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ABSTRACTS

SESSIONE 1
FUNCTIONAL PROPERTIES OF SKELETAL MUSCLE FIBERS

1.
TRANSCRIPTIONAL SIGNATURES OF SKELETAL MUSCLE FIBER TYPES

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Vertebrates have evolved muscle fiber types with a wide range of energetic capacities and biophysical properties. This heterogeneity is the results of complex regulatory mechanisms mainly involving orchestrated changes in gene expression. Here we investigated the diversity among fiber types by transcriptomic analysis. Fibers have been isolated from soleus and EDL mouse muscles in order to obtain a comprehensive collection of fiber types classified according to myosin heavy chain (MyHC) isoforms. Microarray experiments identified a complete catalogue of differentially expressed genes mainly coding for isoforms of the contractile proteins, metabolic enzymes, or proteins involved in Ca^{2+} homeostasis. Interestingly, cluster analysis revealed 3 major groups of myofibers corresponding to slow, intermediate, and fast phenotypes that only partially fit with MyHC classification. In addition, transcriptional profiles allowed to distinguish the genetic pathways differentially activated with a resolution much higher compared to whole muscle analysis. The correlation between this data and miRNA expression profiles will permit to better understand the complexity of the transcriptional circuits involved in myofiber type specification and this could be useful to shed light on the relationship of skeletal muscle fiber type to certain chronic disease, like obesity and insulin resistance.

2.
MODIFICATIONS IN FUNCTIONAL PARAMETERS AND MYOSIN CONCENTRATION IN AGING AND DISUSE.

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It has been shown that, consistently with the observed loss of specific force in vivo in the elderly, specific force (Po/CSA) of identified types of atrophic muscle fibers of sedentary and

immobilized elderly subjects (EL) is lower than that of young (YO) healthy subjects and that a major determinant of such loss could be a lower myosin concentration within individual muscle fibers. It is unclear if this phenomena occurs in disuse muscle atrophy in YO healthy subjects and in normally active EL subjects. We performed two studies in which torque and muscle volume of the quadriceps muscle were determined in vivo; cross sectional area (CSA), Po/CSA and myosin concentration of isolated muscle fibers from biopsy samples of vastus lateralis were determined in vitro. Study 1: 4 YO subjects were subjected to 4 weeks unilateral lower limb suspension (ULLS); isometric torque and muscle volume by MRI were lower (21% and 10% respectively) post-ULLS compared to pre-ULLS and increased (32% and 15%) post-2 weeks recovery. CSA and Po/CSA of muscle fibers were lower (15% and 13%) post-ULLS compared to pre-ULLS. Study 2: specific force and volume by MRI were lower (18% and 27%) in normally active EL subjects compared to YO controls; muscle fibers from EL subjects had a 14% in CSA and a 25% of Po/CSA decrease compared to YO subjects. The myosin concentration analysis was unchanged in EL subjects. In ULLS subjects myosin concentration is decrease post suspension with an almost complete recover after 3 weeks retraining. Our data indicate that, in moderate atrophy, other mechanism such as post-translational modification may represent the determinant of force loss.

3.
CROSSBRIDGE PROPERTIES DURING FATIGUE IN MAMMALIAN MUSCLE FIBRES

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During periods of intense exercise, tetanic force decreases by up to 15% during the first minute and this is then followed by a phase during which force remains stable or declines slowly until finally force declines rapidly. The initial phase of force loss in muscle fatigue is attributed mainly to the increased intracellular [Pi] from ATP hydrolysis. Increased [Pi] could depress the average force of the individual crossbridges or the number of attached crossbridges or both. The present experiments were made to investigate this point in single fibres or small fibre bundles isolated from *flexor digitorum brevis* (FDB) of C57BL/6 mice at 22-24ºC. Preparations were stimulated every 1.5 sec for 105 consecutive tetanic contractions at 24°C and force and stiffness were measured by applying small sinusoidal length oscillations at 2.5 kHz or 4 kHz frequency. Stiffness data were corrected for tendon and myofilament compliances to isolate the stiffness of the crossbridge ensemble. The results showed that the 20% force decrease occurring after 20 tetani (early fatigue) was accompanied by a much smaller stiffness fall and by an increased rate of both tetanus rise and relaxation. Data analysis showed that early fatigue is mainly due to a reduction of the average crossbridge force. This can be explained by a shift to the left of the reactions AM.ADP.Pi ↔ AM*.ADP.Pi ↔ AM*.ADP + Pi, caused by the accumulation of Pi, which pushes crossbridges into the pre-power stroke state (stiffness generating). Increased [Pi] would also explain the faster tetanic tension rise and relaxation found here and reported previously in skinned preparations and predicted by reaction kinetics.

4.

EXPLORING THE MECHANISMS THAT CONTROL THE ADAPTIVE POTENTIAL OF SKELETAL MUSCLE FIBERS

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A remarkable property of skeletal muscle fibers is their ability to adjust their molecular, functional, and metabolic properties in response to developmental and environmental stimuli. This adaptive potential results in fiber type transitions. As skeletal muscle remodeling occurs frequently both in physiological and pathological conditions, understanding how the cooperation between multiple signaling pathways and the coordinated expression of protein families produce the present variety of muscle fiber types is of great importance. We applied genomic analyses to muscles undergoing plastic physiological changes, since these conditions can markedly and rapidly induce the activation of specific genetic programs. The mouse EDL muscle was subjected to chronic low-frequency stimulation (CLFS) that causes a transition of fiber types but not changes in muscle size. To identify earlier master regulator proteins, microarray gene expression profiles in EDL stimulated muscles were collected after 6 and 12 hours of electrical stimulation. Then, several putative master switch genes were identified. Now we are scaling down this phenotypic approach to the single fiber level in order to reveal complete set of genes corresponding to fiber specific 'expression modules' that could explain how muscle fibers can adapt their contractile properties in response to stimuli.

SESSIONE 2
EXCITATION-CONTRACTION COUPLING

5.

OREXIN A (OXA) ACTIVATES STORE OPERATED CA^{2+} CHANNELS OF MOUSE DUODENUM FIBRES

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Orexin A (OXA) has been reported to influence gastrointestinal motility acting at both central and peripheral neural levels. Recently, it was demonstrated that OXA, in addition to its neurally-mediated influences on gastrointestinal motility, exerts direct muscular effects on the mouse duodenum. OXA determines a transient increase of doudenal motility related to and increase of Ca2+ entry. The primary Ca^{2+} influx increased by OXA is due to receptor operated channels (ROCs) activation and the enhancement of T- and L-type Ca^{2+} channels opening (Squecco et al., J. Physiol., 2011)..
The aim of this work was to evaluate if the increase of intracellular [Ca^{2+}] may, also, be due to an increase of Ca^{2+} released from the sarcoplasmic reticulum in turn inducing a Ca^{2+} influx through store operated Ca^{2+} channels (SOCs).
Experiments used high resistance, 60-70 MΩ, conventional microelectrodes insert in a single fibre of a longitudinal duodenal smooth muscle preparation. The occurrence of the SOC current was assessed in voltage-clamp by evaluating the presence of 2-APB-sensitive currents

and by measuring currents in response to voltage-ramps ranging from -120 to 50 mV over a period of 0.5 s. For analysis, the average of two ramps elicited in high-TEA solution, to block ROC, was used for leak subtraction for the subsequent current records after adding Thapsigargin (Tg), 1μM, to deplete the Ca^{2+} stores, followed by the adding of OXA to evaluate the changes of SOC current induced by OXA. The potentiating action of OXA on SOC current was confirmed by the increased of the 2-APB-sensitive currents and of the voltage-ramp currents after OXA treatment in the presence of Tg.

