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Borgo San Luigi Monteriggioni (Siena)

Abstracts

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SESSION 1
MUSCLE PHYSIOLOGY, BIOPHYSICS AND E-C COUPLING

Architectural and molecular responses to eccentric vs. concentric training in human skeletal muscle: possible contraction-specific adaptations

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The present study focused on the architectural, morphological and molecular adaptations of skeletal muscle to concentric (CON) or eccentric (ECC) loading regimes. Twelve healthy young males were randomly assigned to two groups carrying out either a CON or ECC training protocols (3×wk for 10-wk, 4×8-10 repetitions at 80% of CON and ECC 1-RM). MR imaging (MRI) was used to determine muscle volume while ultrasound was used to analyse muscle architecture (fascicle length, Lf; pennation angle, PA). Additional fourteen subjects performed a single bout of CON or ECC exercise for determining acute signalling responses (assessed by immuno-blotting, biopsies taken from VL muscle 30 min after exercise) of 'remodelling pathways' (i.e. MAP Kinases), inflammation (TNF-signalling), anabolism (mTORc1) and catabolism (MuRF---1/MAFBx). Increase in muscle Vol (+8 CON vs. +6% ECC) and maximal voluntary contraction (MVC) (+9 CON vs. +11 % ECC) was similar in both groups. Lf increased significantly after ECC but not CON (+12 vs. +5%) and PA increased markedly after CON (+30 vs. +5%). Despite the ~1.2 fold greater training load of the ECC group, similar changes in VOL and MVC in were found after training. However, distinct architectural adaptations were found between the two loading protocols. In terms of signalling, while MAPK activation (i.e. p38MAPK, ERK1/2, p90RSK) was exclusive to ECC, neither mode affected AKT-mTOR or inflammatory pathways. These morphologic and architectural changes were associated with distinct acute fascicles mechanical behaviour; we speculate that the different architectural and molecular adaptations may be linked as a result of contraction-specific responses.

Acetylcholine receptors and Ca^{2+} signals in myotubes in vitro

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In skeletal muscle fibres, membrane depolarization and $[Ca^{2+}]_i$ transients trigger contraction, but also have wider biological implications at all developmental stages, regulating events such as myoblasts fusion or initiation of apoptotic pathways in disease. In mature fibres, muscle fibre excitation indicates correct nerve-muscle communication, which is required to maintain neuromuscular junction integrity.
Acetylcholine receptors (AChR), which are expressed by satellite cells soon after the onset of differentiation, orchestrate $[Ca^{2+}]_i$ transients, which in turn regulate myoblast fusion and myotube maturation. In vitro, autocrine AChR activation is granted by an endogenous compound.
It has long been known that IP_3R-mediated Ca^{2+} release also contributes to $[Ca^{2+}]_i$ transients during the early phases of in vitro development of human and mouse myotubes. This pathway likely contributes to muscle fibre damage in several diseases. Our work aims at understanding the specific role of AChR and IP_3R in the control of $[Ca^{2+}]_i$ and downstream events. Specifically, we are focusing on mechanism coupling AChR stimulation to IP_3R activation, which is far from being clarified, in particular in human cells.
To this purpose, we are studying formation in vitro of various types of human and mouse myotubes while interfering with $[Ca^{2+}]_i$ transients. As myogenic differentiation may take place in the absence of myoblast fusion, we analyze myotubes from both a morphologic and a functional point of view, timing the appearance of voltage- and ACh-gated currents. The data collected may help in devising approaches to boost myogenic differentiation when it happens to be impaired, as in aging or disease.

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Exercise and sActRIIB-Fc affects molecular signature of skeletal muscle in mdx mice

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Duchenne Muscular Dystrophy (DMD) is characterized by progressive wasting of skeletal muscle. Soluble Activin receptor Fc (sActRIIB-Fc) or placebo (PBS) was injected 1x/wk to block myostatin/activins with or without voluntary running exercise in young mdx mice, a model of DMD. C57Bl/10ScSnJ mice acted as healthy controls. In a 7-week experiment, sActRIIB-Fc increased muscle mass while exercise enhanced muscle aerobic capacity (e.g. citrate synthase and SDH activities). Microarray analysis was conducted from gastrocnemius muscle. Gene Set Enrichment (GSEA) analysis revealed that many pathways for aerobic metabolism were in the top 10 of the most downregulated gene sets in mdx muscle (FDR<0.005). However, most of these were upregulated by exercise (FDR<0.05). sActRIIB-Fc activated 92 canonical processes/pathways in active mice (sActRIIB-Fc running vs. PBS running), but only one process in sedentary mice (sActRIIB-Fc vs. PBS) (FDR<0.05). The interaction effect of sActRIIB-Fc and exercise was also shown in a protein level by analyzing different proteins by western blotting (e.g. major urinary proteins and the phosphorylation of Stat5).
We report here that exercise can modulate aerobic gene expression profiles or pathways of a dystrophic muscle towards a state of a healthier muscle. Furthermore, ActRIIB-Fc, a myostatin/activin blocker affects differently in exercised and non-exercised muscles.

The complex relationship between ROS and exercise in skeletal muscles

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Physical activity and its related increase in oxygen consumption can lead to a temporary unbalance between production and disposal of ROS (Reactive Oxygen Species). In turn, mode, intensity and duration of training and specific characteristics of the skeletal muscle are expected to determine whether increased ROS production will lead to oxidative stress – i.e. to ROS-induced damages of biologically relevant molecules, or to limited/specific modification of sensor systems, affecting muscle contraction and adaptation. By studying skeletal muscles of rats which underwent moderate aerobic training, and by comparing slow- and fast-twitch muscles, induction of muscle hypertrophy/hyperplasy and fiber-type adaptations were related to ROS signaling, while synthesis of antioxidant and cytoprotective molecules was demonstrated. With a more intense training, protein carbonylation – a recognized marker of oxidative stress - was examined; while exercise training induced such modification only in a host of very specific proteins, a number of proteins were found to be carbonylated in basal – i.e. resting – conditions. In addition, acute intense exercise in untrained rats, expected to markedly increase protein carbonylation, did not appear to lead to such outcome. Although ROS build-up is undoubtedly involved in muscle contractile dysfunction and fatigue, these data, on the whole, support the notion that healthy muscle is endowed of robust homeostatic systems to counteract excess ROS, so that ROS prevalent role appears to be in providing signaling cues to allow adaptation to the increased physical activity. Moreover, the major oxidative modification of proteins – carbonylation- may play a still unrecognized role within such context of ROS-related signaling.

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Short-term dexamethasone treatment leads to impaired force generation in human single skeletal muscle fibers

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Glucocorticoids-induced myopathy is characterized by atrophy and decreased nerve excitability of the muscle fibers. However, little is known about the structural and functional adaptations following short-term administration of glucocorticoids, which is a very frequent condition. Five healthy, young males (mean age = 32.8 ± 5.8 years) were recruited for the daily administration of dexamethasone (8mg/d) for one week. Muscle fiber cross-sectional area (CSA), maximum velocity of unloaded shortening (V_o) and maximum force normalized to cross-sectional area (specific force) were measured in single skinned muscle fiber segments from pre- (n = 137) and post-steroid (n = 139) biopsies. Force-time characteristics of the knee-extensors were determined using isometric contractions. Myosin Heavy Chain isoform concentration and percentage distribution were measured using SDS-PAGE. Intracellular signaling pathways controlling muscle mass were studied. A dexamethasone-induced decline of specific tension (~33%; $p < 0.05$) reflected by reduced myosin concentration (~41%; $p < 0.05$) was observed in fast- but not slow-twitch fibers. No change was observed in fiber CSA, V_o and the relative proportion of different myosin isoforms. Knee extension torque in vivo was higher (~16%; $p < 0.05$) after treatment probably reflecting an adaptation of neural control. Decreased serum levels of creatine kinase and myoglobin, suggesting lower protein synthesis, and no activation of intracellular pathways controlling muscle protein breakdown were found. The data suggest that glucocorticoids cause a very early impairment of force-generating capacity at the cell level, which is at least in part explained by reduced amount of contractile machinery.

Crossbridge properties during fatigue and recovery in mammalian intact skeletal muscle fibres at physiological temperature

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Fatigue occurring during exercise can be defined as the inability to maintain the initial force or power output. Fatigue during repetitive tetanic stimulation at 24°C occurred in an initial phase during which individual crossbridge force decreased, followed by a later phase during which also crossbridge number decreased (Nocella et al. *J Physiol*, 2011). In present experiments, force and stiffness were measured during a fatiguing protocol of 105 tetani every 1.5 s on fibres from FDB mouse muscle. We compared fatigue and recovery from fatigue between 24 and 35°C, close to the *in vivo* temperature of FDB muscle during an intense exercise. The results showed that, during first phase, tension declined less and more slowly at 35°C than at 24°C, whereas the force decrease during second phase was greater at 35°C. The initial force decline occurred without great reduction of fibre stiffness and was attributed to a decrease of the average force per attached crossbridge. Force decline during the later phase was accompanied by a proportional stiffness decrease and was attributed to a decrease of the number of attached crossbridge. Similarly to fatigue, force recovery occurred in two phases: the first associated with the recovery of the average force per attached crossbridge and the second due to the recovery of the pre-fatigue attached crossbridge number. These changes, symmetrical to those occurring during fatigue are consistent with the idea that initial phase of fatigue is due to the inhibitory effect of $[P_i]_i$ increase whereas later phase could be due to reduction of Ca^{2+}-release and/or reduction of Ca^{2+}-sensitivity of the contractile apparatus at longer times during fatigue.

Exertional-stress triggers lethal hyperthermic episodes in genetically predisposed mice

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In humans, lethal hyperthermic episodes can be trigger by administration of halogenated anesthetics (a disorder known as Malignant Hyperthermia Susceptibility, MHS) and by high temperature and/or strenuous exercise (crises identified as Environmental/Exertional Heat Strokes, EHSs). Many MHS (and a few EHS) cases have been linked to gain-of-function mutations in the ryanodine receptor type-1 (RYR1), the sarcoplasmic reticulum Ca^{2+}-release channel of skeletal muscle. The correlation between

MHS and EHS episodes is strongly supported by extensive work in animal models: both RYR1$^{Y522S/WT}$ (carrying a mutation linked to MHS in humans) and Calsequestrin-1 knockout (CASQ1-null) mice trigger similar lethal crises when exposed to both halothane and environmental heat (41^{o}C).
Here we tested the following hypotheses: a) strenuous exercise in challenging environmental conditions (34°C, 30-40% of humidity) is a stimulus capable to triggers EHS-lethal episodes; b) the molecular mechanisms underlying exertional-crises are identical to those triggered by anesthetics. When RYR1$^{Y522S/WT}$ and CASQ1-null mice were subjected to an exertional-stress protocol (executed on a treadmill), which was well tolerated by WT animals, mortality rate was dramatically increased (80% and 75%, respectively). During such crises, skeletal fibers undergo rhabdomyolysis: ~99% and ~64% of fibers from RYR1$^{Y522S/WT}$ and CASQ1-null mice results severely damaged. Interestingly, pre-treatment of animals with azumolene (a more-soluble analog of dantrolene, the only drug approved to treat MH crises) reduced mortality rate down to 0% and 25% respectively in RYR1$^{Y522S/WT}$ (n=2) and CASQ1-null (n=4) animals, strongly suggesting that exertional-crises share common molecular mechanisms with classic anesthetic-induced MH episodes in humans.

Treatment with N-acetylcysteine (NAC) prevents/reduces formation of structural cores in RYR1$^{Y522S/WT}$ mice

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Central Core Disease (CCD) and Malignant Hyperthermia (MH) are related skeletal muscle diseases often linked to mutations in the ryanodine receptor type-1 (RYR1) gene, encoding for the sarcoplasmic reticulum (SR) Ca^{2+} release channel. CCD is characterized by hypotonia, proximal muscle weakness and presence of amorphous regions of myofibrillar disorganization (cores) lacking mitochondrial activity. In humans, the Y522S mutation in RYR1 is associated with MH susceptibility with cores. Knock-in heterozygous RYR1$^{Y522S/WT}$ mice are viable, suffers of MH, and develop structural cores: previous studies indicated that oxidative stress likely represent a key event in the myopathic cascade. Here we tested the hypothesis that reducing oxidative stress prevents/reduces structural damage and consequent formation of cores. We treated RYR1$^{Y522S/WT}$ mice from 2-to-4 months of age with a potent anti-oxidant, N-acetylcysteine (NAC) provided *ad libitum* in drinking water (1% w/v), and analyzed skeletal muscle preparations by histology, electron and confocal microscopy. Analysis of *extensor digitorum longus* (EDL) muscles of 4-month-old un-treated mice confirmed the presence of severe structural alterations - previously defined as *un-structured* and *contracture cores* - in a significant percentage of fibers: respectively 28% and 17% presented *un-structured* and *contracture cores* (3 muscles; 149 fibers). On the other hand, in age-matched NAC-treated mice - although the fiber structure did not appear completely normal - frequency of fibers presenting the two types of alterations was greatly reduced: only 2 and 5 %, respectively (3 muscles; 335 fibers), strongly suggesting that oxidative stress plays an important role in the development of cores in RYR1$^{Y522S/WT}$ mice.

