited also the members of “*Centro Interdipartimentale di Ricerca per lo Studio dei Segnali Cellulari*” and the guest groups working at VIMM.



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

PROGRAMME

October 23, Friday

8:00 Departure From Padua

09:30 Arrival in Marostica at Hotel Europa

09:30 – 09:50 **Coffee Break at Hotel Europa**

Congress at Castello Inferiore

10:00 – 10:15 *Welcome*

10:15 – 10:45 **Marco Sciacovelli - gr. Bernardi**

Activation of mitochondrial ERK protects cancer cells from death through inhibition of the permeability transition

10:45 – 11:15 **Nicola Barison - gr. Zanotti**

Structural and functional studies of proteins from Helicobacter pylori important for stomach colonization and pathogenesis

11:15 – 11:45 **Giovanni di Maira - gr. Pinna**

Akt/PKB and CK2: functional connections between two pro-survival protein kinases.

11:45 – 12:15 **Elisa Pasqualetto - gr. Battistutta**

Structural biology on the SulP anion transporters STAS domain

12:30 - 14:30 **Lunch at Hotel Europa**

14:30 – 15:15 **Lecture Prof. Stefano Piccolo**

Molecular determinants of metastasis

15:15 – 15:45 **Stefano Ugel - gr. Bronte**

Active and passive immunotherapeutic approaches based on Telomerase antigen

15:45 – 17:00 VIMM General Assembly

17:30 – 19:30 **Poster Session at Castello Inferiore and Coffee Break**

20:30 **Dinner and Music at Ristorante Al Castello Superiore**

October 24, Saturday

09:45 – 10:30 **Lecture Prof. Fabio Mammano**

Hereditary deafness: a glimpse of light at the end of the tunnel (of Corti)?

10:30 – 11:00 **Laura Quotti Tubi - gr. Semenzato**

A new feature of Casein Kinase 2 function: its role in granulocytic differentiation induced by retinoic acid.

11:00 - 11:30 Coffee Break

11:30– 12:00 **Marina Flaibani - gr. Elvassore**

Photopolymerizable, biodegradable hydrogel for regenerating functional skeletal muscle by freshly isolated satellite cells

12:00 – 12:30 **Ligia Gomes - gr. Scorrano**

Mitochondrial fusion is an early and protective step of autophagy

12:30 – 13:00 **Roberta Sartori - gr. Sandri**

Smad2 and 3 transcription factors control muscle mass in adulthood

13:00 - 15:00 Lunch at Hotel Europa

15:00 - 15:30 **Claudia Lodovichi - gr. Lodovichi**

Interplay among cAMP, cGMP and Ca²⁺ in olfactory sensory neurons.

15:30 – 16:00 **Kenneth Dyar - gr. Schiaffino**

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

16:00 – 16:30 **Moira Marcolongo - gr. Alberti**

“A 7 gene Signature (cirrhosis risk score) predicts liver fibrosis progression in patients with initially mild chronic hepatitis C”

17:00 Departure from Marostica

LECTURE ABSTRACTS

Invited speakers

Molecular determinants of metastasisStefano Piccolo¹¹Department of. Histology, Microbiology and Medical Biotechnologies

The genes and signals controlling metastasis are poorly understood. Efforts in this field are critical to develop new tools for cancer prognosis and treatment. I plan to present data on how three common events in human cancers, oncogenic Ras, mutant-p53 and aberrant TGFbeta signaling, all so far independently implicated in metastasis, actually conspire toward the same goal, that is, inactivation of p63. p63 is a master gene for normal stem cells, but prevents cancer stem cells from undergoing metastasis. We also unveiled new genes downstream of this pathway that may be used as prognostic tool for breast cancer.

Hereditary deafness: a glimpse of light at the end of the tunnel (of Corti)?Fabio Mammano^{1,2}¹ Venetian Institute of Molecular Medicine (VIMM), Padua, Italy.² Department of Physics, University of Padua, Padua, Italy.

The genes GJB2 and GJB6, respectively encoding transmembrane proteins connexin 26 (Cx26) and connexin 30 (Cx30) belong in the DFNB1 complex deafness. Up to 50 percent of all patients with autosomal recessive nonsyndromic prelingual deafness in different populations present with mutations or deletions in this locus. Based on data we acquired from mouse models of hereditary deafness, I will discuss the role and function of Cx26 and Cx30 in the inner ear, and the potential for a gene therapy intervention to ameliorate hearing in DFNB1 patients.

ORAL PRESENTATION ABSTRACTS

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

Activation of mitochondrial ERK protects cancer cells from death through inhibition of the permeability transition

Marco Sciacovelli¹, Andrea Rasola¹, Federica Chiara¹, Boris Pantic¹, William S. Brusilow², **Paolo Bernardi**¹

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We have studied human cancer cell models where we detected constitutive activation of ERK. A fraction of active ERK was found to be located in mitochondria in RWPE-2 cells, obtained by Ki-Ras transformation of the epithelial prostate RWPE-1 cell line; in metastatic prostate cancer DU145 cells; and in osteosarcoma SAOS-2 cells. All these tumor cells displayed marked resistance to death caused by apoptotic stimuli like arachidonic acid and the BH3 mimetic EM20-25, which cause cell death through the mitochondrial permeability transition pore (PTP). PTP desensitization and the ensuing resistance to cell death induced by arachidonic acid or EM20-25 could be ablated by inhibiting ERK with the drug PD98059 or with a selective ERK activation inhibitor peptide. ERK inhibition enhanced GSK-3-dependent phosphorylation of the pore regulator Cyclophilin D, whereas GSK-3 inhibition protected from PTP opening. Neither active ERK in mitochondria nor pore desensitization were observed in non-transformed RWPE-1 cells. Thus, in tumor cells mitochondrial ERK activation desensitizes the PTP through a signalling axis that involves GSK-3 and Cyclophilin D, a finding that provides a mechanistic basis for increased resistance to apoptosis of neoplastic cells.

Structural and functional studies of proteins from *Helicobacter pylori* important for stomach colonization and pathogenesis

Nicola Barison^{1,2}, Laura Cendron^{1,2}, Lorenza Sisinni^{1,2}, Sandra Quarantini^{1,2}, Munan Shaik^{1,2}, Yuri Churin³, Thomas F. Meyer³, **Giuseppe Zanotti**^{1,2}

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²Dept. of Biological Chemistry, University of Padua, Padua, Italy.

³Max Planck Institute for Infection Biology, Berlin, Germany

Helicobacter pylori is a gram-negative bacterial pathogen specialized in the colonization of the human stomach. It establishes a life-long chronic infection in more than half of the human population: most infected people are asymptomatic, but, sometimes, *H. pylori* causes gastritis, ulcers, adenocarcinomas and stomach lymphomas [1]. Recently, new pathogenicity factors have been identified, like HP1287, HP1028 [2] and HP0421. The *HP1287* gene encodes for a homologue of TenA protein from *Bacillus subtilis*, an enzyme that catalyzes the metabolism of a 2-methyl-4-amino-5-aminomethylpyrimidine to a direct precursor of thiamine [3]. In spite of the high structural similarity, HP1287 doesn't seem to be active, so the physiological activity is unknown. The structure of HP1287 has been solved at 2.7 Å resolution [4]. HP1028, whose function is unknown, has been cloned, expressed in *E. coli* in high yield and purified for crystallization trials. Since it doesn't present any sequence similarity with a protein of known structure, in order to obtain approximate initial phases, mutant derivatives containing seleno-methionines have been expressed. Crystals of both native and derivative forms have been grown and crystal optimization is in progress. HP0421 is a 45-kDa cholesterol- α -glucosyltransferase that is critical in *H. pylori* for phagocytosis escape [5]. HP0421 protein has been cloned and expressed in *E. coli* cells. Purification trials are in progress.

[1] Montecucco, C. (2001). *Molecular Cell Biology*. 2, 457-466.

[2] Baldwin, DN. (2007). *Infection and Immunity*. 75, 1005-1016.

[3] Jenkins, AH. (2007). *Nature Chemical Biology*. 3, 492-497.

[4] Barison, N. (2009). *FEBS Journal*. Epub. Sep 23.

[5] Wunder, C. (2006). *Nature Medicine*. 12, 1030-1038.

Akt/PKB and CK2: functional connections between two pro-survival protein kinases.

Giovanni Di Maira^{1,2}, Francesca Brustolon^{1,2}, Giorgio Arrigoni^{1,2}, Oriano Marin^{1,2}, Stefania Sarno^{1,2}, **Lorenzo A. Pinna**^{1,2}, Maria Ruzzene^{1,2}

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² Department of Biological Chemistry, University of Padova, Padova, Italy

The two protein kinases CK2 and Akt (PKB) are both crucial in mediating the transmission of antiapoptotic and proliferative signals.

CK2 is a constitutively active and pleiotropic S/T kinase with tetrameric structure consisting of two catalytic (alpha and/or alpha') and two regulatory (beta) subunits. Akt is activated by a variety of growth factors through a phosphorylation mechanism, which involves two key residues, T308 and S473; they are targeted by the kinases PDK1 and mTOR/Rictor, and by the phosphatases PP2A and PHLPP, respectively. In this study we disclose a new functional connection between CK2 and Akt, demonstrating that CK2 phosphorylates Akt at S129 either *in vitro* or *in vivo*. This phosphorylation is responsible for a hyperactivation state of Akt, but also modulates the level of Tp308. This effect could be due to a regulation on the kinase (PDK1) or on the phosphatase (PP2A) acting on T308. Our results indicate that S129 does not directly influence the activity of these enzymes; instead, it increases the affinity of Akt for the chaperone protein Hsp90, thus facilitating the formation of the Akt/Hsp90 complex; this association prevents the PP2A-dependent dephosphorylation of T308 maintaining a high level of Tp308.