6.
JUNCTOPHILINS INTERACTIONS IN SKELETAL MUSCLE

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The sarcoplasmic reticulum (SR) of skeletal muscle is dedicated to the regulation of intracellular Ca^{2+}. Within the SR, two regions can be distinguished: the terminal cisternae and the longitudinal tubules. Two terminal cisternae facing a t-tubule form the so-called triadic junction, fundamental for the excitation-contraction (e-c) coupling. During e-c coupling the depolarization signal is transmitted from the voltage sensor DHPR to RyR1 located in SR terminal cisternae, causing its opening and Ca^{2+} release.
The spacing between T-tubules and terminal cisternae is maintained by a class of proteins named Junctophilins (JPs). Four JPs subtype with different tissue-specific distribution have been identified: skeletal muscle co-express JP1 and JP2, while in cardiac and smooth muscles is present only the JP2 isoform. JPs anchor the SR/ER to the plasma membrane contributing to the formation and stabilization of triadic junctions. In addition to this structural role, JPs are involved also in Ca^{2+} homeostasis: JP2 interactions with TRPC3 and with RyR2 has been found to regulate store-operated Ca^{2+} entry and intracellular Ca^{2+} release activity, respectively.
We recently observed that in skeletal muscle JP1 and JP2 interact with DHPR through a region spanning amino acids 230-369 of JP1 and amino acids 216-399 of JP2.
Moreover, studies in C_2C_{12} cells knockdown for both JP1 and JP2 revealed that DHPR and RyR signals were rather diffused and less organized in cluster. In agreement, functional experiments showed that down-regulation of JPs resulted in a reduction of intramembrane charge movement and L-type Ca^{2+} current accompanied by a reduced number of DHPRs at the plasma membrane, while there was no substantial alteration in Ca^{2+} release from the SR. Altogether these data suggest that JP1 and JP2 can facilitate the assembly of DHPR with other proteins of the e-c coupling machinery. To further investigate other JPs interactors, we are using the yeast two-hybrid technique with different portions of JP1 and JP2.

7.

THE LESSON OF CALSEQUESTRIN-1 ABLATION IN VIVO: MUCH MORE THAN A BUFFER, AFTER ALL.

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Calsequetrin-1 (CASQ1), the main sarcoplasmic reticulum (SR) Ca^{2+} binding protein, plays a dual role in skeletal fibers: a) provides a large pool of rapidly-releasable Ca^{2+} during excitation-contraction (EC) coupling; and b) is involved in modulating the activity of ryanodine receptors (RYRs), the SR Ca^{2+} release channels. We have generated a mouse lacking CASQ1, instrumental to collect further information about CASQ1 role in muscle. Contrary to initial expectations, the CASQ1-null mutation was not lethal and was compatible with normal motor activity, in spite of moderate muscle atrophy. Lack of CASQ1 determined, though, profound remodeling of the EC coupling apparatus and impairment of contractile function, i.e. prolonged time course, fast SR depletion, and inability of muscles to sustain prolonged tetani. All modifications were more evident in EDL than in Soleus muscles, possibly because the latter expresses higher amounts of CASQ2. Surprisingly, we also discovered that CASQ1-null mice were susceptible to trigger lethal episodes in response to halothane and heat, a phenotype remarkably similar to human malignant hyperthermia (MH) and environmental heat stroke (EHS). With increasing age CASQ1-null animals also develop a myopathy, which is likely initiated by abnormal mitochondrial proliferation and excessive oxidative stress, as shown by a) decreased GSH/GSSG ratio and b) increased production of mitochondrial superoxide flashes at physiological temperature. Finally, we tested the hypothesis that excessive Store Operated Ca^{2+} Entry (SOCE) could play a central role in the pathogenesis of MH: in myotubes lacking CASQ SOCE current was indeed greater compared to WT.

8.

GRADUAL FORMATION AND ACCUMULATION OF TUBULAR AGGREGATES IN FAST-TWITCH MUSCLE FIBERS: SERCA AND CALSEQUESTRIN INVOLVEMENT.

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Tubular aggregates (TAs), ordered arrays of elongated sarcoplasmic reticulum (SR) membranes, are present in skeletal muscle fibers from patients with various myopathies. TAs have been also described in ageing wild type (WT) mice, where they display a dependence on sex (male), and fiber type (fast twitch). The mechanism(s) leading to TAs formation are, though, not yet clear. Here, we investigated the sequential stages leading to maturation of TAs in extensor digitorum longus (EDL) from male WT and calsequestrin knockout (CASQ-null) mice. Initially, the SR Ca^{2+} binding protein CASQ accumulates specifically at the I band level of the sarcomere causing swelling of free SR cisternae. In the second stage, the enlarged SR sacs mature into multiple and longitudinally oriented tubules containing CASQ, which

elongates into the A band. Tubules gradually acquire cylindrical shape and uniform size, apparently in concert with partial crystallization of sarco(endo)plasmic reticulum Ca^{2+} ATPases (SERCA) on its surface, as suggested by freeze-fracture (FF) evidence. Finally, multiple small TAs associate to form fewer mature aggregates of very large size. Interestingly, in fibers from CASQ1-knockout mice abnormal aggregates of SR tubules have different conformation and never develop into ordered aggregates of straight cylinders, possibly due to lack of CASQ accumulation. Based on these results, we suggest a novel interpretation of TAs presence and we provide a fuller understanding of the critical processes and contributing proteins in skeletal muscle aging.

9.

EXERCISE COUNTERACT THE AGE-RELATED DECAY OF EC-COUPLING AND MITOCHONDRIAL APPARATUSES IN HUMAN SKELETAL MUSCLE.

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At the most basic level, muscle contraction requires Ca^{2+} and ATP and, thus, is under direct control of two major intracellular organelles: Ca^{2+} release units (CRUs) and mitochondria. Ca^{2+}-mediated communication between these two myoplasmic organelles is thought to be required for efficient ATP production. We have recently shown that: a) mitochondria in striated muscle are structurally tethered to CRUs; and b) ageing is associated with a significant decrease in frequency of both CRUs and mitochondria.
In order to investigate if the regular physical exercise can reduce the age-related decay of EC coupling and metabolic machineries, we structurally analyzed biopsies from Vastus Lateralis collected from two groups of male subjects: elderly-sedentary (n=7, 67-80y) and age matched individuals which had practiced endurance and/or resistance training for many years (n=11, 65-79y). Our findings shows that: i) the frequency of CRUs is significantly higher in elderly-trained (21.6/100 μm^2) than in elderly-sedentary (15.2/100 μm^2); and b) even greater is the increase in the number of mitochondria: 51.9/100 μm^2 vs. 11.1/100 μm^2 in trained and sedentary, respectively. We have also assessed the positioning of mitochondria with respect to myofibrils: a higher percentage of mitochondria is positioned at the A band in elderly-sedentary (12.6%) compared to elderly-trained (6.3%). The increase of CRU and of the mitochondrial population result in a dramatically augmented frequency of CRU/mitochondrion couples, which functionally will mean more efficient ATP production: 11.5/100 μm^2 in trained vs. 2.5/100 μm^2 in sedentary.

SESSIONE 3
CELL BIOLOGY OF MUSCLE DISEASES-I

10.
IL-6 IS A PIVOTAL PATHOGENIC FACTOR AND A POTENTIAL THERAPEUTIC TARGET OF MUSCULAR DYSTROPHY

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Animal research on muscular dystrophy relies on the use of the mdx mouse strain. Skeletal muscles of mdx mice undergo extensive necrosis early in life. However, in contrast with human Duchenne muscular dystrophy (DMD), the affected muscle rapidly regenerates and regains structural and functional integrity, presumably due to the proliferative capacity of murine satellite cells. One of the pathogenic events associated with dystrophin−deficient muscles is a chronic inflammatory response. A vast body of evidence supports a pivotal role of inflammation in the progression of muscle loss. Among inflammatory cytokines, interleukin−6 (IL-6) is a pivotal mediator in the induction and maintenance of muscle inflammation. IL-6 plays a major role in orchestrating recruitment and retention of activated mononuclear cells in inflamed tissues, inducing the transition from an acute neutrophil infiltrate to a chronic type mononuclear cell infiltrate.
In this study, we provided evidences that increased levels of IL−6 exacerbate the pathological phenotype of adult (24 weeks of age) mdx dystrophic mouse, increasing muscle damage and inflammatory response, similar to that observed at early stage of the diseases and in DMD human patients, promoting muscle atrophy and reducing the life span of dystrophic mice.