Muscle activity controls the association between Ca^{2+} release units and mitochondria in skeletal fibers

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At the most basic level, skeletal muscle contraction requires Ca^{2+} and ATP and, thus, is under direct control of two major intracellular organelles: Ca^{2+} release unit (CRU), and mitochondria. CRUs are the sites of excitation-contraction coupling, the process responsible for triggering Ca^{2+} release from intracellular stores, i.e. sarcoplasmic reticulum (SR), in response to a propagating action potentials in the T-tubule membrane. Mitochondria are the powerhouse of the cell, being responsible for aerobic production of ATP. CRUs and mitochondria in skeletal fibers are functionally and structurally coupled: a) entry of Ca^{2+} into the mitochondrial matrix is able to stimulate the respiratory chain; b) we have recently discovered that, in adult skeletal muscle fibers, mitochondria and CRUs are structurally linked to one another by small stands, or *tethers*.
Here we tested the following hypothesis: muscle activity improves/maintains the correct association between CRUs and mitochondria, which is challenged

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by ageing and inactivity. Using electron and confocal microscopy, we studied: a) ageing human/mouse muscle fibers and b) denervated rat muscle (by nerve crush). Our quantitative analysis shows that ageing (in humans and mice) and transient denervation (14 days, in rats) results in decreased association between CRU and mitochondria (2-to-3 folds decrease), whereas exercise and re-innervation either maintains or rescues the association between the two organelles (up to control levels). Functional implication of maintained/rescued correct-association between CRUs and mitochondria is potentially large: indeed, efficient Ca^{2+} uptake into mitochondria likely depend on their position in respect to sites of Ca^{2+} release.

Toward the realization of a sarcomere like machine

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We report a preliminary stage toward the realisation of a synthetic sarcomere like machine (MYOMAC) consisting of a single actin filament interacting with an array of motor proteins regularly distributed on an inorganic nano-structured surface. A Dual Laser Optical Tweezers system (DLOT, range 0.5-200 pN force, and 1-10,000 nm displacement) has been setup to measure the mechanical output of the bio-machine under either length or force feedback. The correct polarity of the actin filament (5-15 μm long) is controlled by attaching its barbed end to a trapped bead *via* gelsolin protein. The protocols for the preparation of myosin II from frog skeletal muscle (Elangovan et al., *J. Physiol.* **590**:1227-1242, 2011) have been further refined to produce its proteolytic fragments, HMM and Subfragment-1 (S1). Mechanical measurements have been done with a simplified version of MYOMAC, where the motor proteins (HMM) are randomly adsorbed on the flat tip of an etched optical fibre (diameter ~5 μm), the position of which is controlled by a piezoelectric nano-positioner. When the actin filament is pulled away in the direction perpendicular to the motor deposited surface, the rupture events show that the rupture force of a single actin-HMM bond is 12.85 ± 0.35 pN. Alternatively, when a steady force of ~8 pN is imposed on the motors, the lifetimes of the actin-HMM bonds show a time constant of ~1 s, in agreement with the value reported for mammalian myosin S1.
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SESSION 2
REGULATION OF MUSCLE HOMEOSTASIS AND DIFFERENTIATION (I)

Creatine supplementation enhances the mitochondrial function in oxidatively injured myoblasts

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Numerous studies suggest that Creatine (Cr) supplementation may help to treat various neuromuscular diseases in which mitochondrial impairment plays a causative role. Our purpose is to focus on the effect of Cr on mitochondrial responses in oxidatively-stressed, differentiating C2C12 myoblasts. Mitochondrial morphology, network and function were monitored using cytofluorimetric, ultrastructural, and molecular techniques. Oxidative insult with H_2O_2 (0,3 mM-1h) induced severe ultrastructural damages to the majority of mitochondria, showing typical swelling of the matrix and loss of cristae. Cr-supplementation, 3 and 10 mM Cr, 24 h prior to H_2O_2 treatment, was found to significantly prevent these effects in both prealoading conditions. Confocal microscopy allowed imaging of mitochondrial network morphology that in oxidatively injured myoblasts appeared fragmented. Cr supplementation seems to partially preserve the network organization in both Cr prealoading concentrations. Flow cytometric analysis of NAO/cardiolipin interaction showed that H_2O_2 treatment caused a significant peroxidation of cardiolipin within 24 hours post-oxidative injury; 3 and 10 mM Cr pre-loading prevented the oxidation with a protection rate of 32% and of 18%, respectively. Cytofluorimetric data also revealed either a modulation in mitochondria membrane potential per mitochondria unit using a tetramethyl rhodamine methyl ester (TMRM) as potentiometric fluorescent dye and mitotracker green (MTG) for mitochondrial mass (TMRM/MTG ratio), or mitochondrial dysfunction

masked by a ATP synthase reverse mode operating after addition of oligomycin (5uM), an ATP synthase inhibitor. In particular, only the Cr-treated myoblasts showed an increase in TMRM /MTG ratio compared to oxidatively-stressed myoblasts, after 1 and 24h. Furthermore a significant higher ratio of oligomycin-insensitive fraction was found in 3 mM Cr-pretreated cells (1h), as compared to trolox (a reference antioxidant), strengthening the TMRM /MTG ratio data observed in Cr-preloading conditions. Thus, Cr not only reduces H_2O_2-induced mito dysfunction, but also acts in the absence of a relevant contribution of ATP-synthase, differently from trolox condition with slighlty differences between the two Cr concentration conditions used. Expression of nuclear encoded genes controlling mitochondrial biogenesis (PGC-1α, NRF-1α and Tfam) were also studied by RT realtime PCR as well as mitochondrial proteins profile and western blot of specific metabolic target such us AMPK and its downstream signaling substrate acetyl-CoA carboxylase (ACC). Analysis of C2C12 cells treated with H_2O_2 and H_2O_2+Cr, showed that PGC-1α expression levels were markedly increased immediately after oxidative stress in both conditions as compared to controls, and positively correlated with the severity of mitochondrial cell damage, probably due to a compensatory cell response. Interestingly, Cr preloading increased the phosphorylation of AMPK and especially ACC. On the other hand, Cr stimulates AMPK a key energy-sensitive target that controls numerous metabolic and cellular processes. On the whole, our data indicate that under oxidative stress conditions, where the expression of PGC-1α and mitochondrial biogenesis are likely to be stimulated, Cr preloading may favour an enhanced adaptive mechanism which allows myoblasts to increase the regulation of cellular energy metabolism and minimize oxidative damage in the course of myogenesis.

Melatonin action in preventing skeletal muscle cell death. A preliminary study

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Melatonin (Mel) has a wide range of physiological functions among which the protection against oxidative stress, due to its ability to act as free radical scavenger and to stimulate antioxidant enzyme production (Allegra et al., 2003). Oxidative stress is a major player in initiating apoptotic cell death in skeletal muscle too, which is essential for skeletal muscle development and homeostasis. In fact, its misregulation has been frequently observed in a number of skeletal muscle disorders (Loro et al., 2010). Several authors and our research group also demonstrated that Mel can exert an anti-apoptotic action in a variety of cell models (Luchetti et al., 2006).

In this work, Mel activity has been investigated in C2C12 skeletal muscle cells, undergoing various apoptotic chemical treatments, all determining significant ROS increase (Salucci et al., 2013). Cells were pre-treated with Mel and then exposed to H_2O_2, staurosporine or etoposide. By means of a multiple technical approach we demonstrated that Mel prevented the appearance of apoptotic patterns in the majority of conditions. In addition, caspase-3 activation decreased in samples pre-treated with Mel as well as DNA cleavage. Several TUNEL positive nuclei appeared after chemical agent exposure and their reduction was, again, evident in Mel pre-treated specimens. This behavior has been also confirmed at ultrastructural level.

These preliminary results confirm Mel ability to act as an antioxidant and anti-apoptotic molecule in muscle cells too, thus suggesting a potential strategy in approaching myophaties involving apoptosis misregulation.

Constitutive inhibition of muscle differentiation by senescence-activated DNA damage signaling

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Upon exposure to genotoxic stress, skeletal muscle progenitors coordinate DNA repair and the activation of the differentiation program by the DNA damage-activated "differentiation checkpoint" (DC), which prevents the transcription of differentiation genes during the DNA repair. We have shown that in myoblasts, DNA damage-activated cAbl phosphorylates a tyrosine (Y30) at the N-terminal activation domain of MyoD that transiently inhibits MyoD-dependent transcription following DNA damage, and is reversed possibly upon the successful repair of the lesion (1). The presence of a cAbl consensus site discriminates MyoD from the functional paralog Myf5 and from other muscle bHLH proteins in executing the DC during development (2). Our recent observations reveal that the DNA damage-activated ABL-MyoD signaling contributes to DNA repair in skeletal myoblasts, conferring a dual role to MyoD as

transcription factor and component of the repair machinery (3).
In this study we show that the constitutive, endogenous DNA damage signaling associated with cellular senescence triggers a persistent differentiation checkpoint that constitutively inhibits MyoD-driven myogenic program. Indeed, replicative senescent fibroblasts become progressively resistant to MyoD-mediated myogenic conversion, in coincidence with the senescence-activated DNA damage signaling. Interestingly, a cAbl phosphorylation-resistant MyoD mutant (Y30F) that escapes DNA damage signaling can restore the myogenic program in senescent fibroblasts. Activation of muscle gene transcription by MyoD Y30F in senescent fibroblasts coincides with the induction of the S phase and culminates with the formation of multinucleated myotubes that retain unrepaired DNA lesions.
Our data indicate a close coordination between DNA damage-activated checkpoints, cell cycle and control of tissue-specific gene expression, and reveal a novel role of the DNA damage signaling as a key mediator of the functional antagonism between cellular senescence and terminal differentiation. Moreover our data underscore an emerging new role of MyoD during S phase for the maintenance of genomic integrity and setting the epigenetic landscape conducive for determine cellular fates.
(1) Puri PL, et al. Nat Genet. 2002;
(2) Innocenzi A, Latella L, et al. EMBO Rep 2011
(3) Simonatto M, Marullo F, Chiacchiera F, Musarò A, Wang JYJ, Latella L, Puri PL. DNA damage-activated ABL-MyoD signaling contributes to DNA repair in skeletal myoblasts (*Under revision*)

p38α kinase controls Polycomb Repressive Complex 2 (PRC2) distribution, activity and homeostasis during muscle differentiation

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Polycomb group (PcG) proteins are critical regulators of embryonic and adult stem cell differentiation that operate via repression of gene transcription. In vertebrates, PcG proteins form two main groups of multi-protein complexes, PRC1 and PRC2. EZH2, the catalytic subunit of PRC2, methylates lysine 27 of histone H3 (H3-K27me3), a hallmark of PRC2-mediated gene repression. In undifferentiated muscle cells, EZH2-mediated H3-K27me3 contributes to maintain the chromatin of muscle genes in a conformation repressive for transcription. At the onset of differentiation, down-regulation of EZH2 allows the binding of the muscle regulatory factor MyoD and the recruitment of the positive co-activators to generate a chromatin conformation permissive for transcription. We have previously shown that p38α kinase phosphorylates human EZH2 at T372 in muscle stem (satellite) cells exposed to regeneration cues. EZH2 phosphorylation by p38α promotes PRC2-mediated repression of the satellite cell lineage marker Pax7, an event that is necessary for cell cycle exit and induction of the myogenic program. Here we present data that p38α-mediated phosphorylation of EZH2 also directs chromatin re-distribution of the protein into a more compact chromatin compartment. Using a novel combined approach that couples chromatin fractionation to Chromatin Immunoprecipitation (ChIP) we unveiled a striking correlation between the phosphorylation status of T372, the localization of the protein within discrete chromatin compartments and the H3-K27-me3 status of specific subset of genes. Further, we present evidence that p38α phosphorylation is the signal targeting EZH2 to proteosome-mediated degradation at the onset of muscle differentiation. Altogether our data led us to propose a model by which p38α, which is activated by inflammation cues such as TNF in regenerating muscles, phosphorylates EZH2 on T372 and this regulates PRC2 chromatin re-distribution, activity and levels during the transition from proliferating myoblasts to differentiated myotubes.

Skeletal muscle HSP60 expression is fiber-type specific and increases after endurance training

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Heat shock protein (Hsp) 60 is primarily localized inside mitochondrial, plays a key role in the translocation of proteins and cytoprotection, and its levels increase in skeletal muscle upon exercise. The purpose of this study was to examine muscle fiber specificity of HSP60 at rest and after an endurance training program of 6 weeks.

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Forth-eight young (7-weeks old) healthy mice (BALB/c) were subdivided into sedentary and trained groups. Training was performed over a period of 6 weeks on a rota-rod, at a gradually increasing duration and speed. Eight mice of each group were sacrificed after 15, 30 and 45 days. Two days after the last exercise session all mice were sacrificed by cervical dislocation and posterior (gastrocnemius, soleus and plantaris) muscles group of hindlimb were dissected, weighed and frozen in liquid nitrogen or embedded into paraffin. Immunohystochemistry and immunofluorescence analysis showed that skeletal muscle type I fiber expressed high levels of Hsp60. The western blotting analyses of the entire posterior muscle group did not show any difference in the protein levels after endurance training, while the analysis of soleus muscle (reach in type I fibers) showed an over expression of Hsp60 after 30 and 45 days of endurance training. These results indicated that Hsp60 is fibre type–specific. This may be due to the differences in mitochondrial content between slow and fast fibres. Anyway Hsp60 may be localized also in the cytoplasm, in the outer membrane, in the interstitium and in the blood stream, hence the role of this protein in endurance training should be elucidated.