In conclusion, our data indicates that CK2, phosphorylating Akt at S129, positively contributes to its catalytic activity by a double mechanism, a direct activation and the potentiation of the T308 activatory function.

Structural biology on the SulP anion transporters STAS domain

Elisa Pasqualetto^{1,2}, Rosa Aiello^{1,2}, Greta Bonetto^{1,2}, Graziano Lolli^{1,2}, **Roberto Battistutta**^{1,2}

¹ Venetian Institute of Molecular Medicine, Padova, Italy

² Dept Chemical Sciences, University of Padova, Italy

The Sulfate Permease SulP is a large and ubiquitous family of anion transporters. In mammals, the members of the SulP family, known as SLC26, have important roles in normal physiology and human pathophysiology. The clinical relevance of the SLC26 gene family has been highlighted with the identification of pathogenetic mutations in four genes, which have been shown to lead to human disorders. SulP proteins show a similar structural organization: a hydrophobic core with a variable membrane topology and a C-terminal cytoplasmic portion that includes a STAS domain, which plays a fundamental role in the proteins function. The STAS domain of prestin is essential for the plasma membrane targeting and function. In *A. thaliana*, the STAS domain of SULTR1.2 is crucial for the whole sulfate transport activity.

In order to shed light into their role in the context of the SulP transporters, we have cloned and produced sixteen recombinant STAS variants, in milligram amounts of purified material, enough to perform structural studies with both X-ray crystallography and solution NMR. We are currently working on STAS domains from distance-related species: mammalian prestin and pendrin, plant SULTR1.2 and bacterial Rv1739c. These proteins have been characterized in solution by SEC, DLS and CD, and important information on the aggregation propensities, relevant for both NMR and crystallography, have been obtained. Very recently, we have succeeded in the crystallization of a C-terminally deleted variant and in its 3D structure determination.

Active and passive immunotherapeutic approaches based on Telomerase antigenStefano Ugel¹, **Vincenzo Bronte**^{1,2}¹ Venetian Institute of Molecular Medicine,² Venetian Oncological Institute I.R.C.C.S., Padova.

Availability of an antigen shared by tumors of different histologic patterns and conserved during tumor progression gives the chance to investigate how to adapt vaccine strategies to the different patterns and biological properties of many tumor model known. A prototype antigen for this class of common shared tumor antigen is telomerase, a reverse transcriptase primarily implicated to the elongation of telomeres in mammalian cells. TERT, the protein component of the telomerase complex, plays an essential role to sustain tumor-cells proliferation. In fact, TERT is shut down in most human somatic tissues but re-activated in 85% of tumors. We firstly show the over-expression of telomerase in the tumor lesions during tumor progression by immunohistochemistry analysis both in a genetically-engineered and in a chemically-induced tumor models. We demonstrate that TERT genetic vaccination induces telomerase specific CD8 T cell able to infiltrate tumor lesions inducing a significant but modest prolongation of tumor bearing-mice's survival. We investigate also a passive immunotherapeutic approach based on cytotoxic T lymphocytes (CTLs) specific for TAA transfer after host preconditioning by lymphodepletion. This approach, called Adoptive Cells Transfer (ACT), represents the most effective treatment in cancer immunotherapy: it can mediate objective cancer regression in approximately 50% of patients with metastatic melanoma. We found that myeloablation followed by ACT with high affinity TERT-reactive mouse and human T lymphocytes could treat different types of established tumors. In TRAMP mice, which develop prostate cancer, TERT-based ACT halted progression to more aggressive, poorly differentiated tumors and significantly prolonged mouse survival. ACT also caused immunopathology, marked by a transient B cell depletion in primary and secondary lymphoid organs. Moreover, we demonstrated that human cancer stem cells are targeted *in vivo* by TERT-specific CTLs. These data, finally, represent a good opportunity for enhancing and extending the ACT approach to therapy of a wide variety human cancers.

A new feature of Casein Kinase 2 function: its role in granulocytic differentiation induced by retinoic acid.Laura Quotti Tubi^{1,2}, Carmela Gurrieri^{1,2}, Maria Ruzzene^{2,3}, Marianna Gnoato^{1,2}, Kendra Tosoni^{2,3}, Anna Cabrelle², Lorenzo A. Pinna^{2,3}, Francesco A. Piazza^{1,2}, **Gianpietro Semenzato**^{1,2}¹Department of Clinical and Experimental Medicine, Haematology and Clinical Immunology Branch; ²Venetian Institute of Molecular Medicine and ³Department of Biological Chemistry, University of Padua.

The Ser/Thr kinase CK2 promotes cell survival and proliferation but it also modulates molecules involved in cell development. In this study, we aimed to study a potential novel role of CK2 in myeloid cell differentiation. We took advantage of the well-established model of acute promyelocytic leukemia cells (APL) maturation induced by retinoic acid (RA).

Both CK2 expression and activity were high in APL cell lines. We observed a different modulation of CK2 α and CK2 β subunits expression consequent to RA. CK2 inhibition, with chemicals or by RNA interference 1) prevented RA-induced cell cycle arrest in the G1 phase 2) blocked the phenotypical, morphological and functional differentiation of APL cells 3) impaired the upregulation of RA receptor α (RAR α) target genes p21 and CEBP ϵ . Western blot analysis showed co-immunoprecipitation of RAR α with CK2 α both at basal condition and after RA exposure; on the contrary, CK2 β was not detectable in the immunoprecipitate. Interestingly, CK2 blockade determined delocalization of RAR α from nucleus to cytoplasm even in presence of RA: the translocation was confirmed both with immunofluorescence and western blot.

Moreover, after CK2 inhibition, lower-molecular weight RAR α bands appeared, which likely correspond to dephosphorylated forms of the receptor. Remarkably, the combined treatment with proteasome inhibitor MG132 led to a consistent accumulation of these lower forms.

Taken together our results indicate that CK2 is essential in granulocytic differentiation upon RA treatment and could operate at several levels: (1) modulation of RAR α transcriptional activity; (2) retention of RAR α in the nucleus; (3) phosphorylation of RAR α and consequent RAR α localization, stability, and preservation from proteasome-mediated degradation. Further investigation is needed to clarify the mechanism of CK2-RAR α interactions; the kinase could perform its activity in a direct or indirect way or it could also maintain a pre-existing state of phosphorylation through the inhibition of phosphatases.

Photopolymerizable, biodegradable hydrogel for regenerating functional skeletal muscle by freshly isolated satellite cells

Marina Flaibani^{1,2}, Carlo Alberto Rossi^{3,4}, Bert Blaauw^{2,5}, Michela Pozzobon³, Elisa Figallo¹, Carlo Reggiani⁵, Libero Vitiello⁶, Paolo De Coppi^{3,4}, **Nicola Elvassore**^{1,2}

¹ Chemical Engineering Dept, University of Padova, Padova, Italy;

² Venetian Institute of Molecular Medicine, Padova, Italy;

³ Stem Cell Laboratory, Dept of Pediatrics, University of Padova, Padova, Italy;

⁴ Surgery Unit, Institute of Child Health, University College London, London, United Kingdom;

⁵ Dept of Human Anatomy and Physiology, University of Padova, Padova, Italy;

⁶ Dept of Biology, University of Padova, Padova, Italy;

In this work we aimed at design a simple, robust and clinically suitable strategy to restore mass loss in partially ablated muscle. The success of skeletal muscle reconstruction depends on finding the best way to delivery highly myogenic cells through biocompatible scaffolds. Satellite cells (SCs) represent so far the best source for muscle regeneration. Here we designed and developed an injectable and *in situ* photo-crosslinkable hyaluronan-based hydrogel (HA-PI) for delivering SCs through minimally invasive techniques.

The HA-PI was characterized *in vitro* by measuring the apparent viscosity of solution before light exposure and the mechanical properties after crosslinking. For *in vivo* experiments, tibialis anterioris of C57BL/6J mice were partially ablated and engrafted with GFP+ve cells embedded in HA-PI. The cell-hydrogel suspension was crosslinked *in situ*. Through histological and immunohistochemical analyses we investigated the HA-PI degradation rate, the contribution of engrafted cells to the mass recovery and to the replenishment of the SCs compartment, and the reconstitution of neuro- and vascular networks. The muscle functional recovery was monitored by contractile force measurements. Using the *in vitro* data, the operative parameters (HA-PI concentration, injection rate, and light exposure duration) were set to ensure high cell survival, metabolite diffusion through the hydrogel, and repeatability of results. *In vivo* experiments showed that the delivery of only 250 freshly isolated SCs through HA-PI allows the reconstruction after severe muscle ablation (about 15% w/w) with normal muscle architecture, up to 90% of GFP+ve fibers formation and the constitution of muscle GFP+ve SCs within the whole muscle volume. The reconstructive processes were associated with functional recovery, re-innervation of the newly formed fibers and re-vascularization. We strongly believe that this approach to skeletal muscle tissue engineering could be advantageous for the reconstruction of diseased or damaged muscles and could improve the functional recovery that has been associated with previous strategies of muscle replacement.

Mitochondrial fusion is an early and protective step of autophagy

Lígia Gomes^{1,2}, **Luca Scorrano**^{1,3}

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³Dept of Cell Physiology and Metabolism, University of Geneva Medical School, Geneve, Switzerland

Autophagy is a catabolic process, often associated with several diseases, ranging from cancer wasting to genetic and degenerative conditions, that allows the recycling of components of the cell under conditions of nutrient depletion. Autophagy has been long regarded as an unselective process, but under some circumstances specific organelles like mitochondria are selectively engulfed by autophagosomes. We therefore explored whether mitochondrial morphological changes were associated with the onset of autophagy. Starvation, a powerful trigger of autophagy, induced mitochondrial elongation which correlated with increased fusion rate and required the core mitochondrial fusion proteins, as substantiated by a genetic analysis. A combination of real time imaging and small molecule inhibitors indicated that cAMP-PKA axis mediates starvation-triggered mitochondrial elongation by blocking translocation of the pro-fission protein Drp1 to mitochondria. Elongation protected against mitophagy during periods of starvation and the ablation of the required pro-fusion genes converted mitochondria into sinks for ATP and caused starvation-induced death, suggesting a protective role for these morphological changes during periods of limited substrate supply. Thus, mitochondrial shape changes play an important role in the regulation of the fate of cells undergoing autophagy.