11.
THE NATURAL OCCURENCY OF DYSTROPHIN POSITVE MUSCLE FIBRES IN DYSTROPHIC MUSCLE: A TIME-COURSE STUDY IN THE MURINE MODEL OF DUCHENNE MUSCULAR DYSTROPHY

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The presence of revertant fibers in *mdx* mice is a factor that complicates the evaluation of any therapeutic approach for dystrophin restoration. In fact, even though on average the number of dystrophin-positive fibers in untreated animals is fairly low, the inter-individual variability can be quite significant and some animals display several dozens of positive fibers per muscle section. Therefore, in an experimental setting for the evaluation the efficiency in restoring the dystrophin synthesis, in which the levels of dystrophin restoration could have potentially been low, the baseline of spontaneous reversion had to be taken into account for the correct interpretation of the results.
In the literature, no quantitative data regarding the average number of revertant fibers in different *mdx* mice muscles was available, therefore we carried out immunofluorescence analysis in a sample of 40 untreated *mdx* animals in order to calculate the average number of revertant fibers in 11 different muscles. The animals were divided into 4 age groups (group 1:

6-8 weeks; group 2: 6 months, group 3: 12 months, group 4: ≥18 months) to assess the possible correlation between the total number of revertant fibers and the age of the animals. In order to gain some indications about the biological mechanisms underlying the formation of revertant fibers, we also evaluated if and how the formation of clusters of revertant fibers correlates with animals' ages. Last but not least, we are now attempting to study the transcripts leading to dystrophin re-expressions within single clones of satellite cells obtained from *mdx* mice.

12.
HISTONE DEACETYLASES: CRUCIAL REGULATORS OF SKELETAL MUSCLE HOMEOSTASIS

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Histone deacetylases (HDACs) regulate various biological processes by repressing gene transcription and by affecting the acetylation status of many proteins, thereby regulating their activity. To define the specific roles of HDACs in vivo, we used a genetic approach and generated mice mutant for HDAC1 and 2, or HDAC4 and 5 specifically in skeletal muscle. Mice lacking both HDAC1 and HDAC2 in skeletal muscle (HDAC1;2 dKO) show a block in autophagy flux which causes perinatal lethality of about 40% of mutant mice, probably due to an impairment in energy production. The remaining 60% of HDAC1;2 dKO develop a progressive myopathy characterized by muscle degeneration and regeneration, and a shift in metabolism toward a more oxidative status. Conversely, ectopic expression of HDAC1 and HDAC2 in skeletal muscle is sufficient to induce autophagosome formation and promote autophagy flux. Strikingly, feeding HDAC1;2 dKO mice with high fat diet prevents the onset of myopathy and releases the block in autophagy flux. In contrast to HDAC1;2 dKO mice, mice mutant for HDAC4 and 5 in skeletal muscle (HDAC4;5 dKO) show no obvious phenotype under normal conditions. However, when denervated, HDAC4;5 dKO mice are resistant to muscle atrophy. We show that HDAC4;5 dKO mice are unable to up-regulate myogenin upon denervation, and therefore do not over-express the muscle E3 ubiquitin ligases, MuRF1 and atrogin-1, which promote muscle proteolysis and atrophy. Collectively, these studies reveal highly specific functions of individual HDACs in skeletal muscle turnover and homeostasis. Further elucidation of the functions of HDACs in vivo will facilitate the development of HDAC inhibitors to ameliorate muscular diseases.

13.
CHARACTERIZATION OF MECHANISMS CAUSING MYOPATHY ASSOCIATED TO THE NEUTRAL LIPID STORAGE DISEASE (NLSDM).

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The Neutral Lipid Storage Diseases with Myopathy (NLSDM) is a newly recognized disease caused by massive accumulation of triglycerides in the muscles due to mutations in the ATGL gene. The pathogenic mechanisms underlying muscle damage in NLSDM is completely unknown. Taking advantage of ATGL knockout (ATGL-/-) mouse model (kindly

provided by Dr. Zechner) we have investigated at histological, functional, and molecular level the potential pathogenic events associated with NLSDM. Histological analysis, performed on different muscle types (tibialis anterior, soleus, EDL, diaphragm) of 2.5 months old mice, revealed a reduction in the cross sectional area of the single myofibres in ATGL-/- EDL and tibialis anterior muscle, compared to WT. This was associated with the presence of several myofibres with central nuclei, indicating either the activation of a compensatory, but defective, regenerative mechanism or the alteration in the maturation process. To further address this point, we have analyzed the expression markers of the myogenic program such as desmin (proliferation) and myogenin (commitment), which expression normally decrease at the stage of mature muscle. RTqPCR and western blot analysis revealed a significant increase in gene and protein expression of both desmin and myogenin in ATGL-/- diaphragm muscle compared to WT. Moreover we have analyzed, by RTqPCR, the expression of the subunit gamma of acetylcholine receptor that is undetectable or low in innervated adult active muscle. We noted an up regulation of the expression of this subunit in ATGL -/- diaphragm muscle compared to WT, indicating probably an alteration of the neuromuscular junction. Functional analysis revealed a decrease in the specific and maximum force of EDL, but not soleus, muscle of ATGL-/- mice compared to WT. This observation correlates with the histological analysis. These data suggest that different muscles are differently affected by the lack of ATGL.

14.
MECHANISMS UNDERLYING EXERCISE IN THE CONTEXT OF CANCER CACHEXIA

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Cachexia is characterized by severe muscle atrophy and fatigue, thus accounting for poor prognosis and worsening patients' quality of life. To identify cellular and molecular mechanisms of cancer cachexia, we exploited mice bearing ectopically-implanted with C26 colon carcinoma as an experimental model of cancer cachexia. These mice display all the classical features of cachexia, including premature death, severe weight loss, and muscle atrophy and weakness. In addition, we induced mice to exercise, by hosting them in wheel-equipped cages. In mice which start to exercise at the onset of the pathology we observed beneficial effects, including rescue of muscle wasting and resistance, appetite stimulation and increased life span. We propose a gene expression-based mechanism, both within the fibers and the surrounding satellite cells, to explain the exercise-mediated rescue of muscle wasting. In particular, we note that exercise promoted protein synthesis by mTOR activation and attenuated protein degradation by downregulating Atrogin1 in muscle fibers from tumor-bearing mice. We also show that the muscle microenvironment was affected in cachexia, inasmuch as muscle fiber damage occurred, suggesting that repair mechanisms were activated in an attempt to cope with fiber loss. Satellite cell proliferation was indeed stimulated in cachectic muscle but their fusion into muscle fibers was hampered. Failure to downregulate Pax7 expression is responsible for the uncoupling between satellite cell activation and differentiation in cachexia, however, exercise downregulates Pax7 expression. We found that tumor-bearing mice display elevated levels of circulating pro-inflammatory cytokines (such as IL-6) and decreased levels of pro-myogenic factors (such as Vasopressin). Thus, we are

currently investigating whether exercise alters the balance between pro-inflammatory cytokines and pro-myogenic factors.