SESSION 3
REGULATION OF MUSCLE HOMEOSTASIS AND DIFFERENTIATION (II)

Role of PKCzeta on skeletal muscle homeostasis

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PKCzeta (protein kinase C zeta) is a kinase belonging to the atypical subgroup of PKC superfamily. The atypical PKCs are involved in several biological processes such as apoptosis, protein synthesis and glucose metabolism (promoting the GLUT4 translocation to the cellular membrane)(1, 2). Little is known on the role of PKCzeta on skeletal muscle homeostasis. We have overexpressed PKCzeta in mouse skeletal muscles by means of the well established tecnique of in vivo transient transfection and observed marked hypertrophy in PKCzeta positive myofibers. Moreover we have preliminary data indicating that PKCzeta is able to protect muscle from denervation-induced atrophy. It is known that PKCzeta is involved in mitochondrial calcium uptake (3). Thus, we hypothesized that muscle hypertrophy induced by PKCzeta could be due to modulation of MCU (mitochondrial calcium uniporter) activity.

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GAP-43 knockout mice show delayed ultrastructural maturation in skeletal muscle fibers

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The Growth Associated Protein 43 (GAP-43) is involved in neuronal plasticity during development and regeneration. Our recent data revealed that this protein is expressed in both myoblasts and myotubes, and its cellular localization changes dramatically during differentiation. In muscle fibers, GAP-43 localization is found nearby the calcium release units (CRUs) suggesting a functional role for this protein (Guarnieri et al. PlosOne 2013). During skeletal muscle development, CRUs change from longitudinal to transversal orientation pattern according to T tubules maturation (Takekura et al. Developmental Biology 2001). In order to define the role of GAP-43 protein in skeletal muscle, we performed an ultrastructural analysis on diaphragm and EDL (Extensor Digitorum Longus) muscles from GAP-43 knockout (KO) mice. KO mice body weight was significantly lower in respect to WT and the cross sectional area of diaphragm and EDL myofibrils and fibers was smaller in KO versus WT animals. Electron microscopy analyses showed a reduction of transversely oriented CRUs and a reduced triads *per* intermyofibrillar space ratio both in KO neonatal (24h) diaphragm and in 21-days-old EDL. The same analysis performed on the 45-days-old EDL revealed no significant differences between KO and WT mice. These preliminary data suggested that GAP-43 expression could be important for the normal timing of mouse skeletal muscle development.

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Altered insulin-like growth factor 1 signaling in the muscle of knock in SBMA mice

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Spinal bulbar muscular atrophy (SBMA) is a motor-neuron disease characterized by degeneration of lower motor neurons. SBMA is caused by expansion of polyglutamine (polyQ) tract in the gene coding for androgen receptor (AR). Patients develop progressive face and limb skeletal muscle fasciculation, weakness, and atrophy. We and others provided evidence in support of the idea that muscle atrophy in SBMA results not only from denervation, but also from direct myopathic changes exerted by mutant AR in skeletal muscle. We have shown that overexpression of IGF-1 selectively in the skeletal muscle of SBMA mice leads to activation of Akt, phosphorylation and degradation of mutant AR by proteasome, and attenuation of disease manifestations. We tested the hypothesis that alteration of IGF-1/Akt pathway in SBMA muscle contributes to disease pathogenesis. We used a knock in mouse model of SBMA. We found that mTOR expression levels and phosphorylation are increased in skeletal muscle of SBMA mice. We confirmed mTOR activation through studying the phosphorylation levels of mTOR target genes that are increased in this context. We found that FOXO3a expression levels and phosphorylation are also increased mice, but this modulation is detected subsequently to mTOR activation. Importantly, we found that pathways leading to new protein synthesis and protein degradation are activated in SBMA muscle. We propose that mutant AR-induced atrophy results in compensatory hypertrophy in adult SBMA mouse, which occurs through induction of Akt-dependent activation of new protein synthesis. We propose that unbalance between these different pathways leads to skeletal muscle atrophy in SBMA.

SMAD 1/5/8 regulates the stemness of skeletal muscle progenitors

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During proliferation and differentiation of myogenic precursors, e.g. satellite cells, the critical role of bone morphogenetic protein (BMP) signaling has already been established [1]. Mesoangioblasts (MABs) are vessel-associated stem cells [2] and participate to skeletal muscle regeneration. However, MAB myogenic commitment still remains poorly characterized. The present study aims at evaluating whether BMP signalling impacts the myogenic potential of adult MABs (aMABs) or dystrophic MABs (dMABs), both *in vitro* and *in vivo*. Our results suggest that BMP signalling acts as myogenic inhibitor during both *in vitro* and *in vivo* MAB commitment, and maintains their undifferentiated state. Moreover, once injected into dystrophic muscles, aMABs participate to fiber regeneration and partially restore Sgca protein. Finally, MAB therapeutic effect is enhanced by BMP signalling antagonists. In future, additional analyses will be needed to assess whether BMP signalling inhibition significantly ameliorates MAB engraftment and functional outcome during regeneration of the dystrophic skeletal muscle.

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Mesodermal induction and myogenic commitment of induced pluripotent stem cells derived from Spina Bifida somatic cells

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Prenatal diagnoses of congenital anomalies are the main reasons of pregnancy terminations. Spina Bifida (SB), a type of neural tube defect, is one of the most common congenital disorders with abortion rates of nearly 65 percent (1). Causes of SB may be nutritional (folic acid), genetic, or environmental, however, the molecular mechanisms behind SB are yet to be deeply clarified and it is still an open question why Pax3-deficient mice (Splotch) that lack limb muscles, suffer from spina bifida. We hypothesized that induced pluripotent stem cells (iPSCs) as SB disease modelling (2) could offer an extraordinary tool to study the molecular mechanisms responsible for the human syndrome.

In this study, we generated and characterized human iPSCs derived from fibroblasts and myoblasts of SB abortions. In addition, we used several protocols based on embryo body (EB) formation and upon optimization with specific growth factors; we were able to induce mesodermal differentiation. In fact, under these conditions, iPSCs expressed primitive streak markers (Mixl1, T- Brachyury) in early stage of differentiation and mesodermal induction peaked between 7-9 days, when pluripotency markers were strongly downregulated. Then the following plating of cultured cells on gelatine/collagen coated flasks resulted in PDGFR alpha and beta upregulation. In conclusion, we provided evidences that human iPSCs from SB somatic cells elicited pluripotency as documented by teratoma assay, and they were able to form EBs similarly to wt pluripotent cell counterparts. We also set up a protocol for mesodermal lineage inductions in order to proceed further for myogenic commitment, highly dependent on downregulation of pluripotency markers.

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Mitochondrial calcium signalling in the control of skeletal muscle homeostasis

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Skeletal muscle atrophy is due to a number of causes, such as disuse, denervation, aging, fasting, cancer cachexia. In recent years, lots of efforts have been made to understand the molecular pathways, and thus the potential therapeutic targets, of muscle atrophy. Mitochondria play a central role in muscle homeostasis, being the major source of ATP in oxidative myofibers. They have the ability to accumulate Ca^{2+}, behaving as buffers of the cytosolic [Ca^{2+}] increase occurring during contraction. In addition, mitochondrial Ca^{2+} stimulates aerobic metabolism and ATP production, essential for muscle activity. Finally, excessive Ca^{2+} accumulation in mitochondria can trigger cell death. We wish to dissect what is the prevalent effect of mitochondrial Ca^{2+} uptake elicited by muscle contraction, and we aim at verifying whether novel therapeutic strategies against muscle atrophy may be forecasted by modulating Ca^{2+} uptake. The recent molecular identification of the Mitochondrial Calcium Uniporter (MCU), the highly selective channel responsible for Ca^{2+} entry into the mitochondria, has paved the way to novel experimental approaches, in which mitochondrial Ca^{2+} accumulation can be tightly regulated. Moreover, direct measurement and modulation of MCU expression and activity in different physiopathological conditions can now be performed. Our preliminary data demonstrate that overexpression of MCU in mouse skeletal muscle in vivo causes fiber hypertrophy. Most importantly, MCU overexpression protects mouse skeletal muscle from denervation-induced atrophy. These results suggest that mt Ca^{2+} uptake plays a crucial role in muscle trophism and represents a possible target of clinical intervention in diseases in which muscle atrophy is predominant.

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The Histone H3 Lysine 9 Methyltransferases G9a/GLP Regulate Polycomb Repressive Complex 2-Mediated Gene Silencing

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Skeletal muscle regeneration depends on the ability of adult muscle stem cells to enter the myogenic program and differentiate into new mature myofibers. Stem cell differentiation requires a selective activation and silencing of specific transcriptional programs that is achieved epigenetically and involves concerted intervention of several chromatin-modifying enzymes.
G9a/GLP and Polycomb Repressive Complex 2 (PRC2) are two major epigenetic silencing machineries, which in particular methylate histone H3 on lysines 9 and 27 (H3K9 and H3K27), respectively. Components of the two pathways have been separately demonstrated to play crucial roles in the control of skeletal muscle differentiation. However, evidence of a direct crosstalk between H3K9 and H3K27 methylations remained elusive.
Here, we show that PRC2 and G9a/GLP interact physically and functionally. Combining different genome-wide approaches, we demonstrate that Ezh2 and G9a/GLP share an important number of common genomic targets, encoding developmental regulators. Furthermore, we show that G9a enzymatic activity enhances PRC2-mediated H3K27 tri-methylation in vitro and modulates PRC2 genomic recruitment to a subset of its target genes. Taken together, our findings demonstrate an unanticipated interplay between two main histone lysine methylation mechanisms, which cooperate to maintain silencing of a subset of developmental genes.
Since many inhibitors of such chromatin modifying enzymes are already used in clinical trials (i.e. as anti-cancer agents), the data presented in this work will be instrumental to devise strategies to manipulate the regenerative potential of muscle stem cells.

SESSION 4
MUSCLE ATROPHY (I)

Exploiting Vasopressin signaling in muscular atrophy and dystrophies

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Vasopressin (AVP) induces differentiation in myogenic cell lines and primary satellite cells. By interacting with V1aR, the AVP receptor expressed in skeletal muscle, AVP activates phospholipases C and D, regulates cAMP levels, and increases cytosolic [Ca^{2+}], thereby activating CaMK and calcineurin pathways and inducing the formation of multifactor complexes on the promoter of muscle specific genes. In an experimental model of muscular atrophy induced by TNF over-expression, stimulation of AVP pathways counteracts the negative effects of TNF both enhancing regeneration and promoting the resolution of inflammation. Molecular analysis for the expression levels of early and late regeneration markers (Pax7 and MyoD or myogenin and MHC, respectively) suggested an impairment of regeneration in muscles over-expressing TNF. This effect was counteracted by V1aR overexpression. The positive effects of V1aR on muscle homeostasis are due to the promotion of the calcinuerin-IL-4 pathway and by the inhibition of atrophic genes expression mediated by Akt-dependent FOXO phosphorylation. *In vitro* data showed an effect of the stimulation of AVP-dependent pathways even in epigenetic regulation: AVP treatment induces the acetylation of H3 histone, modulating HDAC4 activity and allowing the expression of muscle specific gene, such as myogenin. We are now analyzing the effects of AVP signaling stimulation in mouse models of muscular dystrophies. Preliminary data demonstrate that stimulation of AVP-dependent pathways ameliorates inflammation and regeneration processes. This study highlights a novel *in vivo* role for the AVP-dependent pathways which may represent a potential gene therapy approach for many diseases affecting muscle homeostasis.

Targeting integrin signalling prevents autophagy induction in muscle via JNK/p38 mediated up-regulation of miR-21

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Components of the extracellular matrix are crucial regulators of cell proliferation, migration and survival. Integrin receptors functionally link extracellular matrix components, such as collagen and laminin, to intracellular signalling transducers, such as Focal Adhesion Kinase and Src, to initiate intracellular response. In muscle, lack or defects in extracellular

matrix components leads to severe myopathies, such as Congenital Muscular Dystrophies. Recently, using the ColVI -/- mouse as a model of the Bethlem CMD, ineffective autophagy has been described as the primary defect leading to muscle damage, highlighting the crucial role of the autophagy machinery to maintain muscle homeostasis. In this study we investigated the role of integrin signalling in controlling autophagy in muscle cells. We show that pharmacological inhibition of FAK prevented starvation-induced Akt de-phosphorylation, and in turn Foxo and LC3 activation, thus preventing starvation-induced autophagy. These effects were due to the parallel up-regulation of mir-21 expression, inhibiting the expression of the AKT repressor PTEN. On the other hand, mir-21 up-regulation was dependent on the strong up-regulation of both p38 and JNK activities when FAK was inhibited during starvation.
Indeed, FAK inhibition together to p38 and/or JNK inhibition failed to induce mir-21 expression, and in turn Akt maintenance and autophagy inhibition during starvation. Importantly, same pathways were active in muscle derived from starved ColVI -/-mice, thus identifying mir-21 as a key player in the outside-in integrin signalling, mediating p38/JNK and Akt cross-talk.