Smad2 and 3 transcription factors control muscle mass in adulthood

Roberta Sartori^{1,2,3}, Giulia Milan^{1,2,3}, Maria Patron¹, Cristina Mammucar^{1,3}, Bert Blaauw¹, Reimar Abraham¹, **Marco Sandri**^{1,2,3}

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²Dulbecco Telethon Institute, Padova, Italy

³Department of Biomedical Sciences, University of Padova, Padova, Italy

Loss of muscle mass occurs in a variety of diseases, including cancer, chronic heart failure, acquired immunodeficiency syndrome, diabetes, and renal failure, often aggravating pathological progression. Preventing muscle wasting by promoting muscle growth has been proposed as a possible therapeutic approach. Myostatin is an important negative modulator of muscle growth during myogenesis, and myostatin inhibitors are attractive drug targets. However, the role of the myostatin pathway in adulthood and the transcription factors involved in the signaling are unclear. Moreover, recent results confirm that other transforming growth factor-beta (TGF-beta) members control muscle mass. Using genetic tools, we perturbed this pathway in adult myofibers, *in vivo*, to characterize the downstream targets and their ability to control muscle mass. Smad2 and Smad3 are the transcription factors downstream of myostatin/TGF-beta and induce an atrophy program that is muscle RING-finger protein 1 (MuRF1) independent. Furthermore, Smad2/3 inhibition promotes muscle hypertrophy independent of satellite cells but partially dependent of mammalian target of rapamycin (mTOR) signaling. Thus myostatin and Akt pathways cross-talk at different levels. These findings point to myostatin inhibitors as good drugs to promote muscle growth during rehabilitation, especially when they are combined with IGF-1-Akt activators.

Interplay among cAMP, cGMP and Ca²⁺ in olfactory sensory neurons.

Mara Pietrobon¹, Micol Maritan¹, Tullio Pozzan^{1,2,3}, **Claudia Lodovichi**^{1,3}

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²Dept of Biol Science, Padova,

³CNR.

Although cAMP is the primary second messenger produced upon activation of the odorant receptor, cGMP is also produced and takes part in several key processes such as adaptation, development and long term responses to odorant stimulation. However, the mechanism of cGMP production in olfactory sensory neurons (OSN) is poorly understood and many aspects of the regulation of cGMP 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, although with different kinetics. 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, suggesting that this signaling pathway may be relevant for both local events (pathfinding, neurotransmitter release) and more distal processes involving gene expression regulation.

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

Kenneth A. Dyar^{1,2}, Bert Blaauw², Stefano Ciciliot^{1,2}, Reimar Abraham², Giorgia Pallafacchina^{1,2}, Jana Tothova^{1,2}, Carla Argentini^{1,2}, Miika Ahdesmäki⁴, **Stefano Schiaffino**^{1,2,3}

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In mammals, the suprachiasmatic nucleus (SCN) of the hypothalamus acts as a master pacemaker, controlling circadian rhythms of behavior and metabolism and synchronizing the temporal organization of gene expression and organ function via molecular clocks in peripheral tissues. Recent studies have highlighted that functional circadian timing in peripheral tissues is essential for the normal execution of many important physiological processes, with circadian clock control often exerted at key rate-limiting steps in various pathways. In skeletal muscle the SCN plays a dual role, as it entrains both the skeletal muscle circadian clock and the circadian rhythm of contractile activity, as shown by SCN ablation and transplantation experiments. While muscle contractile activity is strictly dependent on nerve activity, a role of neural signals in circadian clock regulation is unclear. In order to elucidate the contribution of nerve activity on circadian gene expression patterns in muscle, and to identify which pathways in skeletal muscle are under circadian control, we have examined the influence of both neural and non-neural signals on circadian gene expression patterns, focusing on phase entrainment of the core clock genes *Bmal1*, *Per1* and *Per2*, and on the transcription factors NFATc1 and c3, which are known to act as activity sensors in skeletal muscle. Our results indicate that both activity-dependent and -independent factors are responsible for orchestrating circadian gene expression in skeletal muscle. We show that NFATc1 and NFATc3 show circadian variations in nucleocytoplasmic shuttling and transcriptional activity which are abrogated by denervation. In contrast, the phase of core clock gene expression is independent of activity, as neither motor denervation, nor elimination of peripheral sympathetic nerve fibers with 6-hydroxydopamine significantly altered expression phase. Interestingly, our genomic and genetic analyses show that activity plays an important role in modulating overall expression levels of many cycling genes. In these genes expression phase and peak/trough ratio was essentially unchanged by denervation, whereas absolute expression levels were significantly up- or down-regulated. This suggests that both activity and the skeletal muscle core clock coordinately control the regulation of many cycling genes, and provides insight into how the circadian clock and locomotor activity may cooperate to exert control over specific physiological pathways in skeletal muscle.

A 7 gene Signature (cirrhosis risk score) predicts liver fibrosis progression in patients with initially mild chronic hepatitis C

Moira Marcolongo^{1,3}, Bradford Young⁴, Francesca Dal Pero³, Giovanna Fattovich², Laura Peraro², Maria Guido⁵, Giada Sebastiani³, Giorgio Palù¹ and **Alfredo Alberti**^{1,3}

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³Venetian Institute of Molecular medicine, Padova, (Italy)

⁴Celera Alameda, Ca (USA)

⁵Department of Diagnostic Medical Sciences and Special Therapies, University of Padova (Italy)

Fibrosis progression is the main determinant of liver disease outcome in chronic hepatitis C, (CHC) being influenced by environmental and host factors. Recently, a Cirrhosis Risk Score (CRS) based on 7 single-nucleotide polymorphisms (SNPs) was proposed as genetic predictor of cirrhosis in hepatitis C. To assess the role of CRS in predicting fibrosis progression in patients with initially no/minimal to moderate fibrosis, we investigated 271 untreated patients with CHC having an initial liver biopsy showing F0 (104 cases) or F1 (101 cases) or F2 (59 cases) METAVIR stage, who had been followed-up without antiviral therapies for at least 60 months (mean 108.5±71.5 months), and had a liver biopsy at the end of this observation period. 24.4% showed no histologic progression, 75.6% progressed by at least 1 stage, 45.0% by at least 2 stages and 10.3 % by >2 stages. Mean CRS was significantly higher (p=0.005) in patients with fibrosis progression compared to those without progression, and this difference was particularly evident (p=0.002) with F0 in initial biopsy. Mean CRS scores were not associated with degree of fibrosis progression. The relative risk of fibrosis progression increased with increasing CRS values. This association was significant in males but not in females and most evident in males with F0 in initial biopsy, Odd ratio for fibrosis progression being 16.5 (95%CI 1.6-166, p=0.02) in presence of high CRS. Multivariate analysis confirmed the significant association of the CRS score with fibrosis progression. The predictive value of CRS was confirmed in HCV patients admitting significant alcohol intake. Conclusions: Host genetics defined by CRS predict fibrosis progression in male patients with initially mild chronic hepatitis C and may become a useful parameter for prognostic evaluation and treatment decision.

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

1. Mesenchymal stem cells extend the survival and promote the compartmentalization of malignant clone in B-CLL patients.

Elisa Ave^{1,2}, Carlo Alberto Giorgi², Alessandra Bernard², Federica Lessi¹, Federica Frezzato^{1,2}, Cristina Gattazzo^{1,2}, Anna Cabrelle², Monica Facco^{1,2}, Livio Trentin^{1,2}, **Gianpietro Semenzato**^{1,2}

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²Venetian Institute of Molecular Medicine (VIMM) Padova

B-cell Chronic Lymphocytic Leukemia (B-CLL) is characterized by the accumulation of mature clonal CD5+ B lymphocytes in peripheral blood, bone marrow and lymphoid tissues. The evidence that these malignant cells rapidly undergo spontaneous apoptosis *in vitro*, suggests that the selective survival advantage enjoyed by leukemic B-cells also depends upon external signals. Increasing data indicate that different types of stromal cells protect leukemic clone when co-cultured and are an integral part of CLL microenvironment. In the present study we focused our attention on mesenchymal stem cells (MSCs) in order to evaluate their role in the survival and the localization of neoplastic clones. MSCs has been isolated from the bone marrow of 26 B-CLL patients, *ex vivo* expanded and characterized through cytofluorimetric analysis and differentiation cultures (adipocytes and osteocytes). While MSCs from patients exhibited normal phenotype and differentiation capacities, when co-cultured with neoplastic B cells they exerted a notable anti-apoptotic effect. After 7 days CLL-MSCs co-cultures, we observed a relevant extended survival of leukemic cells (60% \pm 17% with MSCs vs 14% \pm 12% without MSCs; Δ = 46%), but not of normal B lymphocytes (30% \pm 15% with MSCs vs 6% \pm 5% without MSCs; Δ = 24%). The same anti-apoptotic effect was observed on B-CLL cells isolated from 3 patients treated with pro-apoptotic compounds, suggesting an involvement of MSCs in drug-resistance (48% \pm 16% with MSCs vs 21% \pm 18% without MSCs; Δ = 27%). Transwell experiments confirm that the anti apoptotic effect is mediated by soluble factors produced by MSCs. Finally, chemotaxis tests showed the ability of MSCs to produce and release molecules promoting the migration and the localization of neoplastic B cells in bone marrow. Taken together, these findings suggest that CLL-MSCs provide survival signals to neoplastic cells extending their lifespan and producing chemotactic factors favouring their accumulation in the bone marrow.