SESSIONE 4
CELL BIOLOGY OF MUSCLE DISEASES-II

15.
MIGRATING TUMOR CELLS AND MUSCLE WASTING IN GIST TRANSPLANTED NUDE MICE

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Cachexia is a fatal underestimated syndrome consequence of many chronic and acute disease states, including cancer, chronic kidney disease, AIDS, sepsis and burns. Skeletal muscle atrophy is a main feature of cachexia. Muscle wasting is due to a catabolic pathway activation which leads to MuRF1 and Atrogin1 upregulation, responsible for muscle protein degradation[1]. Several proinflammatory cytokines have been implicated in the pathogenesis of muscle wasting (TNF-α, IL-1β, IL-6, IFN-γ, TGFβ)[2] as potential mediators of muscle atrophy[2]. However, crosstalk between proinflammatory *stimuli* and ubiquitination of muscle proteins is still not fully understood. We have recently developed a new animal model of cachexia based on subcutaneously injection of human gastrointestinal stromal tumour (GIST) cells in nude mice. Surprisingly, GIST treated nude mice developed an inflammatory-independent cachectic phenotype. Moreover, several tumor cells were found fused in cachectic muscle fibers. This phenomenon occurred without metastasis insurgence. Our data strongly suggest that GIST cells can migrate from tumor mass toward skeletal muscles and fuse within the fibers. In vitro analysis performed with cells derived from different tumors recapitulate the migratory properties observed with GIST. We postulate a general mechanism by which a specific cellular subpopulation within the tumor can be implicated in GIST-induced cachexia.

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[2]Acharyya S. Clin Cancer Res. 2007 Mar 1;13:1356-61.

16.
THE MECHANISMS OF SKELETAL MUSCLE ATROPHY FOLLOWING 35-DAYS BED REST

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Ten healthy sedentary young subjects were subjected to 35-days Bed rest (BR). Needle biopsy samples of vastus lateralis were collected pre-BR, post-7d and post-35d BR. At 35 days BR, but not at 7 we found cellular atrophy, decrease of myosin content and a shift towards MHC-

2X myosin isoform. The general down-regulation of myofibrillar proteins found both at 7 and 35 days BR suggest an early unbalance between protein synthesis and degradation. For this reason we studied by RT-PCR and Western Blot analysis intracellular signaling pathways involved in protein turnover (ubiquitin-proteasome and AKT/mTor) and in autophagy (Beclin1, P62); the up-regulation of Beclin1 post-35d BR indicate that the autophagy system is active and could contribute to progression of atrophy. At 35-days BR atrogin-1 and MuRF1 were not activated suggesting that the ubiquitin proteasome could not be a major determinant of the atrophy progression. Several proteins involved in antioxidant defence systems was down-regulated post 7 and 35d BR and NRF2, a sensor of cell redox balance, was up-regulated post 35d BR therefore the oxidative stress could occurs and cause disuse atrophy. Finally we found a down-regulation in proteins involved in cellular energy production and an expression change of PGC-1α and SREBP1 master controller of muscle metabolism. A decrease in PGC-1 α per se as well as an impairment of metabolism might contribute to muscle atrophy generating insulin resistance. Moreover, as mitochondrial dysfunction produces ROS, PGC-1 α down-regulation might be cause redox unbalance, which in turn could be responsible of protein oxidation and of an impairment of antioxidant defense systems.

17.

THE TIME COURSE OF DISUSE ATROPHY IN A SLOW AND FAST MUSCLE OF HINDLIMB UNLOADED MICE.

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In order to understand the mechanisms underlying disuse atrophy we studied the adaptation of fast e slow muscles (Gastrocnemius and Soleus) at different time of suspension (3 and 7 days).The time course of skeletal muscle adaptations in a fast and a slow muscle (Gastrocnemius and Soleus) of Hindlimb Unloaded (HU) mice at 3 (n=6 animals) and 7 (n=6) days. Gastrocnemius showed a similar decrease of CSA (~18%) at 3 and 7 days of suspension. We found an up-regulation in gene expression of ubiquitin-proteasome system (Murf1), autophagy system (P62) and oxidative stress (Nrf2, Mt1) at 3 days and an increase in SOD1, Catalase and Hsp70 (proteins of antioxidant defense systems) at 3 and 7 days. Markers of oxidative and glycolitic metabolism were also studied, but they were unchanged. Soleus showed type 2A muscle fibers atrophy at 3 (6%) and at 7 days (14%); whereas 1 type fiber atrophy was found at 7 days (9%) only. In Soleus Murf1, Nrf2, Mt1 were over-expressed at 3 days; the expression and the expression of PGC1α, the main coordinator of oxidative metabolism was down regulated at 3 days. In a previous study we showed metabolic impairment after 14 days of suspension in soleus. The latter observation combined with the present data suggest that metabolic impairment could contribute to atrophy process.

18.

COMBINATION OF ENDURANCE TRAINING AND ERYTHROPOIETIN PREVENTS CANCER-INDUCED MUSCLE ALTERATIONS

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Cancer cachexia is a syndrome characterized by loss of skeletal muscle protein, depletion of lipid stores, inflammation, anorexia, weakness, and perturbations of the hormonal homeostasis [1]. In addition to nutritional approaches, exercise training (EX) was proposed as a suitable tool to manage cachexia, in view of recent observations suggesting that decreased physical activity plays a role in the onset of muscle atrophy in cancer patients [2]. Aim of the present work was to verify if endurance training coupled to erythropoietin (EPO) administration could prevent the wasting process in Lewis Lung carcinoma(LLC)-bearing mice. LLC mice were got used to a treadmill for 5 days before tumor injection (10^6 cells s.c.) and then exercised 5 days/week (45 min,14m/min). At the end of the experimental protocol (28 days after tumor implantation), tumor-bearing (TB) mice were characterized by a marked body and skeletal muscle weight loss, resulting in impaired muscle strength. Moreover, tumor growth induced a dramatic anemia (50% hematocrit reduction) and, likely consequently, heart hypertrophy. The combination of EX with EPO (100U/mouse, i.p., weekly) partially counteracted tumor-induced hematocrit reduction and prevented heart hypertrophy. Although in the EX-EPO group skeletal muscle mass was similar to the sedentary TB mice, grip strength was significantly increased. Ultrastructural analysis of the EDL and soleus muscles of TB mice showed inter-myofibrillar mitochondrial swelling and reduced sub-sarcolemmal glycogen storage; both alterations were prevented in EX-EPO mice. Overall, the present data suggest that endurance exercise can be an effective tool to be included in combined therapeutic approaches against cancer cachexia. Further ongoing studies will unravel the molecular mechanisms underlying the reported effects.

References:
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19.

AUTOPHAGY IS REQUIRED TO MAINTAIN MUSCLE MASS

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The ubiquitin-proteasome and autophagy-lysosome pathways are the two major routes for protein and organelle clearance. In skeletal muscle both systems are under FoxO regulation and their excessive activation induces severe muscle loss. Although altered autophagy has been observed in various myopathies, the specific role of autophagy in skeletal muscle has not

been determined by loss-of-function approaches. Here, we report that muscle-specific deletion of a crucial autophagy gene, Atg7, resulted in profound muscle atrophy and age-dependent decrease in force. Atg7 null muscles showed accumulation of abnormal mitochondria, sarcoplasmic reticulum distension, disorganization of sarcomere and formation of aberrant concentric membranous structures. Autophagy inhibition exacerbated muscle loss during denervation and fasting; moreover aged Atg7Ko mice show even worse myopathic features. Thus autophagy flux is important to preserve muscle mass and maintain myofiber integrity. Our results suggest that inhibition/alteration of autophagy can contribute to myofiber degeneration and weakness in muscle disorders characterized by accumulation of abnormal mitochondria and inclusions bodies.