Overexpression of PGC-1alpha prevents disuse muscle atrophy in hindlimb unloaded mice

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To define the contribution of redox imbalance to the regulation of cell signaling pathways involved in disuse atrophy we studied the adaptations of soleus and gastrocnemius to hindlimb unloading (HU) in the early phase of disuse (3 days) with and without antioxidant treatment (trolox). HU resulted in reduction of CSA, induction of the ubiquitin proteasome (MuRF1 and atrogin-1 mRNA up-regulation) and autophagy (Beclin1 and p62 mRNA up-regulation) and redox status alteration (NRF2, SOD1 and catalase up-regulation) in both muscles, whereas reduction in protein synthesis (pAKT and pS6Rp decrease) was observed in soleus only. In addition, alterations of oxidative metabolism (PGC-1alpha and mitochondrial complexes down-regulation) were found in unloaded mice, more in soleus than in gastrocnemius. Trolox did not prevent atrophy and catabolic systems induction in unloaded muscles, suggesting that oxidative stress is secondary phenomenon. Furthermore PGC-1α reduction persisted after trolox, suggesting that a metabolic program could have a primary role in the pathogenesis of atrophy. To define the link between mitochondrial dysfunction and disuse muscle atrophy, mice overexpressing PGC-1α (TgPGC-1α) were unloaded for 14 days. The TgPGC-1α mice showed a dramatic resistance to HU muscle atrophy, i.e. atrophy in gastrocnemius was completely prevented while, in soleus, both slow and 2A fast fibres lost significantly less mass (9.8% and 12% respectively) during the suspension phase compared with the same fibres of HU14-WT (20% and 35% respectively). An almost complete recovery of the muscle mass in unloaded TgPGC-1α mice indicate that PGC-1α and mitochondrial alteration play a major role in disuse atrophy.

The power of mitochondrial shaping machinery in controlling muscle mass

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Skeletal muscle is a tissue with high energy demand that requires an extremely organized and functioning mitochondrial network. Therefore, mitochondria dynamics play a critical role in muscle homeostasis and function. However, only few genetic studies have explored the role of fusion and fission machineries in muscle physiology. Here we have investigated the role of DRP1 by generating a muscle specific knockout mouse. Ablation of DRP1 gene results in 100% lethality at newborn age. DRP-null muscles are smaller in size than controls because of inhibition of protein synthesis and activation of protein breakdown. DRP1 null muscles show also an alteration of myogenesis that contributes to the weakness. Knockout animals showed accumulation of lipid droplets and of abnormal mitochondria that are bigger in size with normal cristae. Therefore, mitochondrial shaping machinery is critical for muscle homeostasis and open a new set of potential therapeutic targets against muscle wasting.

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SESSION 5
MUSCLE ATROPHY (II)

The FOXO signature in muscle wasting. Definition of the gene network that controls protein degradation

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Under stress conditions mammalian cells activate compensatory mechanisms to survive and maintain cellular function. During catabolic conditions, such as low nutrients, systemic inflammation, cancer or infections, protein breakdown is enhanced and aminoacids are released from muscles to sustain liver gluconeogenesis and tissues protein synthesis. Proteolysis in muscle is orchestrated by a set of genes named atrophy-related genes. A system that is activated both in short and prolonged stress conditions is the family of Forkhead Box (FOX) O transcription factors. Here, we report that muscle-specific deletion of FoxO members resulted in protection from muscle loss because FoxO family is required for induction of autophagy-lysosome and ubiquitin-proteasome systems. Importantly, FoxO is required for Akt activity but not for mTOR signalling underlining the concept that FOXOs are upstream mTOR for the control of protein breakdown when nutrients are lacking. Moreover, FoxO family controls the induction of critical genes belonging to several fundamental stress response pathways such as unfolded protein response, ROS detoxification and translational regulation. Finally, we identify novel FOXO-dependent ubiquitin ligases including the recent discovered MUSA1 and a novel one, which we named Specific of Muscle Atrophy and Regulated by Transcription (SMART1). Our findings identifies the critical role of FoxO in regulating a variety of genes belonging to pathways important for stress-response under catabolic conditions.

Msy-3/Csda regulates muscle wasting progression upon skeletal muscle denervation

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The Y-box protein MSY-3/Csda regulates postnatal repression of the myogenic transcription factor myogenin, which plays a key role in regulating pathways involved in muscle maturation and degeneration. MSY-3 binds a highly conserved DNA cis-acting element located upstream of the myogenin promoter (MyogHCE). Recently, it has been demonstrated that myogenin deleted mice are resistant to muscle atrophy following denervation, suggesting a crucial role of myogenin for proteosome-mediated protein degradation. These evidences suggest that MSY-3 mediate modulation of the myogenin regulatory pathway during muscle atrophy and it is a potential therapeutic target for therapies for muscle degeneration-associated diseases. In denervated MSY-3 KO mice we found a clear involvement of this gene in regulating muscle degeneration progression through the transcriptional regulation of myogenin and atrogenes. Muscle fibers of denervated MSY-3 mutant mice showed a substantial reduction of the cross sectional area compared to the WT mice. By electroporation in vivo of BAC constructs in denervated muscle, we also tested the response of the myogenin regulatory locus WT and mutated at the myogHCE. We found that the myogenin WT construct is silenced, contrary to the mutated one, which is instead activated. By high-throughput technology we genome-wide analyzed MSY-3 DNA binding and expression profile in innervated and denervated muscle of WT and MSY-3 knock out mice. The genome–wide analysis highlights a group of genes possibly modulated by the MSY-3 regulatory complex during muscle maturation and degeneration and involved in processes of AChRs assembling on muscle fibers and protein degradation associated to muscle wasting.

The intraluminal domain of junctin contains junctional sarcoplasmic reticulum targeting sequences

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Junctin is an integral membrane protein of the sarcoplasmic reticulum (SR) encoded by the Aspartyl beta-hydroxylase (AβH) gene. The AβH gene can undergo different alternative splicing events, generating at least three distinct proteins, the Aspartyl beta-hydroxylase, junctin and junctate, all sharing the same transmembrane domain and a short highly charged acidic luminal region, but carrying distinct intraluminal tails. Interstingly, junctin and junctate are both expressed in skeletal muscle, but only junctin was shown to interact and co-localize with the Ca2+ release

complex in the junctional SR, whereas junctate displays a typical longitudinal SR distribution. These two proteins may thus represent an ideal molecular model to investigate the presence of specific regions responsible for protein targeting to the junctional SR.
To this aim, junctin deletion mutants were tagged with a green fluorescent protein (GFP) and expressed in primary myoblasts. The results obtained showed that even deletion of small regions within intraluminal domain of junctin resulted in protein mislocalization, thus indicating that the entire luminal sequence is required for protein targeting to the junctional SR. Interestingly, the same region, if inserted downstream the cytoplasmic and trasmembrane domains of sarcolipin, a resident protein of the longitudinal SR, is able to completely re-localize the protein to the junctional SR, further confirming that the intraluminal domain of junctin may actually contain minimal junctional SR targeting sequences.

Role of autophagy, SQSTM1, SH3GLB1, and TRIM63 in the turnover of nicotinic acetylcholine receptors

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Removal of ubiquitinated targets by autophagosomes can be mediated by adaptor molecules, like SQSTM1, in a mechanism referred to as selective autophagy. While cytoplasmic protein aggregates, mitochondria and bacteria are the most well known targets of selective autophagy, its role in the turnover of membrane receptors is scarce. We here show that fasting-induced wasting of skeletal muscle involves remodeling of the neuromuscular junction by increasing the turnover of nicotinic acetylcholine receptors (AChR) in a TRIM63-dependent manner. Notably, this process implied enhanced production of endo/lysosomal carriers of AChR, which also contained the membrane remodeler SH3GLB1, the E3 ubiquitin ligase, TRIM63, and the selective autophagy receptor SQSTM1. Furthermore, these vesicles were surrounded by the autophagic marker MAP1LC3A in an ATG7-dependent fashion, and some of them were also positive for the lysosomal marker, LAMP1. While the amount of vesicles containing endocytosed AChR strongly augmented in the absence of ATG7 as well as upon denervation as a model for long-term atrophy, denervation-induced increase in autophagic AChR vesicles was completely blunted in the absence of TRIM63. On a similar note, in TRIM63-/- mice denervation-induced upregulation of SQSTM1 and LC3-II was abolished and endogenous SQSTM1 did not colocalize with AChR vesicles as it did in wildtype. SQSTM1 and LC3-II co-precipitated with surface-labeled/endocytosed AChR and SQSTM1 overexpression significantly induced AChR vesicle formation. Taken together, our data suggest that selective autophagy regulates the basal and atrophy-induced turnover of the pentameric transmembrane protein, AChR, and that TRIM63, together with SH3GLB1 and SQSTM1 regulate this process.

The role of S6K in the regulation of skeletal muscle mass and function

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The kinase Akt is a very important regulator of skeletal muscle mass, with its activation stimulating protein synthesis and blocking protein degradation leading to muscle hypertrophy. Akt has numerous downstream effectors which might contribute to increase muscle mass. The main kinases downstream of Akt responsible for increases in protein synthesis are mTORC1 and S6K1. Treatment with rapamycin, a specific inhibitor of mTOR, completely blocks Akt-induced hypertrophy in regenerating rat skeletal muscle (Pallafacchina et al., 2002). Furthermore, rapamycin treatment only reduced Akt-induced hypertrophy in S6K1/S6K2 double k.o. myotubes (Ohanna et al., 2005). In this project we want to examine through which pathways activation of Akt

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leads to hypertrophy and improved adult muscle function in vivo, focusing on S6K1 and S6K2.
In preliminary experiments, we transfected a plasmid coding for a constitutively active Akt (myr-Akt) into adult S6K1 and S6K1/S6K2 double k.o. mice using electroporation, measured fiber size and compared this to the hypertrophy seen after transfection in wildtype animals. We found that Akt induces a similar hypertrophy in tranfected fibers from both k.o. mice as compared to wildtype animals. To examine if Akt-induced hypertrophy is dependent on mTORC1 we electroporated wildtype animals while treating them with rapamycin. This lead to a reduction in the hypertrophy of the transfected fibers of 50%, however did not prevent it. In order to better understand which function S6K1 fullfills in skeletal muscle hypertrophy, we have generated a new transgenic mouse line by crossing a transgenic line in which Akt can be inducibly expressed in skeletal muscle, with the S6K1 k.o. line, leading to the generation of the myrAkt-S6K1 k.o. Activation of Akt in myrAkt-S6K1 k.o. for three weeks leads to an almost twofold increase in fibersize, confirming the results obtained by electroporation. Normalized muscle force however is significantly decreased and hypertrophic muscles show areas of muscle degeneration. Taken together this work shows that S6K1 is not required for increasing muscle mass, but is fundamental for increasing muscle force.

SESSION 6
MUSCLE AGING AND SARCOPENIA

There is a need to count in vivo total myofibers in an aging muscle?

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Although denervation has long been implicated in aging muscle, the degree to which it causes loss of myofibers is an open issue in humans, since reinnervation events may long-term compensate motor neuron loss in spinal cord and/or axonal abnormalities along peripheral nerves [1]. Recent experimental study in rodent provides quantitative assessment of the contribution of denervation to myofiber changes in aging muscle, suggesting that it explains the majority of the observed atrophy [2]. This striking result suggests that a renewed focus should be placed on denervation in seeking for its clinical relevance. Translation of animal results to humans asks for methods to count in vivo total number of myofibers in anatomically identified muscles, thus corroborating electrophysiological evidence of loss of motor units. This will be of main value in longitudinal approaches to test extent of denervation/reinnervation induced loss of myofibers and its relevance in prevention and rehabilitation strategies. In collaboration with Landspitali Hospital, we are designing and planning a non-invasive approach based on Gray Value analysis[3], but using µCT technologies [4]. As a first step we will analyze ex-vivo rodent muscles, segmenting myofiber morphometry of isolated leg muscles using the General Electric nanotom x-ray µCT system, which have up to 200 nm detail detectability. Validation of this approach may provide the final evidence to establish improved prevention/rehabilitation strategies to delay/reverse age-related muscle changes and complications due to accompanying diseases.

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MOBILITY in Aging: interim results of 10 weeks of electrical stimulation or leg press trainings in 70 years old sedentary elderlies

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Aging is a multifactorial process that is characterized by decline in muscle mass and performance. Our recent results from a peculiar group of well-trained seniors that exercised regularly in their previous 30-40 years, suggest that decades of high level physical exercise have beneficial effects on age related decay not only affecting muscle trophism and phenotype, but also counteracting denervation atrophy by promoting reinnervation.
Based on this findings, 70 years old seniors with normal life style were recruited (n=28) and trained for 10 weeks either with home-based functional electrical stimulation (h-b FES) or leg press (LP). Before and after training period, all the subjects were submitted to mobility functional tests and muscle biopsies from *Vastus Lateralis* of both legs. No signs of degeneration, and/or of inflammatory cells infiltrates were observed in muscle biopsies after the training. Interim analyses of muscle biopsies (n=38, subjects n=20), show that, despite a very slight decrease of the overall mean myofiber diameter after the training (pre 53.39±16.46 μm vs post 52.14±16.94 μm, $p<0.0001$), statistically significant improvements in functional testing were observed. A significant decrease of slow type fibres was observed either in leg press (59% vs 54%, $p< 0.001$) or h-b FES (56% vs 50%, $p<0.001$) trained subjects. Interestingly, only h-b FES was effective to maintain the trophism of fast fibre after the training (pre 48.52±14.34 vs post 48.22+/-16.72 μm, p=n.s.), demonstrating the effectiveness of h-b FES as a safe home-based method to counteract fast type fiber atrophy associated with ageing and to improve the performances of ageing muscles.