2. Identification of bacterial factors able to promote a Th17 response in gastric mucosa of *Helicobacter pylori*-infected patients

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Helicobacter pylori is a spiral-shaped Gram negative bacterium that colonizes the human gastric mucosa and chronically infects more than half of the human population. During *H pylori* infection, there is a pronounced specific acquired immuno response, characterized by production of antibodies, and by differentiation and activation of effector T cells(1). *H pylori* infection preferentially stimulates a Th1 immune response(2); however, emerging experimental evidence demonstrates that *H pylori* triggers also the development of the Th17 subset(3). Th17 cells release IL-17 and IL-22, and are implicated in the induction of numerous autoimmune and inflammatory responses. Their differentiation is driven primarily by TGF- β , IL-1 and IL-6 cytokines, whereas IL-23, originally thought to be the master regulator, seems to be important for maintenance of Th17 responses(4). The aim of our project is the identification of the factors produced by *H pylori* endowed with the ability to promote the differentiation of T helper cells towards the Th17 phenotype. To this purpose, we have purified bacterial extracts by chromatography, obtained pools of few proteins and tested them for the ability to induce the expression of IL-1, IL-6, IL-23 and TGF- β in monocytes. We have identified a pool, that we are currently identifying by mass spectrometry, that is particularly immunomodulant. Once defined, we plan to express these proteins recombinantly and to use them for verifying the presence of specific Th17 cells in the gastric mucosa of *H pylori* positive patients.

3. Regulation of FoxO3 transcription factor during muscle atrophy

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FoxO proteins are transcription factors that control cell cycle progression, DNA repair, muscle atrophy, stress resistance and apoptosis. These divergent functions are carefully regulated by post-translational modifications including phosphorylation, acetylation and ubiquitination, these mechanisms seem to coordinate the localization, activity and longevity of FoxO protein in a time-dependent manner to ensure a fine regulation.

During muscle atrophy FoxO3 is negatively regulated by AKT phosphorylation, which determines its nuclear localization and activation.

We studied the role of acetylation on FoxO3 in adult skeletal muscle, a post translational modification that modulates FoxO activity. To fulfill this purpose, we have generated different FoxO3 mutants which prevent acetylation (KR) or mimic acetylation (KQ). By a Luciferase assay, we discovered that mutants show the opposite behavior in activating the promoter, notably acetylation-mimicking mutants displayed a lower transcriptional activity. Moreover iperacetylation reduces FoxO-dependent muscle atrophy by causing re-localization of FoxO proteins into the cytoplasm.

Finally we found out that along with the quantity of the acetylated residues their position is important as well. In particular K262 seems to be a key residue for the nuclear export.

In conclusion, we can assert that our findings shed light on the complex regulation of FoxO transcription factors, explaining how acetylation negatively modulates FoxO activity.

4. Hyperinsulinaemia reduces the 24 hour virological response to PEG-interferon therapy in patients with chronic hepatitis C and insulin resistance.

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Background/Aims: Insulin resistance reduces response to pegylated-interferon/ribavirin in chronic hepatitis C. Pathogenetic mechanisms are still undefined. We examined the relationship between baseline insulin levels, the main component affecting HOMA-IR for assessment of insulin resistance in non-diabetic patients, and the “acute” virological response to pegylated-interferon measured 24 hours after the first injection and taken as correlate of intracellular interferon signalling. *Methods* In 62 patients treated with Pegylated-interferon/Ribavirin, serum insulin and HOMA-IR were assessed at baseline while HCV-RNA was measured at baseline, and 24 hours, 1, 2, 4, 12 weeks after treatment initiation. Sustained virological response was examined 24 weeks after therapy discontinuation. *Results:* Mean baseline insulin was 11.52±8.51 U/L and mean HOMA-IR was 2.65±2.01 being both significantly higher with advanced liver fibrosis. HCV-RNA decay observed 24 hours after the first injection of Pegylated-interferon was significantly lower (0.7±0.8 log) in patients with HOMA ³ 3 compared to those with HOMA <3 (1.7±0.8, p=0.001). A highly significant (r = - 0,42) inverse correlation was observed between baseline insulin levels and the 24-hour HCV-RNA decay. The difference in early viral kinetics between patients with HOMA ³ 3 or <3 resulted in a significant difference in the percentage of patients achieving rapid (week 4) and sustained virological response. Multivariate analysis , inclusive of patient age, HCV genotype and fibrosis stage , identified baseline insulin levels as the main independent variable affecting the 24-hour response to Pegylated-interferon. *Conclusions:* Hyperinsulinaemia reduces the cellular response to Pegylated-interferon in chronic hepatitis C with insulin resistance. Strategies to reduce insulin levels before initiation of treatment should be pursued to improve efficacy of antiviral treatment.

5. Hereditary hearing loss due to a single point mutation in the PMCA2 Ca²⁺-pump gene: a functional study in mice.

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Plasma-membrane Ca²⁺-ATPase isoform 2 (PMCA2) is the only system that exports back to the extracellular fluid Ca²⁺ ions entered through hair cell stereocilia during mechano-electrical transduction of sound and acceleration. Ablation or missense mutations of the pump cause deafness in humans and mice, but we have only few clues to the molecular basis of the disease generation. A guanine to adenine change at base 1750 in the coding sequence of *Atp2b2* gene of the pump was found in the *Tommy* mutant mouse, causing profound hearing impairment since postnatal day (P) 18 in the homozygous mice, while moderate deafness was found in the heterozygous. In the homozygous, loss of auditory function is followed, between P40 and P70, by a corresponding base to apex progression of hair cell degeneration in the cochlea. We also investigated Ca²⁺ extrusion in hair cells of organotypic cultures of sensory epithelia from P6 neonatal mice. Confocal Ca²⁺ imaging showed that the dissipation of stereociliary Ca²⁺ transients, induced by Ca²⁺ photoliberation, was significantly compromised in the homozygous mice compared to the wild type. We suggest that the reduced stereociliary Ca²⁺ extrusion can trouble the finely tuned control mechanisms of signal transduction in the cochlea, eventually resulting in hair cell death.

6. Impaired autophagy in the skeletal muscle of collagen VI myopathic mice

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Collagen VI is an extracellular matrix protein forming a microfilamentous network in various tissues. Mutations of COL6 genes in humans cause various muscle diseases, including Bethlem Myopathy and Ullrich Congenital Muscular Dystrophy. Collagen VI null (*Col6a1*^{-/-}) mice display a myopathic phenotype affecting skeletal muscles, with loss of contractile strength, ultrastructural defects of sarcoplasmic reticulum and mitochondria, latent mitochondrial dysfunction, and spontaneous apoptosis. Although these studies indicated that collagen VI plays a key role in muscle, the molecular mechanisms linking collagen VI deficiency with organelle alterations and cell death remained unknown. Analysis of pro- and anti-apoptotic Bcl2 proteins did not reveal any obvious difference between *Col6a1*^{-/-} and wild-type muscles. An important survival mechanism in muscle is the mTOR pathway, which in turn is regulated by Akt and AMPK. Although Akt phosphorylation was apparently normal, AMPK was markedly activated indicating an energy defect in *Col6a1*^{-/-} muscles.

Autophagy is an evolutionarily conserved process crucial in the turnover of cell components, and the above findings suggest that autophagy might be activated in *Col6a1*^{-/-} muscles to allow for removal of altered organelles and compensation of energy deficiency. In contrast, we found that *Col6a1*^{-/-} muscles display a striking defect of autophagy, with a block of autophagosome formation.

The persistence of abnormal organelles and the ensuing apoptosis of collagen VI null mice are due to impaired autophagy. Indeed, *Col6a1*^{-/-} muscles display decreased LC3 lipidation, which matches the lower induction of Beclin1 and Bnip3 and the lack of autophagosomes after food starvation. Local transfection of Beclin1 cDNA in muscle was able to decrease apoptosis, and forced activation of autophagy either by prolonged starvation or by low-protein diet allowed to rescue the myopathic phenotype of *Col6a1*^{-/-} mice.

Alteration of the lysosomal degradative process with excess of autophagic vesicles plays a causative role in certain storage diseases and vacuolar myopathies. However, no causal link between defective autophagy and muscular dystrophy was reported so far. Thus, collagen VI-related dystrophies represent the first muscle disease whose pathogenic mechanism involves a failure of the autophagic machinery.

7. Expression of connexin 26 and connexin 30 in the inner ear of Cx26 conditional null mice Cx26 Sox10Cre

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Connexin 26 (Cx26) and connexin 30 (Cx30) are encoded by two genes (GJB2 and GJB6, respectively) that are found within 50 kb in the same complex deafness locus DFNB1. Previous studies showed a coordinated control of Cx26 and Cx30 at the regulatory and functional level in the inner ear (Ortolano et al., PNAS 2008) of p5 mice. In these recent studies, we observed by immunohistochemistry of cochlear sections the expression of both connexins in the inner ear of conditional Cx26 null mice Cx26 Sox10Cre from the age of p5 up to p30. The expression of Cx30 is almost null at p5 but begins to be restored at the age of p9 in the basal turn of the cochlea. In the adult, Cx30 expression level is equivalent to that observed in aged matched Cx26loxP/loxP control mice. Moreover, since the age of p14, outer hair cells of the Organ of Corti (OoC) start to degenerate, and in the p30 mice, which are deaf, there is a strong hair cells degeneration in the basal turn of the cochlea. These data underline the importance of Cx26 and Cx30 for the hearing function and the preservation of the OoC.