SESSIONE 5
MECHANISMS OF SIGNAL TRANSDUCTION-I

20.
SPHINGOSINE 1-PHOSPHATE EXERTS PRO-MIOGENIC AND PRO-MIGRATORY ACTIONS IN MURINE SATELLITE CELLS

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Regeneration of mammalian skeletal muscle following injury is mediated by resident stem cells, named satellite cells (SCs). These cells, are normally quiescent but, after myotrauma, proliferate and differentiate into multinucleated myofibers. The specific microenvironment, named cell niche, controls SC quiescence and is involved in their activation during muscle regeneration.
Sphingosine 1-phosphate (S1P) is a bioactive lipid involved in the regulation of many biological processes, such as cell proliferation, survival and migration. S1P exerts most of its actions by binding to five specific G protein-coupled receptors, namely S1P1-5. We previously demonstrated that in C2C12 myoblasts S1P induces myogenic differentiation through the ligation to S1P2.
Here, to further investigate the role of S1P in muscle repair, we characterized the biological action of S1P in SCs isolated from selected muscles of C57BL/6 mice. Interestingly, 1 µM S1P was found to enhance SC proliferation, evaluated by [^{3}H]thymidine incorporation assay. Moreover, the investigation of S1P receptor involvement in S1P-induced proliferation, accomplished by different means, such as pre-treatment with pharmacological agonists and antagonists and RNA interference, highlighted a major role for S1P3 and a partial involvement of S1P2. In addition, the effect of S1P on SC migration was evaluated by performing wound healing and Boyden chamber assays: 20 nM S1P was identified as pro-migratory cue, mainly acting through S1P1 ligation.
These findings increase the knowledge on the role of S1P in skeletal muscle regeneration, opening new perspectives for individuating new pharmacological tools to improve the regenerative capacity of skeletal muscle.

21.
THE TRANSCRIPTION FACTOR SNAI1 REGULATES EARLY EVENTS OF MYOGENIC DIFFERENTIATION.

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SNAI1 is a transcription factor best known by its ability to trigger epithelial-to-mesenchymal transitions (EMTs) during embryonic development and tumor progression. Snail encodes zinc finger proteins, which bind to the "E-box" motifs (CANNTG), and represses the transcription of several target genes. Although best known as a major EMTs inducer, Snail is involved in a broad spectrum of biological functions, such as cell differentiation, cell motility, cell cycle regulation, and apoptosis. In the mouse embryo the expression of the SNAI1 transcript was observed in the myotome and sclerotome as well as in the limb bud primordium from 9.5 d.p.c., but the role that SNAI1 plays in muscle development is not known. Here we show that

SNAI1 is expressed in C2C12 and primary mouse myoblasts at both RNA and protein levels and that SNAI1 RNAi knockdown in C2C12 reduces myoblasts differentiation and myotubes formation. On the contrary, the induction of a chimeric ER-SNAI1 increases myotubes formation. We also observed that SNAI1 expression is regulated by serum and strongly decreases after induction of differentiation. We show that in proliferating myoblasts SNAI1 is regulated by cell confluence and by factors involved in muscle differentiation such as WNT3A, ATRA and Insulin. Preliminary analysis of gene expression in SNAI1 knockdown cells identified putative SNAI1 target genes, and in particular the tetraspanin protein CD9, a transmembrane 4 superfamily (TM4SF) protein involved in the muscle cell fusion and myotube maintenance. Taken together, these data suggest an important role of SNAI1 in myogenic differentiation.

22.

INHIBITION OF NOTCH PATHWAY IMPAIRS OSTEOGENESIS WHILE ENHANCES MYOGENESIS OF PERICYTE-DERIVED CELLS

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Notch signaling has a crucial role in cell fate determination during embryogenesis and in postnatal life, being involved in cellular processes such as proliferation, progenitor specification, differentiation and apoptosis. The role of Notch signaling in mesenchymal differentiation has been investigated, *in vivo* and *in vitro*,using several cellular models. Pericyte-derived cells represent a multipotent cell population characterized by robust mesenchymal differentiation abilities.
By using DAPT (N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester), a Notch pathway inhibitor, we analyzed both proliferation and mesenchymal differentiation potential of skeletal muscle pericyte-derived cells.
Our results indicated that DAPT treatment reduces proliferation of pericyte-derived cells. The inhibition of Notch pathway markedly inhibited osteogenic differentiation while had no effect on adipogenic differentiation. When myogenic differentiation was assayed both in spontaneous differentiation and in co-culture with C2C12, DAPT treated cells displayed an enhanced myogenesis. Interestingly, we observed that Notch inhibition during osteogenic differentiation was sufficient to block terminal differentiation, while up-regulation of myogenic efficiency was detected only in cells grown in the presence of DAPT for at least two passages before induction of differentiation.
Our results could suggest that in pericyte-derived cells Notch signaling may be involved both in progenitor specification and in terminal differentiation.

23.

RELEASE OF MICRO-VESICLES DURING C2C12 MYOGENIC DIFFERENTIATION PROCESS

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Skeletal muscle is highly plastic tissue able to adapt to different stress, in part due to its remarkable regenerative capacity. Many growth factors have been implicated in the regulation of myoblast proliferation and differentiation to promote muscle regeneration and repair. Tissue specific micro-vesicles (MVs) are exciting candidates for novel signalling factors, in fact, MVs are able to carry proteins, mRNAs and miRNAs contributing to modifying the microenvironment.
The C2C12 cell line was used as a model to investigate a potential role of MV secretion in the myogenic differentiation process. Western blotting analysis, using antibodies against well-defined MV markers, showed that Tsg101, Hsp60 were more abundant in T0 MV extracts than T2; while an opposite trend was found for LAMP-1 and RAB5. Histone 2B appeared only at T1 and T2 demonstrating the presence of apoptotic bodies. Furthermore, TEM analysis of MV size distribution showed that myoblasts released MVs of about 42±8 nm in diameter, while differentiating cells released significant larger MVs.
These data clearly demonstrate that during differentiation myoblasts release a complex mixture of MVs.
Moreover, it was investigated whether MVs contained the myogenic microRNA miR-1, miR-133a, miR-133b and miR-206. This analysis showed different microRNA ratios in MVs respect to cell body.
These results represent an important step forward the understanding of muscle regeneration process shedding light on an underestimated aspect as MV cell-to-cell communication.

24.

THE PRO-MYOGENIC ENVRONMENT PROVIDED BY WHOLE ORGAN SCALE ACELLULAR SCAFFOLDS FROM SKELETAL MUSCLE

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With the aim to generate tissue-engineered, implantable devices, we decellularized skeletal muscles from murine hindlimb. To analyze the *in vivo* biological activity of the acellular scaffold we replaced homologous muscle in syngeneic mice with an acellular scaffold. The implants were analyzed in regard to histocompatibility, bioactivity and degradability at different times from transplantation.
The procedure to generate acellular scaffold maintains the molecular components and the three-dimensional architecture of the extracellular matrix (ECM). Scaffolds can be stored for several weeks at +4°C or +37°C in tissue culture conditions; however, the storage at +4°C allows a better preservation of the degradation of ECM components. Transplantation experiments show that the grafts are stable for several weeks *in vivo*. The transplanted acellular scaffolds are readily colonized by inflammatory cells and myogenic stem cells that lead to *de novo* formation of muscle fibers. Immunosuppressive treatment enhances myogenesis within the implant.
We demonstrate that the acellular scaffold *per se* represents a pro-myogenic environment supporting de novo formation of muscle fibers, likely derived from host cells with myogenic potential. Our work highlights the fundamental role of this niche in tissue engineering

application and unveils the clinical potential of allografts based on decellularized tissue for regenerative medicine.