Age-related and gender-dependent changes in intra-muscular fat deposition and adipokine receptors in human skeletal muscle

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Aging is characterized by changes in body composition and sarcopenia. The etiology of sarcopenia includes physical inactivity and increased inter-muscular adipose tissue. Fat accumulation can also occur as intra-muscular triglycerides (IMTG) deposition in form of lipid droplets, associated with Perilipins (Plins), such as Plin2. Recently, we found that Plin2 expression increases with age and inactivity, and is inversely associated with muscle mass and strength. Adipokines have been proposed to play a role in this accumulation. We studied the expression of the leptin (LEPRb) and adiponectin (AdipoR1) receptors in Vastus lateralis muscle biopsies from young and old healthy donors and patients with lower limb mobility impairment (LLMI). The expression of LEPRb and AdipoR1 resulted dependent on gender: young women express higher levels of these receptors than men. This gender difference disappeared with age, as old men and women showed similar levels. When comparing healthy subjects with LLMI patients, LEPRb and AdipoR1 expression levels resulted higher in patients, suggesting that inactivity synergises with age in triggering the expression of these receptors, which is similar to that of Plin2. No correlation with adiponectin and leptin circulating levels was present.
These data suggest that a gender difference exists regarding a series of muscle molecular parameters, including IMTG and adipokine receptors levels, and, from previous studies, genes involved in apoptosis and inflammation, a condition chronically present in old

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subjects (inflamm-aging). It appears clearly that aging and physical inactivity impinge upon all these parameters, suggesting a role in age-related loss of muscle mass and strength.

Agrin-mediated potentiation of aged human myoblast proliferation: a possible mechanism of action

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The age-related skeletal muscle wasting is characterized by an impairment in tissue regeneration. In particular, satellite cells fail to proliferate and differentiate properly. A change in the concentration of neurotrophic factors, which follows age-associated denervation, could contribute to this phenomenon. Neural agrin is a heparan sulfate proteoglycan, produced by motor neurons and released in the synaptic cleft. Agrin is notably known as a synaptic organizer for the neuromuscular junction. More recently, it has been proposed as positive modulator of the skeletal-type excitation-contraction coupling mechanism and contractile properties. In this study, we investigated the possible role of neural agrin in promoting satellite cell proliferation *in vitro* and compared the effect in old *versus* young myoblasts derived from human healthy donors of different age (5-77 years). We observed that 1 nM neural agrin increased the mean population doublings in "old" myoblasts, while it did not in "young" counterparts. These results were confirmed by bromodeoxyuridine incorporation, which revealed an enhancement in cell cycle activation only in cells from old donors. The senescence phase at the end of the growth curves excluded a possible transformation into cell lines. Western blotting performed in old myoblasts detected the presence of the canonical agrin receptor MuSK. The superfusion of 1 μM imatinib, inhibitor of Abl kinases downstream MuSK, prevented the agrin effect on myoblast proliferation. In conclusion, our results indicate a novel role for neural agrin as local factor controlling the human satellite cell proliferation. We suggest a mechanism of action possibly based on MuSK receptor activation.

Apoptosis contribution in human aged satellite cells

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Adult skeletal muscle is able to regenerate after injury, mainly through muscle-specific stem cells, known as satellite cells (SC). However, in aged muscle, sarcopenia lead to an impaired regeneration: SCs derived from elderly subjects are unable to perform a complete differentiation program to maintain muscle mass and function. Several studies have suggested that both intrinsic and extrinsic factors are involved[1]. This reduced capability seems due to both impairment of myoblasts differentiation and an alteration of gene profile. In particular, specific gene pathways related to modulation of miRNAs and involved in muscle remodeling are entailed. Moreover, we also demonstrated that during the ageing process SCs display an increase of oxidative damage and decrease of scavenger activity[2,3]. This evidence was confirmed by our microarrays analysis showing an age-related dysregulation of some genes involved in SCs antioxidant and repair activity (Polimerase K, SHC1 and FOXO1A)[4]. The last two genes could also be implicated in apoptotic process activation. Annexin V/PI staining and TUNEL technique revealed a significantly higher percentage of apoptotic SCs from old subjects *versus* young ones. Further RT-PCR analysis demonstrated an alteration of differentiation and apoptotic processes as well as miRNAs expression. Collectively, our data suggest that apoptosis could be considered as one of the possible mechanism responsible for the impairment of aged SCs differentiation and muscle regeneration.

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SESSION 7
MUSCLE WASTING AND CANCER CACHEXIA

Growth hormone and exercise effects on myotendinous junction ultrastructure in hind-limb unloaded and hypophysectomized rats

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Myotendinous junctions (MTJs) are specialized sites of muscle-tendon interactions and muscular force transmission from myofibrils to collagen fibrils [1]. The ultrastructural interface between muscle cell membrane and extracellular matrix is highly folded and interdigitated. The aim of our work is to examine the junctional behavior in the atrophic condition, during particular prevention protocols. In this study, the MTJs of plantaris muscles from twenty hypophysectomized rats were processed for electron microscopy [2]. The animals were assigned to one of five groups: control (CTRL), hind-limb suspended (HS), hind-limb suspended and exercised (EX; 3 daily bouts of 10 climbs up a ladder), hind-limb suspended and growth hormone injected (GH; 2 daily injections of 1 mg/kg bw), hind-limb suspended, GH injected and exercised (GH+EX). MTJs of plantaris muscle were analyzed by electron microscopy and the contact between muscle and tendon was evaluated using an IL/B ratio, where "B" is the base and "IL" is the interface length of MTJ's digit-like processes. After 10 days of unloading, the mean IL/B ratio was significantly lower in HS (3.92), EX (4.18) and GH (5.25) groups than in the CTRL (6.39) group. On the opposite, the mean IL/B ratio in GH+EX (7.3) was higher than CTRL. These findings indicate that the interaction EX+GH attenuates the changes in MTJ structure that result from chronic unloading and suggest a potential use of this treatment as a countermeasure to these adaptations.

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miRs profiling in Vastus Lateralis and blood from young men with Unilateral Lower Limb Suspension

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MicroRNAs (miRs) profiling was studied in a model of unilateral lower limb suspension (ULLS) inside the framework of MYOAGE EU project-"Understanding and Combating Age-related Muscle weakness". The main task was to identify the circulating and tissue–specific miRs, post-transcriptional regulators of gene expression, involved in the disuse-related muscle atrophy and its reverse process.
Muscle atrophy was studied in 9 young subjects in a time series experimental design as follows: i. time 0 or pre-ulls; ii. time 1 or post-ulls after three weeks of leg suspension; iii. time 2 or post-training (post-tr) after 3 weeks of rehabilitation with physical exercise. RNA was extracted both from *Vastus Lateralis* biopsy and serum samples obtained from the same subjects. All the samples were provided by the University of Manchester involved in the project. The protocol received the approval of the local ethical committee. miRs profiling was conducted using Applied Biosystems platform (Card A, about 370 miRs). Based on the results obtained by profilings, we identified miRs up-and down-regulated both in muscle and serum comparing all the different times. The myoMiRs -1,-133a, -206 were validated in real-time RT-PCR together with miRs modified both in muscle tissue and blood at different times, such as miR -132, -365,-18a. Circulating miRs resulted to have a very low concentration in comparison with those found in the tissue. Data obtained highlight the relevance of myoMiRs in the skeleton muscle both during the process of atrophy and its reversibility by physical

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exercise. Further studies are on going to validate the most up- and down- regulated miRs, both at tissue-specific and systemic level, aimed to identify the molecular pathways involved in muscle atrophy and its reverse process.

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Dissecting the possible role of CXCR4 pathway in muscle loss induced by cancer

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Cancer cachexia is a life-threatening syndrome characterized by severe body weight loss, due to depletion of adipose tissue and skeletal muscle, and affects up to 80% of patients with advanced cancers. The rapid loss of muscle mass is the main cause of function impairment, fatigue and respiratory complications, leading to death in 20-48% of cases. To date, no effective treatment is available.
Interestingly, previous microarray analysis has identified a subset of genes whose expression is specifically altered in cachectic muscles of hepatoma-bearing rats. The recent development of novel softwares to analyze long list of genes, like Ingenuity Pathways Analysis software, enabled us to find a gene signature suppressed specifically in rat muscles atrophying because of cancer (and not because of diabetes or fasting or disuse): CXCR4 pathway.
To test if suppressing this pathway is sufficient to drive muscle atrophy, we treated fully differentiated C2C12 myotubes with the inverse agonist AMD3100 and measured fiber diameter as index of muscle atrophy. Importantly, neither 48h-treatment with AMD3100 (up to 1 ug/ml) nor with the agonist SDF1 (up to 200 ng/ml) of CXCR4 receptor causes evident myotube toxicity. Interestingly, myotubes treated for 24 or 48h with 0.25, 0.5 or 1 ug/ml AMD3100, but not with SDF1, displayed decreased diameter by 7-18%. Ongoing experiments in cultured myotubes and *in vivo* adult mouse muscles aim at dissecting the possible role of this pathway in muscles during cancer cachexia.
In conclusion, our preliminary data show that attenuating CXCR4 pathway recapitulates muscle atrophy at least in cell culture.

PGC-1α counteracts cancer-induced muscle wasting in female mice

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PGC-1α overexpression in the skeletal muscle produces changes typically associated with endurance exercise including: mitochondrial biogenesis, fiber type switch (fast to slow) and resistance to fasting or denervation-induced atrophy. Aim of this study was to investigate if PGC-1α hyperexpression prevents muscle wasting in experimental cancer cachexia.
C57/BL6 male and female wild-type or PGC-1α transgenic mice were divided into two groups: controls (C) and tumor-bearers (TB). TB received subcutaneously $1*10^6$ Lewis Lung Carcinoma cells. All the animals were sacrificed 28 days after.
In males, tumor mass was significantly higher in transgenic than in wild-type mice. Gastrocnemius weight was significantly reduced in both C and TB transgenic mice vs. wild-type animals. Tibialis mass equally reduced in both wild-type and transgenic TB; in the same muscle, fiber cross sectional area (CSA) was only slightly improved in transgenic vs. wild-type TB. In females, tumor mass was comparable in transgenic and wild-type mice. Gastrocnemius weight was equally reduced in both wild-type and transgenic TB. Tibialis mass was reduced only in TB wild-type mice while was unchanged in TB transgenic mice with respect to C. Consistently, CSA decrease occurring in wild-type TB was prevented in transgenic TB.
These results suggest that the enhanced oxidative capacity induced by PGC-1α overexpression in the skeletal muscle improves cancer-induced muscle wasting in female but apparently not in male mice. Such discrepancy could be partially explained by the different tumor mass. Further studies are needed to clarify the effective role played by PGC-1α overexpression in the prevention of cancer-induced muscle wasting.

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Regulation of the myogenic potential of muscle stem cells in cachexia

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Cachexia, a debilitating condition characterized by extreme skeletal muscle wasting, contributes significantly to morbidity and mortality. Efforts to elucidate the underlying mechanisms of muscle loss have predominantly focused on events intrinsic to the myofiber. In contrast, less regard has been given to potential contributory factors that take place outside the fiber within the muscle microenvironment. By exploiting two different models of cancer-cachexia consisting in mice bearing ectopically transplanted carcinoma cells (i.e. C26-bearing BALB/c and LLC-bearing C57/BL6 mice), we investigated which factors affect myogenic differentiation by a combination of *in vivo* and *in vitro* studies, the latter performed by culturing C2C12 cells in the presence of cachectic mouse serum. We have shown that cachexia is characterized by a deregulation of myogenic cell potential leading to the loss of muscle regeneration, which ultimately contributes to muscle wasting. A chronic up-regulation of the Pax7 gene, which inhibits MRF transcriptional activity, is at least in part responsible for such a loss of stem cell myogenic potential. We have shown that Id3 over-expression is sufficient to inhibit myogenin expression in C2C12 and that TNF upregulates Id3 expression; since the latter is a transcriptional target of Pax7 we hypothesize that is could mediated the Pax7-dependent block of MRF activity. TNF is sufficient to mimic cachectic serum of LLC-bearing mice, in as much as both totally inhibit C2C12 differentiation *in vitro*. Interestingly, this inhibitory effect does not occur in the presence of C26-bearing mice serum, which contains mostly IL-6. Implications from this observation will be discussed.

Are myopathy and altered protein metabolism predictive factors for relapse in colorectal cancer?

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Skeletal muscle is the major reservoir of body proteins and it can be particularly affected in conditions associated to altered protein turnover and metabolism such as cancer. In muscle biopsies from patients (n=50) affected with colorectal cancer at diagnosis, we observed a high percentage of abnormally nucleated myofibers (median 13.1%) in the absence of muscle atrophy and local tissue inflammation, but inversely correlated with node metastasis ($p<0.05$). These features were not observed in a group of muscle biopsies from non oncologic patients (n=25), (median=3%, $p<0.0001$ vs oncologic patients). Analyses on serum samples collected prior to surgery testing markers of systemic inflammation, muscle enzymes, prealbumin, and albumin as marker of protein turnover, showed that in the absence of systemic inflammation, in the prevalence of cancer patients the levels of either prealbumin and albumin were below the normal range and the mean values were significantly lower compared to that detected in non oncologic patients. During the 3 years follow-up, the 42% of cancer patients showed disease relapse. Interestingly, in these patients, the percentage of abnormally nucleated myofibers and the serum levels of protein turnover biomarkers were lower, even though not significantly, compared to that observed in patients with no disease worsening. Up to now, our data indicate that myopathy and altered protein turnover appear to be associated with an early stage of cancer. In addition, myopathy seems not to be associated with disease worsening, while malnutrition seems to be predictive of colorectal cancer relapse.