8. A newly engineered FRET-based biosensor to monitor cAMP in the mitochondrial matrix

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The classic model for cAMP production in mammals involves activation of GPCRs that, through heterotrimeric G proteins, modulate the activity of transmembrane adenylyl cyclases (tmACs). Recently, an additional source of cAMP in mammals, the soluble adenylyl cyclase (sAC), has been identified in a variety of cellular extracts. sAC is structurally and biochemically distinct from tmACs, being insensitive to G proteins and to forskolin, while being directly stimulated by bicarbonate and regulated by calcium. Most catabolic processes converge on the citric acid cycle and on the electron transport chain, the end products of which are CO₂ and ATP. Mitochondria constantly respond to changes in substrate availability and energy utilization to maintain cellular ATP supplies. Reversible phosphorylation of mitochondrial proteins has been proposed to play a fundamental role in metabolic homeostasis but the signaling pathways involved remains unknown. Very recently it has been demonstrate the existence of a CO₂-HCO₃⁻-sAC-cAMP-PKA signaling cascade wholly contained within mitochondria, which would serve as a metabolic sensor modulating ATP generation in response to nutrient availability. To directly monitor cAMP dynamics in mitochondria of living cells we have constructed FRET based sensors targeted to the mitochondrial matrix. Our preliminary results indicate that the sensors are correctly expressed in the matrix and that mitochondrial cAMP increases are detectable in different cells type.

9. Proteome differences between brown and white fat mitochondria reveal specialized metabolic functions

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Mitochondria are functionally specialized in different tissues and a detailed understanding of this specialization is important to elucidate mitochondrial involvement in normal physiology and disease. Mitochondria play a central role in adipose metabolism, as highlighted by mitochondrial impairment in metabolic diseases, neurodegenerative diseases and in ageing. Brown adipocytes are very rich in mitochondria that uniquely express the uncoupling protein UCP1. When activated, this protein dissipates proton motive force in the form of heat. Thus, controlled uncoupling is a potential strategy for the treatment of morbid obesity. Tissue specific functions of mitochondria in white fat are less characterized. We have applied high resolution, quantitative mass spectrometry to directly and accurately compare the in vivo mouse mitochondrial proteomes of brown and white adipocytes. Their proteomes are substantially different qualitatively and quantitatively and are furthermore characterized by tissue-specific protein isoforms, which are specifically modulated by cold exposure. At transcript and proteome levels brown fat mitochondria are more similar to their counterparts in muscle. Conversely, white fat mitochondria not only selectively express proteins that support anabolic functions, but also degrade xenobiotics revealing a protective function of this tissue. In vivo comparison of organellar proteomes can thus directly address functional questions in metabolism.

10. Insulin interferes with the canonical IFN-alpha pathway: a possible link between IR and non-responder HCV infected patients.

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Background and Aim: Insulin resistance (IR) is a frequent feature in CHC (chronic hepatitis C) and seems to decrease the rate of sustained virological response. However the mechanisms by which IR interferes with antiviral therapy are still unclear. We therefore investigated the possible role of insulin in interfering with interferon-alpha (IFN-alpha) pathway in human hepatoma cell line HepG2 by analysing the gene expression of some IFN stimulated genes ISGs (PKR, MxA and 2'-5' OAS). *Methods:* HepG2 were treated with different concentration of IFN-alpha and insulin alone or in combination. Time course analysis of the expression of ISGs was performed by Real Time PCR. PKR protein expression was also evaluated in the same settings by Western-blot analysis. *Results:* IFN-alpha alone enhanced the expression of ISGs. In HepG2 treated with IFN-alpha plus insulin a dose-dependent reduction in PKR, MxA and 2'-5' OAS total mRNA levels were higher compared to untreated cells (p: 0,017, p: 0,103, p: 0,002 respectively with 100 nM insulin of stimuli; p: 0,0017, p: 0,186, p: 0,006 respectively with 1000 nM insulin of stimuli). PKR protein expression showed the same trend of gene expression. Insulin alone had no effects on these genes and protein. *Conclusions:* In HepG2 insulin significantly reduces the induction of three major ISGs, in a dose-dependent manner. The control of insulin levels at baseline before initiation of IFN based therapy should be considered in the clinical practice to improve the antiviral response in CHC.

11. Cyclophilin D modulates Mitochondrial FOF1 ATP-synthase by interacting with the lateral stalk of the complex

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The mitochondrial FOF1ATP synthase is a complex of 600 kDa organized into a catalytic part (F1) and a membrane moiety (FO) linked by central and peripheral stalks. The complexity of such structural organization requires accessory factors, which are not yet completely defined. In the present study we investigated the interactions between the mitochondrial chaperone cyclophilin (CyP) D and the ATP synthase. By blue-native gel electrophoresis purification and immunoprecipitation of FOF1 ATP synthase from bovine heart mitochondria we found that CyPD is associated to the complex. Treatment of intact mitochondria with bifunctional reagents demonstrated that CyPD interacts with the lateral stalk of the ATP synthase; and studies of ATP synthesis and hydrolysis revealed that these interactions have functional consequences on enzyme catalysis. Treatment of MgATP submitochondrial particles or intact mitochondria with phosphate increased CyPD binding and decreased its enzymatic activity; while Cyclosporin (Cs) A displaced CyPD from membranes, and activated both hydrolysis and synthesis of ATP sustained by the enzyme. No effect of CsA was detected in CyPD-null mitochondria, which displayed a higher specific activity of ATP synthase than wild-type mitochondria. Modulation by CyPD binding appears to be independent of IF1, whose association to ATP synthase was not affected by CsA treatment. These findings demonstrate that CyPD association to the lateral stalk of ATP synthase modulates the activity of the complex. CyPD function does not appear to be related to ATP synthase assembly, which is not affected in CyPD-null mitochondria.

12. Defining novel molecules to rescue immunity against cancer: molecular and biological basis for new therapies

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Prostate cancer (PCa) is the second leading cause of malignancy-related mortality in males in the Western world. The available treatments for PCa have demonstrated weak curative efficacy. Immunotherapy may provide a valid alternative therapy but the success of this approach depends on the ability of CTL to kill tumor cells. However, if the tumor environment exerts a suppressive action on TILs, immunotherapy will achieve little, if any, success. Thus, it is paramount to understand modulation of TIL responses by the tumor environment. To analyze the role of the prostate tumor environment we have started a study which allowed us to demonstrate that human PCa are infiltrated by terminally differentiated CTL that are completely unresponsive. The steady-state regulation of the dormant state is dependent on the enhanced intratumoral metabolism of L-Arg, because the addition of ARG- and NOS- specific inhibitors was sufficient to activate them and recover their functions. These results identify a mechanism by which PCa induces in situ immunosuppression and suggest novel strategies for the tumor immunotherapy. Based on our findings, drugs controlling ARG and NOS might be useful to aid immunotherapeutic approaches for the treatment of cancer by creating a favorable tumor environment for the T lymphocyte effector program. On these bases, we have developed and tested a new class of NO-donor molecules. The data obtained from in vitro assays indicate that these compounds can normalize the immune status of tumor-bearing hosts and restore mice lymphocyte responsiveness, in terms of proliferation and effector functions. Moreover, we observed that the leading compound AT38 reduce intratumoral RNS generation in vivo and facilitates TIL infiltration into the tumor site. Thus, this compound could be consider potential candidate as adjuvant in the antitumor immunity elicited by cancer vaccination.

13. Structural plasticity of CK2 hinge region: a regulatory element and a new opportunity for drug development

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The hinge region of protein kinases is involved in binding of ATP. Its destabilization or incomplete formation is associated to instability of ATP binding and loss of catalytic efficiency. In all kinase structures solved so far, this region is in an identical “closed” conformation. Here we present structures of human CK2 alpha subunit in two different conformations: a canonical “closed” hinge region and an atypical “open” conformation. We present evidences that the “closed” hinge region is correctly formed for ATP binding while a decrease in ATP affinity is observed for the “open” conformation. A “relaxed-closed” conformation, as seen in the apo-structure, is compatible with GTP binding as alternative CK2 co-substrate. We propose that CK2 hinge region serves as a regulatory element and, in this respect, constitutes a unique feature in the protein kinase family. The “open” conformation could be exploited for design of new inhibitors that would gain high selectivity against other protein kinases in virtue of the additional space available in the “open” state.

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

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Electrical activity is essential in transforming immature circuits into the organized connections that subserve adult brain functions. The role of neuronal activity in this process has been investigated in other sensory systems, in particular in the visual system, while is still poorly understood in the olfactory system (OS). To address this question we study the influence of neuronal activity on a specific circuitry within the olfactory bulb, the intrabulbar projection (IBP), which connects specifically and reciprocally homologous glomeruli. To accomplish this goal we are studying the development of the IBP in mice lacking afferent activity due to surgical manipulation, such as naris occlusion, and in genetically modified lines of mice in which electrical activity has been abolished due to the overexpression of the channel Kir2.1 (OMP-IRES-tTA/Teto-Kir2.1-IRES-tau-lacZ). We inject neuronal tracer into the glomerular layer on one side of the bulb and we analyze the corresponding projection on the opposite side of the bulb at different stages of postnatal development (P15, P30, >P50) in wild type mice and in lines of mice in which a specific OR is coexpressed with GFP (M71-GFP, P2-GFP). In this latter case the corresponding GFP labelled glomeruli are easily identifiable in the OB allowing to study the IBP between specific homologous glomeruli. Preliminary data indicate that the ratio of the diameter of the injection and the projection is larger in the Kir2.1 mice than in the wild-type mice (data collected at P30).