SESSIONE 6
MECHANISMS OF SIGNAL TRANSDUCTION-II

25.
CAVEOLIN-1 PROMOTES PROLIFERATION AND CONFERS RESISTANCE TO OXIDATIVE STRESS-INDUCED CELL DEATH IN RHABDOMYOSARCOMA CELLS

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Caveolin-1 (Cav-1) is the major scaffolding protein of *caveolae* and is involved in several processes, including cholesterol homeostasis, endocytosis, regulation of signal transduction, and cell senescence induced by oxidative stress. In cancer, Cav-1 plays a dual role, behaving as tumor-suppressor or tumor-oncogene depending on the tumor type. Rhabdomyosarcomas (RMS) are aggressive paediatric malignancies whose tumor-initiating cells are supposed to be mesenchimal precursors committed to the muscle lineage. Though Cav-1 expression has been significantly detected in immature cells of different RMS histotypes, its contribute in this cancer remains elusive.
In the present work we show that increasing Cav-1 expression levels in different embryonal and alveolar RMS cells promotes sustained proliferation and confers a greater resistance to peroxide hydrogen-induced cell death. Conversely, shRNA-mediated loss of Cav-1 expression affects proliferation and triggers increased RMS cell death in the presence of oxidative stress. Overall, these data indicate Cav-1 as a valuable target for treating RMS.

26.
DIFFERENTIAL MYOGENICITY OF SATELLITE CELLS ISOLATED FROM CANINE MUSCLES OF DIFFERENT EMBRYONIC ORIGIN

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Embryologically, the extraocular (EOMs) and jaw-closer muscles develop from prechordal mesoderm rather than somites (limb and trunk muscles). The MyHc in EOM is different from that seen in limb muscles (Rubinstein *et al*, 2000) and they also have differential sensitivity to diseases. We hypothesized that those differences may also endow the muscle associated Satellite Cells/Muscle Progenitor Cells (MPCs). The MPCs were isolated from canine somitic (SDM) (*vastus lateralis, rectus abdominus, gluteus superficialis, biceps femoris, psoas*) and presomitic (PSDM) (*lateral rectus, temporalis- M fiber, retractor bulbi and masseter*) muscles. We studied: population doubling level (pdl), MPC fusion index, the expression myogenic proteins, telomerase activity and intracellular calcium [Ca2+]i homeostasis.

Population doublings (pdl) data showed that SDM-MPC rapidly undergo senescence and stop to proliferate at average of 20 pdl. Consistent with this result, telomerase activity is higher in PSDM-MPC than SDM-MPC although restricted at early passages. Differences have been found among MPC samples for the expression of early (Pax7, MyoD) and late (MyHC) myogenic markers as indicated by immunofluorescence analysis, allowing SDM-MPC to better fuse and differentiate. Moreover, SDM-myotubes reveled a more efficient functionally differentiation with respect to PSDM-myotubes. Our results showed that PSDM-MPCs elicit a stronger stem cell phenotype compared to somitic ones, since PSDM-MPCs proliferate longer and seems to differentiate less efficiently than SDM-MPCs.

27.

EVALUATION OF THE PROTECTIVE EFFECTS OF NATURAL ANTIOXIDANT COMPOUNDS AGAINST SKELETAL MUSCLE IMPAIRMENT DUE TO AGING

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Skeletal muscle performance is impaired by aging process. We previously found a reduction of resting chloride conductance (gCl) important to sustain excitability (Pierno et al., *FEBS Lett.* 449, 12, 1999) and a reduced activity of the ATP-dependent potassium (KATP) channel, a sensor of cell metabolism (Tricarico and Conte Camerino, *Mol Pharmacol.* 46, 754, 1994). The calcium homeostasis and contractile properties were also modified in terms of cytosolic calcium increase and modification of the mechanical threshold (MT) for contraction, an index of excitation-contraction coupling. At the aim to understand if these modifications can be associated with a oxidative damage, proposed to be one of the cause of sarcopenia, we tested the effects of a olive oil-derived antioxidant MIX containing hydroxytirosol, homovanillic acid and gallic acid on skeletal muscles of 27-months-old rats. At the end of 8-weeks treatment we analyzed the resting gCl and the mechanical threshold for contraction by current clamp and point voltage clamp electrophysiological techniques. The KATP channel activity was determined by patch clamp and calcium homeostasis by using FURA-2 cytofluorimetric technique. Resting gCl was lower in aged animals with respect to the adults and was partially restored in treated animals. The KATP channel activity was significantly increased in treated animals toward the adult value. Also the resting intracellular calcium concentration and MT were restored by the antioxidant treatment, although sarcolemma resting calcium permeability was not modified. Also, the response to caffeine in aged animals treated with MIX was similar to that found in the adult. Red-ox studies showed that the increase of the level of malonil dialdehyde (MDA), as oxidative stress index, was slightly counteracted in the brain of treated rats. These findings suggest the beneficial effect of this compound to ameliorate the skeletal muscle functional decline due to aging. (Regione Puglia PE-004)

28.

REACTIVE OXYGEN SPECIES CONTRIBUTE TO PROMOTE THE ATP-MEDIATED PROLIFERATION OF MOUSE SKELETAL MYOBLASTS

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Adenosine 5′ triphosphate (ATP) is not only the primary energy source of cells; if released, it acts in the extracellular microenvironment, exerting autocrine and/or paracrine regulatory functions. In skeletal muscle reactive oxygen species (ROS) represent important signalling molecules regulating various functions in physiological and pathological conditions. Here, we have investigated the possible trophic effect of ATP in cultured skeletal mouse myoblasts and we have tested the possible role of ROS in mediating the effect of ATP on cell proliferation.
10 μM ATP induced transient intracellular Ca^{2+} oscillations in proliferating mouse myoblasts, both in the presence and in the absence of extracellular Ca^{2+}. The Ca^{2+} response was completely blocked by 100 □M, suramine, a broad-spectrum purinergic P2 receptor antagonist. In parallel, we observed that addition of 10 μM ATP to the medium promoted myoblast proliferation by ~ 20% and suramine inhibited this ATP-induced effect on myoblast growth. Adenosine (10 μM) or the adenosine receptor blocker CGS 15943 (100 nM) did not influence the ATP-mediated effects. Catalase (1200 U ml^{-1}) also prevented the trophic effect of ATP. Furthermore, addition of exogenous H_2O_2 (3 μM) mimicked the ATP effects, inducing a similar increase in myoblast growth.
Our results strongly suggest that exogenous ATP controls mouse myoblast proliferation and that H_2O_2 may play a crucial role in mediating the purinergic receptor cascade.

29.

INTRACELLULAR PATHWAYS REGULATING ER-STRESS-INDUCED AUTOPHAGY IN MUSCLE AND MUSCULAR DYSTROPHY

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The endoplasmic reticulum (ER) is a highly dynamic organelle that synthesizes and folds intraorganellar, secretory, and transmembrane proteins. Disruption of ER homeostasis interferes with protein folding and leads to the accumulation of unfolded and misfolded proteins in the ER lumen. This condition is known as “ER stress”, and it activates autophagy. Stress can be triggered by a number of stimuli that perturb ER function, and it is implicated in the pathogenesis of a variety of human diseases, including neuronal degenerative diseases, such as Alzheimer’s disease, Parkinson’s disease, and diabetes. In skeletal muscle, ER-stress occurs physiologically during myoblast differentiation but also during pathological conditions, such as during SLA or myotonic dystrophy type 1, where a high level of autophagy was described, as well as in DMD. Different molecules are involved in ER-stress induced autophagy such as eIF2a, JNK, CaMKKb and PKCθ. PKCθ is a member of the novel Protein Chinase C family widely expressed. Indeed, PKCθ activation and membrane localization may play an important role in the dynamic membrane changes that occur during ER stress-induced autophagy. In this study we dissected both in vitro and in vivo ER-stress induced autophagy pathways in muscle. We focused on the role of PKCθ and demonstrated that. PKCθ is strongly activated in cultured myoblasts during ER-stress induced by different stimuli, such as thapsigargin or tunicamycin treatments. Moreover we showed that PKCθ is actually required for ER-stress-induced autophagy in vitro, since its inhibition and/or lack in myoblasts prevents ER-stress-induced LC3 activation and dots formation. In vivo, lack of PKCθ prevents fasting-induced autophagy and muscle wasting, irrespective of Akt pathway inhibition. We also demonstrated that ER-stress and autophagy are activated in mdx muscle and, also in this case, lack or inhibition of PKCθ prevents autophagy.