SESSION 8
MUSCULAR DYSTROPHIES, RELATED DISEASES AND CANCER (I)

Authophagy in DMD progression and response to HDACi

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Although the genetic basis of Duchenne muscular dystrophy (DMD) has been known for over two decades, the pathogenic mechanism underlying the disease progression remains obscure, and this gap of

knowledge has prevented the development of effective therapeutic approaches. We have shown that pharmacological treatment with histone deacetylase inhibitors (HDACi) improves dystrophic muscles, and this finding inspired the current clinical trial with the HDACi Givinostat in DMD patients. However, a more detailed understanding of the mechanism of action of these drugs is required for further development of combinatorial strategies that optimize the therapeutic efficacy. Emerging evidence indicates that autophagy is crucial in maintaining muscle mass and myofiber integrity by removing dysfunctional organelles and restore tissue homeostasis. Still, the contribution of autophagy in DMD pathogenesis and disease progression has not been elucidated. Our data show deregulated autophagic activity in muscles of mdx mice during disease progression, supporting the hypothesis that the autophagy is involved in DMD pathogenesis. By feeding young mdx mice with a defined diet that increases autophagy we observed a beneficial effects on muscle morphology and regenerative potential of muscle stem cells (MSC). In particular, we observed that diet-induced autophagy enhanced the ability of fibro-adypogenic precursors (FAPs) to promote the myogenic potential of MSC. Moreover, our data suggest that low protein diet in old mdx mice, which are typically refractory to HDACi treatment, might influence MSC regeneration potential. Our data support the notion that autophagy is actively contributing to DMD pathogenesis and modulation of autophagy can be used to enhance the efficacy of pharmacological treatment with HDACi. More in general, our results indicate the importance to optimize a nutritional scheme as a part of the treatment of HDACi.

Reactivation of autophagy by spermidine ameliorates the dystrophic phenotype of collagen VI null mice

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Collagen VI (ColVI) is an extracellular matrix glycoprotein particularly abundant in skeletal muscles. In humans, genetically inherited ColVI deficiency leads to several muscle diseases. ColVI null (Col6a1-/-) mice display an early onset myopathic phenotype characterized by dysfunctional mitochondria, dilated sarcoplasmic reticulum and spontaneous apoptosis. We previously showed that the persistence of altered organelles in ColVI deficient skeletal muscles is due to an autophagy impairment. The drugs individuated so far as potential therapeutic agents, e.g. cyclosporin A and rapamycin, are known to have several side effects which may overcome their beneficial effect during long-term or chronic treatments. Spermidine is a non-toxic, naturally occurring cationic polyamine recently highlighted as a potent autophagy inducer. We treated Col6a1-/- mice with spermidine administered intraperitoneally at 5-50 mg/kg/day for 10 days, or added to drinking water at 3-30 mM for 30 days. Our results show that spermidine is able to reactivate autophagy in a dose-dependent manner, leading to a concurrent amelioration of the histological features and a significant decrease of myofiber apoptosis in dystrophic muscles. Wild-type animals treated with spermidine do not display any major sign of sufferance or muscle changes, except for a slight increase of apoptotic myofibers. Spermidine may be useful not only in the treatment of human ColVI-related muscular dystrophies, but also to counteract other pathologies characterized by an impairment of autophagy. Intracellular concentrations of this drug could be increased not only by direct administration, but also by feeding patients with spermidine-enriched food like soy beans or mushrooms.

Cellular and molecular effectors of HDACi activity in the treatment of Duchenne Muscular Dystrophy

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Previous studies reported on the ability of histone deacetylase inhibitors (HDACi) to exert beneficial effects in dystrophic muscles by promoting endogenous regeneration and preventing fibro-adipogenic degeneration. Preclinical studies in mdx mice demonstrated a dose-dependent efficacy of Givinostat in mdx mice and provided the basis for an immediate traslation of HDACi into clinical studies with DMD patients. However the molecular effectors of such activity are still unclear. We have recently identified a population of muscle-interstitial cells, referred to as fibro-adipogenic progenitors (FAPs), that mediates the beneficial effects of HDACi in young mdx mice. FAPs isolated from young mdx mice or dystrophic children show a "latent" myogenic phenotype that is implemented by HDACi at the expense of their fibro-adipogenic potential. Moreover HDACi enhance FAP ability to promote differentiation of adjacent satellite cells. To investigate the molecular basis of HDACi effects on FAPs, we have used a combination of assays including gene expression microarray, RNA-seq and nuclease accessibility (NA)-seq, that identified an HDACi-responsive network

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composed by HDAC-repressed miRNAs, which control the composition of the SWI/SNF chromatin-remodeling complex and direct its activity to toward promoting a pro-myogenic phenotype instead of fibro-adipogenesis. We show that HDACi de-repress BAF60C and myomiRs (miRs 1.2, 133 and 206) in FAPs from regenerating muscles of young mdx mice, leading to downregulation of BAF60A and B and the preferential formation of BAF60C-based SWI/SNF complex that promotes skeletal myogenesis. We also identified BAF60C as key determinant of the response to HDACi in FAPs from young, but not old, mdx mice. Analysis of BAF60C regulatory regions shows dynamic transitions in chromatin modifications (AcH3, H3K4me3 and H3K27me3) that are highly responsive to regeneration cues and HDACi. In particular, in FAPs of regenerating muscles Baf60c is a bivalent gene sensitive to HDACi that promote histone hyperacetylation and chromatin accessibility leading to Baf60c expression.

Mir34a and mitochondrial impairment in a model of Duchenne muscular dystrophy: identification of a possible novel therapeutic strategy

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During the years we identified Nitric Oxide donors as good candidate molecules for Duchenne Muscular dystrophy therapy.
Nitric oxide regulates mitochondrial activity, morphology and dynamics; many types of dystrophies in which nitric oxide generation is defective display mitochondrial alterations. We decided to evaluate in detail mitochondrial structure and activity in αSG$^{-/-}$ mice. We found a severe reduction in mitochondrial content in both tibialis anterior and diaphragm muscles.
Mitochondrial biogenesis was impaired in αSG$^{-/-}$ mice, also under cold stimulation, with high acetylation levels of PGC1-α and reduced expression of Sirt-1, leading to low Oxphos capacity of fibers. This mitochondrial impairment was associated to high production of Fgf21, a mitokine which is also a biomarker of mitochondrial disease.
We also found that this phenotype was due to high expression of a miRNA, miR-34a, that controls both Sirt-1 levels and Fgf21 functions downregulating the Fgf21 coreceptor β-klotho and leading to dysregulation of Fgf21 signalling. Nitric oxide administration decreased miR-34a levels increasing Sirt-1 expression, enhancing mitochondrial function and triggering a therapeutic fiber switch, with fibers dysplaying higher Oxphos capacity.
After treatment with a nitric oxide donor also the expression level of β-klotho was restored and this accounted for induction, in muscle, of Fgf21-dependent lipid mobilization.
Altogether these results suggest a role of miR-34a in the pathogenesis of muscular dystrophy involving mitochondria and their functions and highlight miR-34a and mitochondria as potential targets of therapies.

Heregulin-induced Utrophin upregulation promotes recovery of muscle homeostasis in mdx mice transplanted with microencapsulated sertoli cells

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Duchenne muscular dystrophy (DMD) is an X-linked genetic disease characterized by progressive muscle degeneration ultimately leading to impaired locomotion and premature death. Due to the associated chronic inflammation DMD patients are treated with antiinflammatory steroids, albeit with limited efficacy and undesired side effects. We found that a single intraperitoneal (i.p.) transplantation of microencapsulated porcine Sertoli cells (SCs) [1] in *mdx* mice, an animal model of DMD, rescues muscle morphology and performance in the absence of pharmacological immunosuppression. Compared with muscles of mock-treated mice, muscles of SC-treated mice show a dramatic reduction of infiltrated inflammatory cells and fibrosis. While modulating the inflammatory response, SCs induce upregulation of the dystrophin paralogue, utrophin in muscle tissue thus promoting sarcolemma stability. We found that heregulin β1, a neuregulin-1 polypeptide capable of transactivating utrophin promoter and to ameliorate the dystrophic phenotype when i.p. injected in *mdx* mice [2], is expressed and secreted by porcine SCs. SC-treated *mdx* mice injected with a heregulin β1 blocking antibody fail to recover muscle morphology but show a marked reduction of infiltrated (MAC3^{+}) macrophages as in the absence of antibody treatment, suggesting that

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the reduction of inflammation is a SC-mediated effect independent of heregulin β1-induced recovery of muscle morphology. Thus, i.p. transplantation of SCs in dystrophic mice restores muscle homeostasis due to combinatorial effects. Our results open new perspectives in the treatment of patients affected by DMD or related diseases.

[1] Luca et al. *Tissue Eng* 13(2007):641-8; [2] Krag et al. *Proc Natl Acad Sci USA* 101(2004):13856-60.

A tissue engineering approach via acellular matrix to reshape the pathological diaphragm of *HSA-Cre, Smn$^{F7/F7}$* mouse model

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Impairment of extracellular matrix (ECM) is one of the major pathological features of myopathies. In particular, myopathic limb and respiratory (e.g. diaphragm) muscles become fibrotic and weak, bringing to death by respiratory failure. *HSA-Cre, Smn$^{F7/F7}$* animals present a specific impairment of the skeletal muscle and their diaphragm displays fibrosis and myofiber loss. Since acellular ECM scaffolds retain important bioactive molecules, we aimed at ameliorate diaphragm muscle condition of *HSA-Cre, Smn$^{F7/F7}$* mice using a decellularized matrix obtained from healthy-mice diaphragm. We characterized the matrix after detergent enzymatic treatment (DET) establishing that 3 DET cycles are a good compromise between DNA content reduction and ECM preservation. After 3 DET cycles, collagen and elastin content was not statistically different from fresh tissue. Importantly, matrix possessed the same thickness and stiffness of a fresh diaphragm.. *In vivo:* decellularized patches were surgically applied to the diaphragm of *HSA-Cre, Smn$^{F7/F7}$* mice and changes in terms of thickness and morphological/cytological aspects were evaluated. New collagen deposition was noticeable 15 days post implantation with evident features that a remodeling process began. The acellular patch was gradually re-populated during the three time points, with an increasing presence of Ki67+ cells. After 30 days the patch was partially reabsorbed. On the other hand, the weak native diaphragm underwent strong remodeling showing muscle regeneration and increase in thickness.

In conclusion, we successfully developed an acellular diaphragmatic scaffold with preserved ECM able to exert a positive effect when applied in a myopathic diaphragm, influencing local cellular activation, turnover and matrix composition.

Muscle stem cells in Spinal Muscular Atrophy

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Spinal muscular atrophy (SMA) is a common human inherited disease characterized by degeneration of motoneurons (MN) and muscle atrophy. This pathology is due to mutations of the survival of motor neuron gene (Smn1) which code for an ubiquitously expressed protein that has been involved in various cellular processes including cytoplasmic assembly of snRNP into the spliceosome and pre-RNA splicing, and more recently in stem cells function.

We are involved in the development of gene therapy strategies for SMA based on the delivery of self-complementary AAV9-SMN1 vectors (scAAV9-SMN1). We and others have demonstrated that a single intravenous injection induced a tremendous rescue of SMNΔ7 mice, a model of SMA. However, despite the restoration of SMN expression in the central nervous system (CNS) and peripheral organs,this strategy did not allow the complete rescue of the treated mice (mean life expectancy ~200 days).

Although SMA has traditionally been considered as a pure lower MN disease, growing evidences suggest that peripheral organs including skeletal muscle, are also vulnerable to reduced levels of SMN.In order to investigate a potential dysfunction of muscle progenitors in SMA, we studied two populations of muscle resident progenitors : satellite cells and PW1+ interstitial progenitors (called PICs), in two mouse models of SMA (the SMNdelta 7 and the more severe hSmn2 model). Our preliminary data strongly support a possible pathogenic role of muscle stem cells dysregulation in SMA.

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Myo-ALS: the role of skeletal muscle in the pathogenesis of neuromuscular diseases

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Mutations in Tar-DNA binding protein-43 (TDP-43) were recently identified in patients with familial ALS and Inclusion Body Myositis (IBM). TDP-43 is a regulator of gene expression and is involved in RNA processing and functioning, including splicing, stability, transport, translation and microRNA maturation. To better understand the specific role of TDP-43 on muscle homeostasis we plan to generate a new transgenic mouse in which the hTDP-43 cDNA, containing the A315T mutation, will be placed under the transcriptional control of muscle specific promoter (MLC). This mouse will be analyzed for histological, molecular and functional parameters. We propose to identify the alterations induced by ALS-associated TDP-43 mutant protein and to monitor the functional and structural alterations of muscle, neuromuscular junction (NMJ), nerves and of major intracellular organelles. Moreover Chromatin Immunoprecipitation (ChiP) analyses with TDP-43 antibody will be carried out to identify specific RNA targets of this protein in skeletal muscle. In addition, to define whether mutant hTDP-43 causes alteration on myogenic program we will analyze transfected C2C12 cell line with the mutated isoform of human Tar-DNA binding protein-43 (TDP-43A315T) driven by MLC promoter.