15. ATP-MEDIATED CELL-CELL SIGNALLING IN THE ORGAN OF CORTI: THE ROLE OF CONNEXIN CHANNELS

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Connexin 26 (Cx26) and connexin 30 (Cx30) form hemichannels that release ATP from the endolymphatic surface of cochlear supporting and epithelial cells, as well as gap junction (GJ) channels that allow the concomitant intercellular diffusion of Ca²⁺ mobilizing second messengers. Released ATP in turn activates G-protein coupled P2Y2 and P2Y4 receptors, PLC-dependent generation of IP3, release of Ca²⁺ from intracellular stores, ensuing in the regenerative propagation of intercellular Ca²⁺ signals (ICS) across these coupled cells. The range of ICS propagation is sensitive to the concentration of extracellular divalent cations and to ectonucleotidase activity. Here we characterized the expression patterns of Cx26 and Cx30 in samples of cochlear tissues obtained from mice aged between P5 and P6. Immunofluorescence revealed an expression gradient along the longitudinal axis of the cochlea, decreasing from the basal to the apical cochlear turn (CT) and more pronounced in outer sulcus (OS) cells than in inner sulcus (IS) cells. Gap-FRAP (fluorescence recovery after photobleaching) assays showed highest degree of GJ-mediated dye coupling in OS cells of the basal CT. Cell-cell coupling was inhibited by the nonselective connexin channel blocker carbenoxolone (CBX), and was absent in hair cells. In response to photostimulation with caged inositol (3,4,5) triphosphate (IP3), we observed prominent transfer of ICS in the lateral direction from IS cells to OS cells across the hair cell region (HCR) of the basal CT. ICS transfer in the reverse direction was most pronounced in the middle CT. In addition, OS cells in the basal CT displayed an impressive rhythmic activity of cytosolic free Ca²⁺ concentration ([Ca²⁺]_i) oscillations, coordinated by the propagation of Ca²⁺ wavefronts sweeping repeatedly through the same tissue area, in the longitudinal direction. This activity was not paralleled in IS cells.

16. Structural interaction between cardiac sympathetic nerve terminals (STs) and target cells and their age-related changes in mouse heart

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Introduction: The autonomic nervous system is the main regulator of cardiac activity. Its sympathetic component modulates coronary vasodilatation, heart rate and contractility. It is known that impairment of sympathetic nervous system (SNS) occurs in aging and in pathological conditions. However, little is known on how synapses between the STs and the target cells are functionally organized. The aims of our project are: i) to provide a detailed structural and physiological description of the interaction between STs, vasculature and cardiomyocytes; ii) to investigate whether changes in these relationships participate in the age-dependent functional impairment of the SNS.

Results: Immunofluorescence analysis of normal adult mouse hearts demonstrated an interaction of the STs with coronary vessels, cardiomyocytes and capillaries. The functional meaning of the latter relationship needs to be clarified. We hypothesized a role of the SNS in the neoangiogenesis process, an hypothesis supported by works demonstrating the role of noradrenaline in VEGF synthesis. To this aim, we are measuring capillary size and density in hearts from sympathectomized mice, which lack of the STs, vs normal hearts. In parallel, a preliminary analysis suggests an age-related alteration of the STs, that appear longer and more branched in young mice as compared to older controls. We are now evaluating if these changes are related to alterations of STs density and distribution with respect to target cells.

17. RNOS-induced chemokine inactivation in cancer

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Tumor microenvironment does not appear to be suitable for T lymphocyte functions and indeed a number of reports indicate that tumor-infiltrating lymphocytes (TIL) are impaired in both signal transduction and effector systems. At the tumor site, the reactions of NO with oxygen (O₂) or oxygen-related reactive intermediates yield numerous reactive nitrogen as well as oxygen species (RNOS). One of the most intensively studied reaction is the one between NO and superoxide anions yielding peroxynitrite (ONOO⁻), which is a potent oxidant. In the past, we provided data showing that RNOS are involved in tumor-induced immunosuppression and we speculated that RNOS might block T lymphocyte infiltration in the tumor.

We decided to study the role of RNOS in tumor-induced immunosuppression. Typically, TILs are unable to reach the core of the tumor mass, and they concentrate at the border of the neoplastic lesion. We speculated that RNOS modify chemokine biology and keep TILs distant from the tumor. Chemokines are small cytokines with selective chemoattractant properties, coordinating the homeostatic circulation of leukocytes as well as their movement to sites of inflammation or injury. Dysregulated expression of chemokines and/or their receptors is involved in the development of many human diseases, including autoimmune and chronic inflammatory diseases as well as immunodeficiency and cancer. We found that the chemoattractants CXCL12, CCL21 and CCL2 lose their ability to recruit T lymphocytes if exposed to peroxynitrite. However, the modified chemokine CCL2 retains its capacity of recruiting myeloid-derived suppressor cells.

18. Role of MRF4 in adult skeletal muscle.

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Muscle regulatory factors are a family of four basic helix loop helix proteins playing a crucial role in myogenesis during embryonic development and muscle differentiation. Experiments of gene targeting in transgenic mice have shown that MyoD, Myf5 act as muscle determination factors while myogenin acts as differentiation factors. MRF4 can subserve both these roles during the embryo development. In the adult skeletal muscle, the expression of MRFs acquires a characteristic profile: Myf5 is not expressed in adult fibers, MyoD and myogenin are expressed respectively in fast muscle and in slow muscles, Mrf4 is the only muscle regulatory factors expressed at high levels in adult skeletal muscle. Although it exists a great amount of literature describing Mrf4 role and regulatory networks during the different stages of myogenesis, very little is known on the effective function of this factor in adult skeletal muscles.

Here we show with different approaches that Mrf4 is expressed at similar levels in slow and fast muscles (soleus and EDL). Conversely its localization is clearly different among the two types of muscles, being almost constitutively nuclear in the soleus slow muscle and mostly cytosolic in the fast EDL. Moreover both expression levels and localization seem to be activity-dependent. To determine the role of MRF4 we exploited the electroporation to either over-express or knock-down this transcription factor in adult slow and fast rat muscles. We demonstrate that: i) Mrf4 acts as a negative regulator of muscle growth, independently of fiber type; ii) Mrf4 is involved in the induction and the maintenance of the slow gene program in regenerating and adult soleus muscles. To further investigate the underlying mechanisms we performed microarray analyses from soleus muscles transfected with RNAi constructs.

19. *Helicobacter pylori*-induced production of APRIL and BLyS by macrophages infiltrating MALT lymphomas.

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Chronic inflammation due to the constant presence of *Helicobacter pylori* (Hp) may produce lymphoid follicles which organize in gastric mucosa. Such a lymphoid tissue associated to the mucosa (MALT) represents a pre-neoplastic condition which, in a limited number of patients, may evolve to a lymphoma containing B cells of low degree of malignancy. According to the present model of the genesis of lymphoma, one or more tumor clones, containing B cells of the peripheral area, take origin from the organized MALT, colonize and replace original follicles, and eventually will destroy gastric glands and produce lympho-epithelial lesions. It is generally accepted that in the early phases of lymphoma development, neoplastic transformation is accelerated by T cells activated by antigens of Hp. In parallel, a particular cytokine environment is expected to be crucial for the activation, proliferation and transformation of B lymphocytes. In this line, we planned to establish whether one or more constituents of Hp are involved in the induction and/or in the maintenance of the neoplastic proliferation of malignant B cells through an action upon the cells of the innate immunity. B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) are involved in normal B cell survival and differentiation and both are produced by cells of innate immunity such as, monocytes, macrophages, dendritic cells and neutrophils. Most importantly, BAFF and APRIL are potent growth factors in B cell malignancies. The present study demonstrates that macrophages infiltrating MALT lymphomas produce both BAFF and APRIL; furthermore, monocytes-derived human macrophages, but not dendritic cells, release the two proteins, once infected with the bacterium.

These results demonstrate for the first time that Hp contributes by itself in creating a milieu promoting the proliferation of B cells, which is expected to sustain the MALT lymphoma development.

20. Structural study of *Helicobacter pylori* HP1454 secreted protein emerging as a new virulence factor

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Helicobacter pylori is a highly successful human-specific gastric pathogen that colonizes more than half the world's population. Infection with this gram-negative bacterium can induce gastric pathologies ranging from chronic gastritis to peptic ulcers and even cancer [1].

Several *H. pylori* virulence factors, such as the *cag* pathogenicity island (PAI), the CagA protein and the vacuolating toxin VacA, have been identified [1]. Recently many research groups have moved their efforts towards the identification of new potential *H. pylori* protein targets, which could compromise the colonization and persistence of the bacterium in the gastric niche. Different approaches have identified new candidates such as secreted factors, enzymes involved in the membrane and peptidoglycan maintenance, and new proteins without any significant homology with other well characterized bacterial factors.

Secreted proteins of human pathogen *H. pylori* are of special interest because they come in direct contact with host tissues and may mediate important pathogen-host interactions [2]. HP1454, a secreted factor whose function is unknown, has been cloned, expressed in *E. coli*, purified and characterized in solution by analytical gel filtration chromatography and Circular Dichroism. Crystals have been grown and a native diffraction data set measured at the ESRF synchrotron facility (Grenoble, France). Since HP1454 does not present any sequence similarity with proteins of known structure, in order to obtain approximate initial phases, mutant derivatives containing seleno-methionines have been produced. Crystals optimization is in progress.

21. A novel role for Opa1, mutated in Dominant Optic Atrophy, in the adaptation of cells to heat stress.

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OPA1 is a large GTPase of the mitochondrial inner membrane (IM). Mutations in OPA1 are associated with autosomal dominant optic atrophy (ADOA), the most common optic neuropathy of genetic origin. Opa1 has genetically distinct roles in mitochondrial fusion and in the control of cristae remodelling and cytochrome c release during apoptosis, and is involved in the ability of the cell to cope with stressful conditions. Here we addressed the role of Opa1 in the control of the cellular response to heat shock. Cells exposed to prolonged, intense heat shock die, whereas the exposure to milder and shorter thermal stress elicits a pre-conditioning that attenuates the response to subsequent death stimuli. While this protection has been ascribed to a mitochondrial event, its molecular mechanisms are largely unknown. During heat shock mitochondria undergo fragmentation, associated with the increase in the levels of Opa1 and its proteolytic partner Parl, but not with changes in the levels of other mitochondria-shaping proteins. Of note, thermally stressed mitochondria display the decrease of the long Opa1 form and accumulation of the short Opa1 form. Moreover, in heat-shocked mitochondria the soluble form of Opa1 accumulates in a Parl-dependent manner. Accordingly, cell lacking Opa1 or Parl are more susceptible to heat shock and do not display any preconditioning, which is conversely rescued by the reintroduction of the soluble form of Opa1 in Parl^{-/-} cells. The importance of the appropriate processing of Opa1 into its soluble form is further substantiated by the accumulation in Opa1^{-/-} and Parl^{-/-} cells of intermembrane space specific aggregates, both in unstressed and heat-shocked cells. Thus, the soluble form of Opa1 is required for protein stability in the intermembrane space and for the mitochondrial response to heat shock.