Taken together these results demonstrate that PKCθ is a key determinant in the ER-stress induced autophagy in skeletal muscle. As being autophagy a physiological process, whose alteration in pathological conditions can lead to muscle wasting, a fine dissection of the molecules and of the signalling pathways involved is a paramount to develop strategies aimed to finely regulate these events.

SESSIONE 7
MYOGENESIS AND REGENERATION-I

30.
TARGETING THE DYSTROPHIC HEART: DIRECT CARDIAC REPROGRAMMING OF GRMD FIBROBLAST

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Improvements in palliative care have resulted in a prolongation of life in Duchenne muscular dystrophy (DMD) patients; a tragic consequence of this improved care is an increase in clinically significant cardiac involvement. Currently, 95% of DMD patients have relevant cardiomyopathy.
So far stem cell based therapies produced limited benefits for post-ischemic conditions and they require invasive surgical procedures to get cardiac biopsies for cell isolation. Therefore alternative strategies have been proposed aiming to switch terminally differentiated cells fate towards cardiomyogenic lineages throughout genetic manipulation. Recently, it has been proved that a combination of three transcription factors, Tbx5, Gata4 and Mef2C, represent the minimal requirements for cardiac fibroblasts reprogramming towards mature and functional induced-cardiomyocytes (iCM). In this work, we extend the cardiac reprogramming protocol to fibroblast isolated from skin biopsies of wt and cardiomyopathic GRMD (Golden Retriever Muscular Dystrophy) dogs and previously corrected with lentiviral vectors carrying the canine mini-dystrophin gene. *In vitro* molecular and immunohistochemical analysis highlighted a cardiac switch consistent with an upregulation of late cardiac markers by wt and GRMD-corrected iCM. *In vivo* xenotransplantations of reprogrammed cells contributed to cardiomyocytes formation in developing heart. Altogether these results provide preliminary evidences of direct cardiac reprogramming using dystrophic somatic cells. Genetically corrected GRMD iCM may provide new insights towards the development of a tailored therapy approach for dystrophy-related cardiomyopathy.

31.
$S1P_2$ RECEPTOR AND SKELETAL MUSCLE REGENERATION

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Sphingosine 1-phosphate (S1P) modulates muscle growth by stimulating specific membrane receptors in muscle fibers and satellite cells. The study explored the role of $S1P_2$ receptor during *in vivo* regeneration in $S1P_2$-null mice and in control mice systemically treated with

JTE-013 (JTE), a selective $S1P_2$ receptor antagonist. Degeneration was induced by notexin injection.
The mean cross sectional area of 4-day regenerated fibers was significantly smaller in $S1P_2$-null and JTE-treated muscles compared to control muscles. The addition of exogenous S1P directly to the regenerating muscle stimulated the growth of untreated soleus, while it was ineffective in the JTE-treated muscle. Accordingly, 1 μM S1P promoted differentiation into myotubes of satellite cells isolated from untreated muscle, but the pro-myogenic effect of S1P was abrogated in satellite cells isolated from $S1P_2$-null muscle. Moreover, the neutralization of circulating S1P, by the systemic injection of a specific antibody, reduced the growth of regenerating untreated muscle whereas it did not affect that of $S1P_2$-null fibers. The reduced growth of JTE-treated soleus was associated to the decreased expression level of myogenin, a specific transcription factor that coordinates skeletal muscle differentiation. Moreover, activation of Akt, a key kinase regulating muscle growth, was significantly smaller in the JTE-treated regenerating soleus compared to the untreated regenerating control.
These results indicate that the absence or inactivation of $S1P_2$ receptor delay the regeneration process and differentiation of isolated satellite cells, suggesting that $S1P_2$ receptor might play a critical role in myogenesis.

32.

SKELETAL MUSCLE REGENERATION IN MICE IS STIMULATED BY LOCAL OVEREXPRESSION OF V1A-VASOPRESSIN RECEPTOR

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Skeletal muscle has a remarkable capacity to regenerate after mechanical or pathological injury. We show that the V1a receptor (V1aR) for vasopressin, a potent myogenic promoting factor that stimulates differentiation and hypertrophy *in vitro*, is expressed in mouse skeletal muscle and modulated during regeneration following experimental injury. We used gene delivery by electroporation to overexpress the myc-tagged vasopressin V1aR in specific muscles, thus sensitizing them to circulating vasopressin. The correct localization on the surface of the fibers of the recombinant product was demonstrated by confocal immunofluorescence directed against the myc tag. V1aR overexpression dramatically enhanced regeneration. When compared with mock-transfected controls, V1aR overexpressing muscles exhibited significantly accelerated activation of satellite cells and increased expression of differentiation markers. Downstream of V1aR activation, calcineurin was strongly up-regulated and stimulated the expression of interleukin 4, a potent mediator of myogenic cell fusion. The central role of calcineurin in mediating V1aR-dependent myogenesis was also demonstrated by using its specific inhibitor, cyclosporine A. This study identifies skeletal muscle as a physiological target of hormones of the vasopressin family and reveals a novel *in vivo* role for vasopressin-dependent pathways. These findings unveil several steps, along a complex signaling pathway, that may be exploited as potential targets for the therapy of diseases characterized by altered muscle homeostasis and regeneration.

33.

TRANSPLANTATION OF MICROENCAPSULATED SERTOLI CELLS IN *MDX* MICE REDUCES MUSCLE INFLAMMATION AND PROMOTES MUSCLE REGENERATION

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Duchenne muscular dystrophy (DMD) is characterized by progressive muscle degeneration associated with chronic inflammation. Activation of inflammation-dependent pathways causes muscle necrosis and fibrosis, which represent the most deleterious outcome of DMD. Indeed, DMD patients are currently treated with antiinflammatory steroids, despite their limited efficacy and undesired side effects. Sertoli cells (SCs) are normally found in the testes where they couple trophic effects with prevention of immune damage to developing germ cells. Based on their ability to produce several immunomodulatory and trophic factors SCs have been successfully implanted to create an ectopic immune-privileged environment that prolongs survival of co-transplanted cells or restores systemic immune tolerance in autoimmune diseases (1). We transplanted SCs encapsulated in highly biocompatible microcapsules (2) in the peritoneal cavity of dystrophic, 4-week-old *mdx* mice. After 3 weeks, diaphragm, tibialis anterior and gastrocnemius muscles from SC- and mock-treated mice were examined. Compared to muscles from mock-treated mice, muscles from SC-treated mice showed: i) a dramatic reduction in necrotic areas and fibrous/adipose tissue infiltration; ii) reduced number of regenerating myofibers and increased number of regenerated and normal myofibers; iii) a marked reduction of infiltrated macrophages, activated ($MyoD^+$) satellite cells and differentiating ($myogenin^+$) myoblasts; iv) reduced expression of RAGE, a receptor that transduces inflammatory stimuli in immune cells and becomes re-expressed in muscle precursor cells and regenerating myofibers during early phases of muscle regeneration (3). Our data suggest that transplantation of microencapsulated SCs in dystrophic patients might be a useful therapeutic approach to counteract inflammation, thus creating a more suitable microenvironment for muscle regeneration.

1) Mital P. et al. 2010. *Reproduction* 139:495-504; 2) Luca G. et al. 2007. *Tissue Eng* 13:641-8; 3) Riuzzi F. et al. 2011. *J Cell Sci,* in revision.