SESSION 9
MUSCULAR DYSTROPHIES, RELATED DISEASES AND CANCER (II)

miR-206 detection on muscle sections by hybridization *in situ* assay

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Previous data show that class 1 and 2 HDAC inhibitors (HDACi) are able to prevent muscle degeneration and promote regeneration in dystrophic muscles of young *mdx* mice *(Minetti et al., 2006).* We have recently isolated and characterized a population of muscle interstitial cells (referred to as fibroadipogenic progenitors – FAPs that mediate the beneficial effect of HDACi on dystrophic muscles *(Mozzetta et al., 2013).* HDACi promote a lineage switch in FAPS by promoting the myogenic phenotype, rather than the fibroadipogenic one. We show that HDACi activate a network of miRNA that changes the composition of the SWI/SNF chromatin remodeling complex in FAPs, by de-repressing myomiRs (miR-1,miR-133 and miR-206), which in turn target two alternative BAF60 subunits (BAF60A and B) thereby favoring the formation of BAF60C-based SWI/SNF complex that promotes the pro-myogenic phenotype. We have set an *in situ* hybridization assay that reveals the expression of miR 206 in FAPs from dystrophic muscles exposed to HDCAi. We propose that myo-miR detection in FAPs can be used a biomarker of HDACi activity that can be used in the currently undergoing clinical trial based on treatment of dystrophic boys with the HDACi Givinostat.

Validation of PTRF/cavin-1 expression and plasma membrane association with caveolin-1 in human cell lines and mouse primary tumor cultures of rhabdomyosarcoma

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PTRF/cavin-1 is a protein that cooperates with caveolins (Cav-1, Cav-2 and Cav-3) to the morphogenesis of membranous caveolar domains in different cell types. In this study we investigated its expression in rhabdomyosarcoma (RMS), an aggressive soft-tissue myogenic tumor that mainly affects children and adolescents. Our expression studies performed by RT-PCR and Western blot analysis showed that PTRF/cavin-1 is mainly detectable, in concomitance with Cav-1 and Cav-2, during the in vitro growth of human RMS cell lines and two primary tumor cultures established from RMS developing in conditional mouse models. In contrast, the variable and limited degree of myodifferentiation in the different RMS lineages was characterized by down-regulation of PTRF/cavin-1, Cav-1 and Cav-2 and by the increment of Cav-3. Confocal microscopy analysis showed that PTRF/cavin-1 has a broad cellular distribution in RMS cells, ranging from nuclei and cytosol to plasma membrane. In addition, immunoprecipitation analysis showed that PTRF/cavin-1 and Cav-1 co-localize and physically

interact at the plasma membrane, suggesting that this close association may play an important role in the progression of RMS.

p38alpha kinase targets the chromatin of rhabdomyosarcoma cells to epigenetically repress microRNA-containing loci and induce muscle-like differentiation

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Rhabdomyosarcomas (RMSs) are a heterogeneous group of fast growing highly malignant tumours that are thought to arise from muscle precursors. Despite expressing the muscle regulatory factor MyoD, RMS cells do not activate the skeletal muscle program in response to differentiation stimuli. We and others previously showed that during muscle regeneration p38alpha kinase converts inflammatory signals into the epigenetic cues that control gene expression. In addition, in some RMS cell lines and tumours an impaired activation of the pathway has been described and it can be restored by over-expressiony of the active form of the upstream kinase, MKK6EE.To investigate the mechanism by which active p3□alpha induces differentiation in RMS we performed ChIP-seq and mRNA-seq analysis in Rh30 RMS cells, over-expressing MKK6EE. ChIp-seq analisy showed that the majority of phospho-p38alpha binding sites are included in a small number of loci encoding for clusters of microRNA (miRNA). MiRNAs are small non-coding RNAs that mediate post-transcriptional repression through sequence-specific binding to target mRNAs. A careful analysis uptaking miRNA expression database of RMS patients (http://www.oncomiR.umn.edu/SMED) indicated that the p38alpha-targeted miRNAs are over-expressed in RMS as compared to normal muscle. Further, we show here that, consistent with the induction of a muscle-like phenotype, ectopic activation of p38alpha in Rh30 cells represses the expression of these miRNAs through an epigenetic mechanism that involves the Polycomb Repressive Complex 2 (PRC2). Altogether our data suggest that p38alpha-regulated miRNAs may contribute to RMS pathogenesis and could represent a novel pharmacological target against these tumours.

Novel experimental tools to study the interplay between muscle and motor neurons

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One of the crucial systems affected in several neuromuscular diseases is the loss of connection between muscle and nerve, leading to a pathological non-communication between the two tissues. One of the best examples of impaired interplay between the two tissues is the disease Amyotrophic Lateral Sclerosis (ALS). This project intends to dissect the molecular mechanisms underlying the functional connection between muscle and nerve. The main goals are: a) to identify the molecular alterations taking place in muscle and nerves associated with ALS; and b) to define how the progression of a pathological condition in skeletal muscle will eventually affect NMJ and motor neurons. To achieve this we will develop new cell culture technologies to study this interplay. Myoblasts as C2C12, C2C12 SOD1 G93A and skeletal muscle primary cultures from wild type and transgenic animals will be cultured in vitro with motor neurons isolated from the ventral spinal cord from embryonic day 15-16 mouse. We will use two different systems of co-culture: in the first motor neurons will be seeded on myotube cultures while in the second we will use compartmented cultures. Motor neurons will be also cultured with a three dimensional culture system (X-MET), able to reproduce the structure and function of adult skeletal muscle without using any scaffold or any specific chemical layer. All these co-cultured systems will be analyzed by both immunocytochemical and electrophysiological techniques in order to study their biological and electrical properties.

Sex steroid hormones, microRNAs and IGF-1 signalling as regulators of muscle aging

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Aging-related deterioration in endocrine function, especially decrease in systemic female sex hormone

estradiol (E_2), affects skeletal muscle properties. Another important pathway regulating muscle aging is insulin like growth factor 1 (IGF-1), which interacts with E_2-signalling. MicroRNAs (miRs) are regulatory elements likely to mediate muscular responses to, e.g., physical exercise and hormones. However, the role of miRs in the crosstalk between E_2 and IGF-1 signalling is unknown. We hypothesized that E_2-sensitive miRs regulate the activity of IGF-1 pathway thereby affecting muscle aging in females. Two mouse models were used: sham-operated (CO) and ovariectomized (OVX) young mice and ovarian senescent (OVF) and OVF plus E2-supplemented old mice. MiR and gene expression were studied in gastrocnemius muscles. Our preliminary data indicate E2-responsiveness of miR-142-3p but not miR-223. However, the IGF-1R, which is known to be targeted by miR-223, was affected. The expression of IGF-1R was significantly lower in old mice without and with E2 compared to the young OVX mice ($p=0.035$ and 0.018, respectively). The mRNA expression of FOXO1, predicted target for miR-142-3p and -223, did not follow their expression. FOXO1 expression was significantly higher in OVF with E_2 compared to OVX mice ($p=0.033$). Furthermore, the mRNA expression of AKT, a kinase which phosphorylates FOXO1 thereby inactivating it, was highest in samples with lowest FOXO1 expression. Our results indicate that miR-based regulation is involved in muscle aging. Although in addition to miR-142-3p and -223 there seems to be additional factors affecting E2 and IGF-1 signaling in mouse muscle. Therefore further studies are warranted.

Role of HSPB8 in a muscular model of amyotrophic lateral sclerosis (ALS)

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The aggregation of misfolded, mutated proteins (Superoxide-Dismutase-1, SOD1; TAR-DNA-binding-protein-43, TDP-43) is a pathological hallmark of familial forms of Amyotrophic Lateral Sclerosis (ALS), suggesting that the protein quality control system (molecular chaperones and the degradative systems ubiquitin-proteasome and autophagy) is saturated or not sufficient to maintain a correct proteostasis. Motoneurons are particularly sensitive to misfolded protein toxicity, but also other cell types could be affected, suggesting that ALS is a non-cell autonomous disease. So far, whether motoneuron and muscle degeneration involve the same mechanisms is not clear. Our previous data strongly suggest that muscle cells better manage misfolded mutated-SOD1 species than motoneuronal cells due to their higher degradative power and that mutated-SOD1 largely accumulates in spinal cord, but not in muscle of transgenic ALS mice. In the present study we compared the capacity of motoneuron and muscle cells to respond to proteotossic stress (e.g: proteasome and autophagy inhibition). We found that muscle cells activate the autophagy markers LC3 and p62 and the chaperone HSPB8 at higher extent than motoneuron cells. Interestingly, the overexpression in muscle cells of mutated-TDP43 (which does not aggregate) increases the levels of HSPB8 and the knock-down of HSPB8 increases the aggregation of both wild-type and mutated-TDP43, suggesting its primary role in TDP43 turn-over. These data together with the observation that HSPB8 is upregulated in muscles of ALS transgenic mice only at symptomatic stage strongly suggest HSPB8 as a potential modulator of ALS and that its boosting may represent a valid therapeutic approach.

GRANTS: AriSLA; Foundation Thierry Latran, France; AFM-France; Regione Lombardia.

SESSION 10
SATELLITE CELLS AND MUSCLE REGENERATION

Lysophosphatidic acid stimulates migration of murine satellite cells: a role for sphingosine kinase/S1P signaling axis.

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Literature data support the concept that the pleiotropic bioactive lipid lysophosphatidic acid plays a role in the regulation of skeletal muscle cell biology, even if data available so far are largely incomplete. In this study we report that LPA acts as powerful chemoattractant of satellite cells isolated from mouse tibialis anterior muscle, suggesting that by favoring migration of activated satellite cells at the site of muscle injury, LPA can play a critical role in the complex biological process of skeletal muscle repair. Among the known LPA-specific receptors, LPA1 and LPA3 were found mainly responsible for transducing the signal that evokes the observed biological response. Moreover, sphingosine kinase/S1P signaling axis was found

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implicated in the transmission of the pro-migratory action of LPA, since pharmacological inhibition or genetic silencing of sphingosine kinase-1 fully inhibited the effect exerted by LPA. These results for the first time demonstrate that cross-talk between LPA and S1P signaling pathway plays a role in the regulation of biological activity of satellite cells, reinforcing the notion that control of endogenous bioactive lipid biosynthesis and signaling is critical for the physiological functioning of skeletal muscle cell precursors.

MEF2C phosphorylation: a regulatory mechanism in satellite cells

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MEF2C belongs to the family of Myocyte Enhancer Factor 2 transcription factors, which activate the muscle-specific gene expression program synergistically with the bHLH Muscle Regulatory Factors. In skeletal muscle cells MEF2C is expressed already at the proliferating stage, but its transcriptional activity is silent, highlighting that its function is finely regulated at several levels, including alternative splicing patterns, post-translational modifications and protein-protein interactions with regulators. Several studies showed that phosphorylation of MEF2 factors at so-called Ser/Thr-Pro motifs can modulate protein function through the induction of conformational changes by the peptidyl–prolyl cis/trans isomerase Pin1. We identified two novel critical phosphorylation sites in MEF2C, Ser98 and Ser110, located in the alternative spliced exon α1 and essential for the binding with the negative regulator Pin1. Using two polyclonal phosphor-specific antibodies, we observed that the phosphorylation of Ser98 and Ser110, which negatively affects MEF2C function, decreases upon induction of muscle differentiation, coherently with the hypothesis of phosphorylation-based negative regulation of MEF2C. We confirmed the same regulatory mechanism also in primary myogenic stem cells (SCs) by the combined analysis of the dynamic of MEF2C phosphorylation with the study of the alternative splicing pattern in satellite cells retained in their niche associated with isolated myofibers. Taken together our results lead us to suppose that MEF2C phosphorylation on the Pin1 binding sites and the consequent interaction with Pin1 might contribute to keep silent the MEF2C-dependent transcription of muscle specific genes in proliferating SCs avoiding their premature differentiation and allowing the expansion of the activated SCs pool.

SoxF Genes as Important Transcriptional Regulators of Satellite Cell Behavior through their Interaction with β-Catenin

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Around birth, fetal muscle progenitor cells adopt a satellite cell position. The emerging satellite cells enter quiescence, a molecular state poorly characterized *in vivo*, which can be altered during post-natal growth or in response to injury. We were interested in identifying new molecular pathways involved in the progression from a proliferating population to a quiescent post-natal progenitor cell population. Pax3 is a paired-homeobox transcription factor expressed in muscle progenitor cells throughout development, including post-natal satellite cells. After performing expression profiling of *Pax3*$^{GFP/+}$ cells during development and post-natally, we have identified new transcription factors, the SoxF genes (Sox7, Sox17, Sox18), which are induced at the onset of satellite cells emergence.