22. Multi-parametric screening of pathological conditions on hESC-derived cardiomyocytes through microstructured cultures and a microfluidic platform

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Cell therapy is a promising approach for the treatment of infarcted hearts, but the hostile environment, characterized by oxidative stress, inflammatory cytokines and fibrous scar tissue, limits the survival and the functional engraftment of transplanted cells.

We aimed at developing an *in vitro* system for screening the effects of the major pathological conditions of an infarcted myocardium on human cardiomyocytes (hCM) viability and functional properties. This *in vitro* cell model should fairly represent the human heart physiology and be a valuable tool to predict hCMs fate after *in vivo* implantation. In this perspective, the system is based on microstructured hCM culture coupled with a microfluidic platform (μ FP) for multi-parametric spatial-temporal control of *in vitro* pathological environment.

hCMs are one of the most promising cell source for cardiac cell therapy and were derived from the human embryonic stem cell line HES2, following a 20 days differentiation protocol. Microstructured cultures were obtained through hCMs seeding onto polyacrilamide hydrogel microcontact printed with laminin and a geometry of circular islands (300 μ m diameter, 700 μ m center-to-center spacing, 10x10mm area). The multilayered μ FP was fabricated using lithographic techniques and molded in PDMS; its microfluidic channels allowed to selectively delivery fluids to the cultured cells.

The cell viability and the maintenance of typical cardiac markers (TroponinT, Connexin43, α -actinin, Nkx2.5) were verified via immunofluorescence after 7 days of culture, while morphometric measurements allowed to quantify both the spontaneous or induced (via electrical stimulation) contractile activity of the cell islands. The μ FP reproduce the temporal patterns of pathology evolution, thus allowing to study cell behavior under well defined disease-like conditions.

hCMs were exposed to H₂O₂ (0.01; 0.1 and 0.5mM) both in static culture and within the μ FP. In static culture, the exposure to 0.1mM H₂O₂ for 16 hours maintained cell viability, but inhibited both spontaneous and induced contractions; whereas 0.01mM did not affect functional properties of hCM.

23. Structural characterization of the acid stress response YceI protein (HP1286) from *Helicobacter pylori*

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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 (1).

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.

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 lipid-binding protein.

Crystals of HP1286 enzyme have been obtained and diffraction data measured at the ESRF synchrotron. Crystals belong to the P212121space group, with one dimer per asymmetric unit. The structure has been solved by molecular replacement. Small crystals of the other two proteins have been grown and their optimization is in progress. We expect that the structural knowledge of these proteins, essential for the survival of the bacterium, are useful in designing new inhibitors, potentially useful against the pathogen.

24. Analysis of SOCS3 expression in patients with T-Lymphoproliferative Disease of Granular Lymphocytes

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In patients with T-Lymphoproliferative Disease of Granular Lymphocytes (T-LDGL) proliferating granular lymphocytes (GLs) display high levels of the transcription factor STAT3 that likely contributes to the accumulation of the leukemic GL clone. Activation of STAT3 induces the constitutive expression of SOCS3 (suppressor of cytokine signalling), that in normal cells works as an inhibitor of STAT3 itself. Using Real Time PCR analysis in a series of patients with T-LDGL, we investigated STAT3 and SOCS3 expression in proliferating GLs. We confirmed that STAT3 was overexpressed in patients with respect to controls, while SOCS3 expression was similar between malignant GLs and resting normal GLs. These data prompted us to investigate whether SOCS3 might be unresponsive to physiological stimuli in T-LDGL. To address this issue, we analyzed SOCS3 expression in GL cultures after treatment with some cytokines crucial for LDGL proliferation (IL-15 and IL-2) or for induction of STAT3/SOCS3 transcription (IL-6). Interestingly, we observed that, in the presence of IL-6, SOCS3 was overexpressed in normal GLs, whereas remained unchanged in leukemic GLs, despite the further activation of STAT3 transcription. These data revealed that in T-LDGL patients SOCS3 shows low expression levels, consistent with what is found in resting cells, thus resulting in an uneffective block of STAT3 activation. In conclusion, our results suggest that the aberrant activation of JAK/STAT pathway in T-LDGL is associated with uncoupling of counteracting signals, suggesting that the lack of inhibitory signal might play a role in the pathogenesis of disease.

25. The p23 co-chaperone protein is a novel substrate of CK2 in Arabidopsis, involved in salicylic acid (SA) Signaling

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Protein kinase CK2, a constitutively active Ser/Thr protein kinase, is essential for cell viability, playing a special function in tumor cells as a pro-proliferative and anti-apoptotic kinase. It is well conserved in all eukaryotes, and is essential also in plants, where it is involved in several crucial processes. Recently, we demonstrated that the production of nitric oxide induced by salicylic acid (SA) requires the presence of active CK2, since specific CK2 inhibitors suppress the response. However, CK2 does not change its global catalytic activity in response to SA; this suggested to us the possibility that CK2 intervenes in SA signaling by specifically modulating the phosphorylation level of only one or few proteins among its many substrates. Therefore, we performed this study, looking for putative CK2 targets, which become phosphorylated only in response to SA. This proteomic approach allowed the identification of a new CK2 substrate in Arabidopsis, the co-chaperone p23 protein, a component of the Hsp90 chaperone complex, involved in several signaling pathways. We demonstrated that p23 is efficiently phosphorylated by recombinant CK2 and by endogenous CK2 in Arabidopsis; in-gel kinase assays confirmed that CK2 is the major Arabidopsis kinase phosphorylating p23. We also found that a physical association occurs between p23 and CK2 in vitro and in vivo. All together, our data suggest that p23 is a good candidate to mediate the CK2 involvement in SA signaling.

26. Isoform specific phosphorylation of p53 by protein kinase CK1 - Identification of Ser20 as a major target for the δ isoform

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The ability of three isoforms of protein kinase CK1 (α , γ 1 and δ) to phosphorylate individual residues in the N-terminal region of p53 has been assessed using as substrates either recombinant GST-p53 or a synthetic peptide reproducing its 1-28 sequence with suitable substitutions. The wild type peptide is readily phosphorylated at seryl residues by CK1 δ and to a lesser extent by CK1 α , while CK1 γ 1 proved nearly inactive on it. By replacing alanine for critical residues it was possible to show that the main target of CK1 δ is S20 and that E17 is essential for its recognition. If however S15 is substituted by phosphoserine the peptide is readily and almost exclusively phosphorylated at threonine 18 by all the three isoforms of CK1. Similar to the peptide full length p53 is also susceptible to the δ and α but not to the γ 1 isoforms of CK1: kinetics however reveal an affinity for the protein 3-orders of magnitude higher than for the peptide (Km 0.82 μ M and 1.51 mM, respectively). By combining kinetic analysis with immunodetection of individual phosphoresidues S20 was found to display the highest affinity (Km 0.65 μ M) as compared to S9, S15 and S37 (Km: 1.4, 6.7, 15.1 μ M respectively). The E17 to Ala mutation drastically reduces S20 phosphorylation. No phosphorylation of S6, S46 and S392 could be detected. Collectively taken our biochemical data support the concept that non primed phosphorylation of p53 by CK1 is an isoform specific reaction preferentially affecting S20 by a mechanism which is grounded both on a local consensus and on a remote docking site.

27. Opa1 and steroidogenesis during syncytialization of trophoblast cellsMichal Wasilewski^{1,2}, **Luca Scorrano**^{1,2}¹Dulbecco-Telethon Institute²Venetian Institute of Molecular Medicine, Padova, Italy

Optic Atrophy 1 (Opa1) is a key regulator of inner mitochondrial membrane (IMM) structure, and of cristae remodeling during apoptosis in order to facilitate the release of cytochrome c. However, it is unclear if dynamic changes in the structure of the cristae play a role also in processes other than amplification of cell death. In human placenta, production of progesterone, the key pregnancy hormone, is sustained by syncytiotrophoblasts, a population of fused trophoblast cells. This biosynthesis depends on the transport of cholesterol to the IMM, where the first step of steroidogenesis takes place. While in most cells a cholesterol-transporting protein shuttles this lipid from the outer to the inner membrane, syncytiotrophoblasts lack it and require that the IMM directly uptakes cholesterol from the outer. Intriguingly, this is associated with modification of mitochondrial morphology, resembling the morphology of organelles with remodeled cristae. We therefore investigated the possible role of Opa1-dependent changes in cristae shape in steroidogenesis. We observe that in a cellular model of trophoblast syncytialization, the level of specific Opa1 isoforms decreases concomitantly to the onset of mitochondrial morphology changes and progesterone production. Progesterone production, but not syncytialization of trophoblasts, is impaired in cells where levels of Opa1 are kept steady. We propose that remodeling of the IMM driven by changes in Opa1 levels enables efficient transport of cholesterol to the IMM, and therefore allows mitochondria for steroidogenesis.