SESSIONE 8
MYOGENESIS AND REGENERATION-II

34.
ROLE OF MAGIC-F1 RECOMBINANT PROTEIN IN CARDIAC DEVELOPMENT OF TRANSGENIC MICE

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Magic Factor-1 (Met-Activating Genetically Improved Chimeric Factor-1 or Magic-F1) is an HGF-derived, engineered protein able to interact with c-Met receptor. The major consequence of c-Met activation is improving myogenenis, resulting in myocyte hypertrophy both *in vitro* and *in vivo* [1]. In transgenic mice the myosin light chain 1F promoter controls the expression of MAGIC-F1, which is active in fast-twitch fibers. Muscular hypertrophy is found in fast-twitch fibers of Magic-F1 transgenic mice. Furthermore, MAGIC-F1 can interfere positively in muscle regeneration, triggering satellite cells activation. Although MAGIC-F1 expression is limited in skeletal muscle tissues due the specificity of the promoter, preliminary results indicate that MAGIC-F1 also has an effect on the maintenance and regeneration of cardiac muscle. For this reason we investigate the morphology and dimensions of Magic-F1 transgenic hearts, where the recombinant protein is not expressed. However, circulating MAGIC-F1 proteins produced in skeletal muscle tissue can reach the cardiac tissue and interfere with its homeostasis.
The morphometric analysis of adult transgenic cardiac sections indicates that the presence of the recombinant protein determines dramatic morphological changes in the heart. The increased weight, cross-sectional area and perimeter of the heart of transgenic animals suggest a robust remodeling of the heart. Cardiac chamber volumes are also increased in transgenic animals compared to control mice, suggesting the occurrence of heart dilation caused by MAGIC-F1 paracrine effects. These data were confirmed also by the three-dimensional reconstruction of the myocardium [2].
In conclusion the presence of Magic-F1 in the heart of transgenic mice exerts a typical pathological phenotype resembling cardiac hypertrophy and dilation.
To better understand the biological effects of MAGIC-F1 on the morphology and function of cardiac muscle more detailed studies are required.
[1] Cassano M. et al., PLoS ONE, 2008, 3: e3223.
[2] Mattoli F. et al., International Journal of Biomedical Imaging, 2011, 2011: 236854.

35.
PKC THETA ABLATION IMPROVES HEALING IN A MOUSE MODEL OF MUSCULAR DYSTROPHY

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Inflammation is a key pathological characteristic of dystrophic muscle lesion formation that exacerbates the wasting process in dystrophic muscles. Limiting immune response is thus one

of the therapeutic options to improve healing, as well as to improve the efficacy of gene- or cell-mediated strategies to restore dystrophin expression, and the search of new downstream molecular targets is an increasingly growing area of interest. PKCθ is a member of the PKCs family highly expressed in both immune cells and skeletal muscle; given its crucial role in adaptive, but also innate, immunity, it is being proposed as a valuable pharmacological target for T-cell dependent immune disorders. In our study we asked whether targeting PKCθ could represent a valuable approach to efficiently prevent inflammatory response and disease progression in a mouse model of muscular dystrophy.
We generated the bi-genetic mouse model *mdx*/$\theta^{-/-}$, where PKCθ expression is lacking in *mdx* mice, the mouse model of Duchenne muscular dystrophy. We found that muscle wasting in *mdx*/$\theta^{-/-}$ mice was greatly prevented, while muscle regeneration, maintenance and strength was significantly improved, as compared to *mdx* mice. This phenotype was associated to reduction in inflammatory infiltrate, pro-inflammatory gene expression and pro-fibrotic markers activity, as compared to *mdx* mice. Moreover, BM transplantation experiments demonstrated that the phenotype observed was primarily dependent on lack of PKCθ expression in hematopoietic cells.
These results demonstrate a hitherto unrecognized role of immune-cell intrinsic PKCθ activity in the development of DMD. Although the immune cell population(s) involved remain unidentified, our findings reveal that PKCθ can be proposed as a new pharmacological target to counteract the disease, as well as to improve the efficacy of gene- or cell- therapy approaches.

36.

SYNTHETIC SCAFFOLDS MAY BE USED FOR THE ORIENTATION OF CARDIAC STEM CELLS PROPERTIES, DIFFERENTIATION AND EXTRACELLULAR MATRIX INTERACTIONS IN CARDIAC TISSUE ENGINEERING: *IN VITRO* AND *IN VIVO* STUDIES

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Cardiovascular diseases are characterized by the progressive loss of functional cells and the subsequent heart failure. When the pharmacological approach no longer complies with the disease evolution, organ transplantation appears to be the only treatment able to rescue the patient life. Cell therapy promises to be clinically efficient and would allow circumventing many limitations of organ transplantation, such as organ low availability, major surgical procedures, high costs and longterm immunosuppression.
Intramyocardial injection of stem cells is by far the most-used delivery technique in preclinical studies, the use of a scaffold may improve the success of the surgical approach, the localization of cardiac stem cells to the infarcted or damaged area and let us use only a few cells, reducing the amount of time between an endocardial biopsy and the cell implantation.
We designed porous Poly-Lactic Acid (PLLA) and Fibroin scaffolds to deliver CPCs in the heart, we isolated and characterized CPCs for the expression of c-Kit, MDR-1 and Sca-1 by flow cytometry, we tested their degree of differentiation *in vitro* studying the expression of all known rat sarcomeric proteins by real-time PCR and their differentiated morphology on

Electron Microscopy samples. A particular attention was given to the expression of microRNA, because their role in the differentiation process of cardiac precursors is emerging. We also tested the host reaction to scaffolds, CPCs, and CPCs/scaffolds. *In vivo*, almost all the used scaffolds induced a foreign body reaction in *nude* mouse and rats, but not in SCID mice. Cardiac stem cells a T cell-mediated immune response induced in *nude* mice, letting us suppose that, differently from Mesenchymal Stem Cells, they express MHC molecules on their surface.
The degree of differentiation, the expression of ECM and integrin proteins, and the expression of several sarcomeric proteins were dependent on the type of scaffold and the polymer used.

37.
GAP-43: A NEURON-SPECIFIC PROTEIN FOUND IN SKELETAL MUSCLE

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In the past twenty years, many evidences suggest that the Growth Associated Protein 43 (GAP-43), also named phosphoprotein B-50, isolated from rat brain, could be considered a neuronal marker directly involved in neurite branching, cytoskeleton remodelling, neuronal development and protection. Vice versa only few papers reported the presence of GAP-43 in skeletal muscle fibres in relation to some phases of fibre growth and/or regeneration (Heuss D, Acta Neuropathol. 91:409-15, 1996; Ma et al, J Orthop Res. 25:1498-505, 2007). Also considering these poor evidences, there is no concluding hypothesis concerning its localization or relationship with other muscle proteins.
The aim of our study was to investigate GAP-43 protein expression and distribution in C2C12 cells and mice skeletal muscle fibres, using immunoblot and immunofluorescence protocols for confocal mycroscopy.
Immunoblot analyses showed the presence of the protein in C2C12 cell homogenates as well as in mature muscle fibres. Immunofluorescent images revealed that the protein was localized nearby the nuclear membranes in C2C12 myoblasts, while in C2C12 myotubes, it was detected on muscular streaks, in a regular double strand pattern. This localization was found also in fibres (EDL, Soleus, Tibialis, FDP) isolated from 1 week, 1 and 24 months old mice. In isolated adult mouse fibres, GAP-43 localization appeared in direct relationship with α-actinin, while no co-localization with RyR, DHPR and mitochondria was detected,
In conclusion, these preliminary data confirm the presence of GAP-43 in the skeletal muscle, and its localization within the filament network supports the hypothesis of a possible functional role in the muscle physiological processes.