SoxF genes are members of the SOX family of transcription factors with a trans-activation domain C-terminal to the HMG box. They are involved in the regulation of embryonic development and in the determination of the cell fate. After functional analysis of these genes in the satellite cells, we observe that their over-expression increase the pool of quiescent Pax7+ve cells, consistent with an increase of proliferating satellite cells when using dominant negative constructs for these genes. Conditional ablation of Sox17 in the myogenic lineage in post-natal mice leads to an increase in the number of muscle fibers but with smaller size, and more interestingly, a reduction of satellite cell numbers due to a loss of quiescence. We further show that this effect is probably mediated by the direct interaction of β-catenin with SoxF genes, through a β-catenin binding site, regulating cell proliferation.

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Towards pharmacological modulation of Cripto in skeletal muscle regeneration

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We have recently provided new insights in TGF-β ligand-specific signaling by Cripto on satellite cells, showing that inactivation of *cripto* in adult satellite cells compromises skeletal muscle regeneration. Moreover we demonstrated that Cripto promotes myogenic committment of satellite cells and that it is mitogenic for satellite cell-derived myoblasts. These notions support the model in which Cripto possesses intrinsic activities as a transacting factor on satellite cells both in cell culture and *in vivo*. In line with this idea, we demonstrated that the viral- mediated overexpression of a soluble form of Cripto (sCripto) is able to rescue the effect of the genetic ablation of *cripto* in the adult satellite cell compartment on muscle regeneration, thus providing direct evidence that sCripto was able to fully recapitulate the function of endogenous membrane Cripto. Furthermore, sCripto overexpression improves muscle regeneration that normally occurrs in a model of acute injury, increasing myofiber CSA area. In order to investigate whether recombinant sCripto could be used as a pharmacological approach to improve skeletal muscle regeneration, we perfomed preliminary experiments by injecting sCripto intramuscularly (IM) or by using biodegradable microcarriers (gelatin hydrogel microspheres), a system that enables the protein to maintain its activity over a prolonged period of time, allowing to control the release kinetics of the protein. Our preliminary results show that myofiber CSA area significantly increased in sCripto treated mice compared to control, supporting the idea of using sCripto as biopharma either alone or in combination with other molecules to stimulate muscle regeneration mobilizing endogenous stem cells.

Influence of cyclosporine A treatment on stemness and apoptosis of muscle satellite cells

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Collagen VI (ColVI) is an extracellular matrix protein forming a microfilamentous network in various tissues. Mutations of ColVI genes in humans cause various muscle diseases, including Bethlem Myopathy and Ullrich Congenital Muscular Dystrophy (UCMD). Mice lacking ColVI (*Col6a1*$^{-/-}$) display a myopathic phenotype with mitochondrial dysfunction, defective autophagy and spontaneous apoptosis of muscle fibers[1,2,3]. Moreover, *Col6a1*$^{-/-}$ mice show impaired muscle regeneration and defective self-renewal ability of satellite cells (SCs) after injury[5]. Previous work on muscle biopsies and primary cultures of UCMD patients revealed the presence of mitochondrial dysfunction and spontaneous apoptosis, which could be normalized by seeding cells onto purified ColVI or by pharmacological treatment with cyclosporine A (CsA). Interestingly, CsA treatment in younger UCMD patients also led to a significant increase in the number of regenerating myofibers[4,5]. Based on these findings, we investigated the effect of CsA on satellite cells, the main player of muscle regeneration. Towards this aim, we performed *in vitro* studies with isolated single myofibers derived from EDL muscles of wild-type and *Col6a1*$^{-/-}$ mice, and cultured in proliferating media supplemented with CsA. Cells were classified into three populations (Pax7^{+}MyoD^{-}, Pax7^{+}MyoD^{+} and Pax7^{-}MyoD^{+}) and the effects of CsA were compared with the known effects of culture on purified collagen VI[5]. Our results show that cyclosporine A promotes the survival of *Col6a1*$^{-/-}$ satellite cells, by decreasing apoptosis without affecting their proliferation. Based on these findings, we will carry out further studies on the effects of cyclosporine A on the survival and stemness of satellite cells *in vivo*.

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HDAC4 is necessary for satellite cell differentiation and muscle regeneration

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In response to injury, skeletal muscle has high capacity to regenerate: quiescent muscle progenitor cells begin to proliferate and express transcription factors for muscle specification. In vitro has been demonstrated that completion of muscle differentiation requires shuttling of histone deacetylase 4 (HDAC4), a member of class IIa HDACs, from the nucleus to the cytoplasm and consequent activation of MEF2-dependent

differentiation genes. In vivo, HDAC4 expression is up-regulated in skeletal muscle upon injury and in muscular dystrophy, suggesting a role for this protein in muscle regeneration. With the aim to elucidate the role of HDAC4 in skeletal muscle regeneration, we generate mice lacking HDAC4 in the satellite cells ($HDAC4^{fl/fl}$;$Pax7^{CE}$ Cre). We show that HDAC4 null satellite cells start earlier but cannot fully complete the differentiation program in respect to controls. In addition, we found that $HDAC4^{fl/fl}$;$Pax7^{CE}$ Cre mice, upon double freeze injury, show impaired regeneration than control mice, highlighting the importance of HDAC4 in satellite cells renewal and/or ability to differentiate. To further study the role of HDAC4 in muscle regeneration, we generate mice with the deletion of HDAC4 driven by myogenin promoter (HDAC4mKO). By analyzing skeletal muscle at different time points after freeze-injury, we found that HDAC4mKO mice show regenerating fibers smaller than control one. These data were confirmed also by molecular analyses of expression of myogenic markers. All together, these data delineate the importance of HDAC4 in muscle regeneration and suggest a protective role in response to muscle damage.

Myogenic microRNAs expression correlates with the ability of pericytes from skeletal and smooth muscle to differentiate in skeletal muscle tissue

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microRNAs mediate several cell processes including cell differentiation. The most studied muscle specific microRNAs, known as myomiRs, are miR-1, miR-133 and miR-206. In this study we investigated whether the expression pattern of these myomiRs could relate with the differentiative potentials of human perivascular pericytes (hPCs) isolated from two different tissues namely skeletal (sk-hPCs) and smooth muscle (sm-hPCs). PCs are thought to represent the ancestor cell population of a wide range of mesodermal precursors situated throughout the body, being able to differentiate along myogenic, osteogenic and adipogenic lineages. As assessed by FACS, IF, RT-PCR and WB we found that sk-hPCs and sm-hPCs express known hPCs markers and do not express myogenic regulators. We found that both sk-hPCs and sm-hPCs are able to differentiate in smooth muscle cells, while only sk-hPCs differentiate in skeletal muscle cells. Quantitative PCR analysis of myomiRs in undifferentiated cell populations indicated that sk-hPCs and sm-hPCs express basal levels of miR-1 and -133. In contrast miR-206 is expressed in sk-hPCs while it is not detectable in sm-hPCs. Following induction of skeletal muscle differentiation miR-1 and miR-133 and miR-206 are significantly upregulated in sk-hPCs while they were significantly downregulated in sm-PCs. It has been recently shown that Linc-MD1 is activated upon myoblast differentiation. Interestingly, we found that Linc-MD1 expression closely correlate with myomiRs expression in sk-hPCs and sm-hPCs.
Taken together our results indicate that skeletal muscle differentiation potential of sk-hPCs and sm-hPCs strictly correlated with the expression of myomiRs and Linc-MD1.

SESSION 11
NOVEL ROUTES TOWARDS THE 'HEART' OF MYOGENESIS

Effects of Relaxin on the electrophysiological properties of fibroblasts: standpoint on cardiac remodelling

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The hormone relaxin (RLX) is a peptide known to influence smooth muscle physiology. RLX is also produced by the heart and has advantageous effects on the cardiovascular system. In addition to the benefits observed in mouse neonatal cardiomyocytes, where it can stimulate their growth, RLX is known to have helpful consequences also in matrix remodelling, implying its involvement in endogenous mechanisms of cardiac regeneration: it should work as an antifibrotic molecule, inhibiting the fibroblast-to-myofibroblast transition. The present work was planned to test the effects of RLX treatment on the electrophysiological properties and different ionic currents of cultured fibroblasts and myofibroblasts, in order to find further evidence for the possible employ of this peptide as an anti-fibrotic agent. Fibroblasts were transformed into myofibroblasts in the presence of 2 ng/ml TGF-β. The electrophysiological features of fibroblasts and myofibroblasts were investigated by the whole-cell patch-clamp. Particularly, we investigated the membrane passive properties and the major ionic currents present in these preparations. Among them, we observed that the inward rectifier K+ current, that is typically increased in size in myofibroblasts respect to fibroblasts, did not change when cells were cultured in the presence of RLX, indicative of its ability to

antagonize the effects of TGF-â on cardiac fibroblast-myofibroblast transition. At the moment, any clinical implications of this research can only be speculated, but the newly RLX- induced condition of the ion channel opening state well correlates with the fibroblast-myofibroblast transition inhibition, suggesting a potential use of RLX in the treatment of myocardial fibrosis.

Optimization of cardiac differentiation methods for pluripotent stem cells

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Patient-specific cardiac tissue is difficult to obtain and not suitable for large-scale screenings. Induced pluripotent stem cells (iPSCs) offer an alternative, patient-specific cell source for basic research and clinical applications. In literature, several protocols have been described to differentiate pluripotent stem cells towards cardiomyocytes in two-dimensional (2D) systems (1). However, these 2D methods show high variability in efficiency both within experimental replicates and among different cell lines and cardiomyocytes are often immature. Recently, a promising cardiac differentiation protocol from pluripotent stem cells has been described by Burridge *et al.* (2) based on embryoid body formation of a defined number of cells in 96-well plates and employing chemically defined media. Here we further optimized this method by timed addition of specific growth factors and/or culture in 3D. We used two human embryonic stem cell lines (ESCs), H1 and H9, and protein-reprogrammed human iPSCs. Preliminary data showed that addition of activin A to 2D-cultured H1 and H9 ESCs during the initial phase of the cardiac differentiation process led to an increased number of spontaneously contracting cardiomyocytes for H9 ESCs. Unexpectedly, the same treatment completely abolished formation of contractile clusters for H1 ESCs. When differentiated in a 3D environment, iPSCs and ESCs displayed higher expression of cardiac myosin heavy chain alpha (MYH6) compared to those differentiated in the typical 2D protocol. However, 3D-differentiated cells still retained expression of pluripotency markers.

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The cardiac differentiation of mesenchymal stem cells from a "PKCe-point of view"

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Protein Kinase Cepsilon **(**PKCε) plays a pivotal role in stem cell proliferation and differentiation and exerts cardio-protective effect in ischemia-reperfusion injury. Although many studies have been performed on physiological and morphological effects of PKCε mis-expression in cardiomyocytes, molecular informations on the role of PKCε in cardiac gene expression are still lacking. We addressed the molecular role of PKCε in mouse cardiomyocytes, rat BMMSCs and adipose advential Sca1+ cells derived from mouse aorta and pulmonary artery. We show that PKCε is modulated in cardiac cells producing an opposite regulation of the genes *nkx2.5* and *gata4* both *in vivo* and *in vitro*. P-ERK1/2 is involved in this pathway, indeed pharmacological inhibition of ERK1/2 rescues the expression levels of both *nkx2.5* and *gata4*, suggesting that a reinforced MAPK signalling is at the basis of the observed inhibition of cardiac gene expression in the PKCε over-expressing hearts. We demonstrate that PKCε is critical for cardiac cell early gene expression evidencing that this protein is a regulator that has to be fine tuned in precursor cardiac cells. On the other side we studied the role of PKCe in differentiation of mesenchymal advential Sca1+ cells towards smooth muscle, cardiac muscle and endothelial fate. We have now preliminary data on this stem cell type whose origin (adipose adventitial tissue) opens new perspectives in cardiac disease therapy. Infact, in humans, adipose tissue is deposited around heart and coronaries. It, thus, could represent a good source for isolation of progenitor cells to regenerate vessels and cardiac tissue.

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MicroRNAs and iPSCs as potential tools for cardiac regeneration in chronic cardiomyopathy

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Muscular dystrophies (MDs) constitute a heterogeneous group of genetic diseases, characterized by progressive degeneration of both skeletal and cardiac muscles. In many MD types, the heart features progressive dilation of the ventricles, hypertrophic remodeling and, eventually, chronic cardiac dysfunction. Muscle degeneration is still not curable, hence novel tools for regenerating the diseased myocardium or for modeling MD cardiomyopathy *in vitro* are gathering growing interest. In this perspective, we are focusing on the translational potential of microRNAs and stem cells. MicroRNAs (miRNAs) are small RNA molecules, able to negatively regulate transcription and/or translation of many key genes in a wide range of biological processes, including the cardiomyogenic fate of somatic and pluripotent stem cells. Many populations of cardiomyogenic somatic stem cells have been described from the mammalian myocardium, including cardiac mesoangioblasts (cMABs). Interestingly, *in vivo* regenerative potential of dystrophic cMABs can be tuned through over-expression of specific miRNAs, both in the short (1) and long term (2). With regards to pluripotent stem cells, an intriguing source for cardiomyogenesis relies recently on induced pluripotent stem cells (iPSCs), obtained by reprogramming of somatic cells. Murine iPSCs can differentiate *in vitro* into beating clusters and mature cardiomyocytes, and their potential is further tunable through small molecules (3). However, little is still known about the translational potential of iPSCs and about putative miRNA-based control of iPSC fate. Therefore, more refined studies are yet required to elucidate *in vivo* relevance of these novel technologies in the context of the regenerative medicine for MDs.

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