28. Elongation and turning behaviour of the olfactory sensory neuron axon termini upon activation of the odorant receptor.Ilaria Zamparol and **Claudia Lodovichi**¹¹Venetian Institute of Molecular Medicine, Padua, Italy

In olfactory sensory neurons (OSN) the odorant receptor (OR) is not only involved in detection of odors but also in axonal convergence, although the molecular mechanism underpinning the latter function remain largely unknown. In a previous study we found that the OR at the growth cone is capable of binding odors and coupled to local increases of cAMP and Ca²⁺. These results strength the hypotesis that the OR at the axon termini can act as an axon guidance molecule. Indeed it has been shown that the levels of cyclic nucleotides and Ca²⁺ play a critical role in the process of elongation and turning of the axon termini. To assess whether gradients of cAMP and or Ca²⁺ can affect the behaviour of the OSN growth cone, we create gradients of pharmacological (forskolin and IBMX) and physiological stimuli (odors) capable of modulating the level of cAMP and Ca²⁺ in the growth cone and we analyzed, in real time imaging, the behaviour of the OSN axon termini. Preliminary data indicated that focal gradient of forskolin and odors can affect the elongation and turning of the growth cone of OSN. We found that odors can bind the OR at the growth cone, although we cannot exclude that molecules present in the olfactory bulb (OB) can activate the OR. To address this question we treated axon termini of OSN loaded with Fura 2 AM with bulb extract (lysed, dialyzed, dialyzed processed through gel filtration chromatography). Preliminary data indicated that in these conditions a clear rise in Ca²⁺ is osservable at the growth cone. These results suggest the presence of moleucules, within the OB, capable of binding and activate the OR.

29. Engineering an *in vitro* 3D model of human skeletal muscle myogenesis

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Myofibers, the basic structural elements of skeletal muscle tissue, are formed and regenerated after injury in a unique series of events that include myoblasts adhesion, fusion and differentiation. In this process a key role is played by morphological, mechanical and biochemical stimuli provided by the extracellular environment *in vivo*. Traditional *in vitro* two-dimensional (2D) cell culture systems have been very useful to elucidate early steps of myogenesis. However, cells cultured on flat substrates differ considerably in their morphology, cell-cell/cell-matrix interaction, and differentiation from those in the physiological three-dimensional (3D) environments. The aim of this work is thus to study the human myogenesis in an *in vivo*-like physiological microenvironment through the development of a new *in vitro* 3D cell culture system. To achieve myoblasts spatial organization and alignment, cells were suspended in Matrigel® ($5 \div 10 \cdot 10^6$ cells/ml) and injected into parallel channels (500µm in diameter and 1cm long) fabricated inside a photo-polymerizable polyacrylamide hydrogel (PA HY). The highly structured alignment of skeletal muscle fibers *in vivo* was achieved. Moreover, the PA HY chemical composition was optimized in order to obtain a soft scaffold surrounding myoblasts and myotubes, with mechanical properties (elastic modulus, E) similar to those of the physiological microenvironment of muscle *in vivo* ($E \approx 12 \pm 4$ kPa). Live and dead analysis showed the hydrogel biocompatibility and the possibility of cell culturing within the micro-channels for several days. The hydrogel ensures mass transport of metabolite and cytokines required for the proper myoblasts growth and differentiation. Immunofluorescence and confocal imaging were used for analyzing 3D myogenic marker expression and sarcomeric proteins scaffolding in differentiating myotubes.

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- Abraham, Reimar; 21; 22
 Ahdesmäki, Miika; 22
 Aiello, Rosa; 18
Alberti, Alfredo; 22; 26; 29
 Angelin, Alessia; 27
 Argentini, Carla; 22
 Arrigoni, Giorgio; 18; 37
Ave, Elisa; 25
Babolin, Chiara; 25
Barison, Nicola; 17; 34
 Basso, Emy; 30
Battistutta, Roberto; 18; 31
 Bernard, Alessandra; 25
Bernardi, Paolo; 17; 27; 30
Bertaggia, Enrico; 26
 Bisetto, Elena; 30
 Bisetto, Sara; 30
 Blaauw, Bert; 20; 21; 22; 27
Bonaldo, Paolo; 27
 Bonecchi, Raffaella; 33
 Bonetto, Greta; 18
 Bonisegna, Sara; 26
Bortoletto, Gladis; 26; 29
Bortolozzi, Mario; 27; 28; 32
Bronte, Vincenzo; 19; 30; 33
 Brusilow, William S; 17
 Brustolon, Francesca; 18
 Bustos, Victor H; 37
 Cabrelle, Anna; 19; 25; 36
 Calabria, Elisa; 33
Carafoli, Ernesto; 27
 Cassatella, Marco; 34
 Cendron, Laura; 17; 25; 34; 36
 Cescon, Matilde; 27
 Chiara, Federica; 17
 Churin, Yuri; 17
 Ciciliot, Stefano; 22; 33
 Cimetta, Elisa; 35; 39
 Ciubotaru, Catalin Dacian; 32
Coletto, Luisa; 26; 27
 Costa, Alex; 37
 Cozza, Giorgio; 37
Crispino, Giulia; 27; 28; 32
 Crosetti, Marco; 30
 D'Elios, Mario Milco; 25
 Dabbeni-Sala, Federica; 30
 Dal Pero, Francesca; 22
De Bernard, Marina; 25; 34
 De Coppi, Paolo; 20
 De Palma, Antonella; 33
 De Siati, Daniele; 27; 28
 D'Elios, Mario Milco; 34
 Delpozzo, Federica; 30
 Derra, Carlo; 43
Di Benedetto, Giulietta; 28
Di Maira, Giovanni; 18
Dyar, Kenneth A; 22
Elvassore, Nicola; 20; 35; 39
 Facco, Monica; 25; 36
 Fattovich, Giovanna; 22
 Figallo, Elisa; 20
Flaibani, Marina; 20
Forner, Francesca; 29
 Forte, Michael A; 30
Franceschini, Lisa; 26; 29
 Frezzato, Federica; 25
 Fromme, Tobias; 29
 Gasco, Albert; 30
 Gattazzo, Cristina; 25; 36
 Giorgi, Carlo Alberto; 25
Giorgio, Valentina; 30
 Giulitti, Stefano; 35
 Gnoato, Marianna; 19
Gomes, LÍgia; 20
 Grumati, Paolo; 27
 Guido, Maria; 22
 Gurrieri, Carmela; 19
Kasic, Tihana; 30; 33
 Keller, Gordon; 35
 Klingenspor, Martin; 29
 Kumar, Chanchal; 29
 Lessi, Federica; 25
 Lippe, Giovanna; 30
 Lo Schiavo, Fiorella; 37
Lodovichi, Claudia; 21; 31; 38
Lolli, Graziano; 18; 31
Lorenzon, Paolo; 31
 Luber, Christian; 29
Majumder, Paromita; 28; 32
Mammano, Fabio; 13; 27; 28; 32
 Mammucari, Cristina; 21
Mann, Matthias; 29
 Mantelli, Barbara; 30
 Maraldi, Nadir; 27
Marcolongo, Moira; 22; 26; 29
 Marin, Oriano; 18; 37
 Maritan, Micol; 21
 Martewicz, Sebastian; 35
 Martines, Diego; 26
 Mauri, Pierluigi; 33

- Mazzariol, Valentina; 32
 Meyer, Thomas F; 17
 Milan, Giulia; 21
 Mirandola, Silvia; 26; 29
Molon, Barbara; 33
Mongillo, Marco; 28; 32
Moretti, Irene; 33
Munari, Fabio; 34
 Noventa, Franco; 26
 Pallafacchina, Giorgia; 22
 Palù, Giorgio; 22
 Pantic, Boris; 17
 Papinutto, Elena; 31
Pasqualetto, Elisa; 18
 Patron, Maria; 21
 Peraro, Laura; 22
 Petronilli, Valeria; 30
 Piazza, Francesco A; 19
Piccolo, Stefano; 13
 Pietrobon, Mara; 21
Pinna, Lorenzo A; 18; 19; 31; 37
 Plebani, Mario; 26
Pozzan, Tullio; 21; 28
 Pozzobon, Michela; 20
Quarantini, Sandra; 17; 34
Quotti Tubi, Laura; 19
 Ranchio, Alessandro; 31
 Rasola, Andrea; 17
Realdon, Stefano; 26; 29
 Redolfi, Nelly; 31
 Reggiani, Carlo; 20
 Rodríguez-Hernández, Laura; 28; 32
 Rossi, Carlo Alberto; 20
Ruzzene, Maria; 18; 19; 37
 Sabatelli, Patrizia; 27
Sandri, Marco; 21; 26; 27
Sanjuan Szklarz, Luiza K; 35
 Sarno, Stefania; 18; 31; 37
Sartori, Roberta; 21
 Savino, Benedetta; 33
Schiaffino, Stefano; 22; 32; 33
Sciacovelli, Marco; 17
 Scimemi, Pietro; 27; 28
Scorrano, Luca; 20; 35; 38
 Scribano, Laura; 26
 Sebastiani, Giada; 22
Semenzato, Gianpietro; 19; 25; 36
Serena, Elena; 35; 39
 Seydel, Anke; 27; 28
 Shaik, Munan; 17; 34
Sisinni, Lorenza; 17; 34; 36
 Soldani, Cristiana; 30; 33
 Soriano, Maria Eugenia; 30
Teramo, Antonella; 36
 Tiepolo, Tania; 27
Tosoni, Kendra; 19; 37
 Tothova, Jana; 22
 Trentin, Livio; 25; 36
Ugel, Stefano; 19; 30
 Urciuolo, Anna; 27
Venerando, Andrea; 37
 Vermi, William; 34
Viola, Antonella; 30; 33
 Vitiello, Libero; 20
Wasilewski, Michal; 38
 Young, Bradford; 22
 Zaglia, Tania; 32
 Zambello, Renato; 36
Zamparo, Ilaria; 38
Zanotti, Giuseppe; 17; 25; 34; 36
Zatti, Susi; 35; 39
 Zonta, Francesco; 28
 Zoso, Alice; 39
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