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Con il supporto e contributo scientifico di:



SESSION 1

MUSCLE DIFFERENTIATION AND REGULATORY MECHANISMS

1. MYOD REGULATES P57KIP2 EXPRESSION BY INTERACTING WITH A DISTANT CIS-ELEMENT AND MODIFYING A HIGHER-ORDER CHROMATIN STRUCTURE

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The bHLH transcription factor MyoD, the prototypical master regulator of differentiation, directs a complex program of gene expression during skeletal myogenesis. The up-regulation of the cdk inhibitor p57kip2 plays a critical role in coordinating differentiation and growth arrest during muscle development as well as in other tissues. p57kip2 displays a highly specific expression pattern and is subject to a complex epigenetic control driving the imprinting of the paternal allele. However, the regulatory mechanisms governing its expression during development are still poorly understood. We have identified an unexpected mechanism by which MyoD regulates p57kip2 transcription in differentiating muscle cells. We show that the induction of p57kip2 requires MyoD binding to a long-distance element located within the imprinting control region KvDMR1 and the consequent release of a chromatin loop involving p57kip2 promoter. We also show that differentiation-dependent regulation of p57kip2, while involving a region implicated in the imprinting process, is distinct and hierarchically subordinated to the imprinting control. These findings highlight a novel mechanism, involving the modification of higher order chromatin structures, by which MyoD regulates gene expression. Our results also suggest that chromatin folding mediated by KvDMR1 could account for the highly restricted expression of p57kip2 during development and, possibly, for its aberrant silencing in some pathologies

2. REGULATION OF ALTERNATIVE SPLICING DURING MYOGENIC DIFFERENTIATION

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Post-transcriptional and co-transcriptional regulation of RNA processing play a crucial role in generating protein diversity, thus modulating many cellular processes such as cellular differentiation, reprogramming, and responses to external stimuli. RNA binding proteins participate to this fine regulation, by binding to cis-regulatory elements (enhancers and silencers), helping the recruitment of the spliceosome and allowing alternative splicing (AS) of a multitude of exons. Genome-wide analyses have recently highlighted an extensive modulation of AS events during myogenic differentiation of C2C12 cells, suggesting a major role for AS in the acquisition of the muscle protein repertoire. By using C2C12 as model system, we found that several splicing regulators undergo dynamic changes in nuclear abundance during myogenic differentiation. For instance, the polypyrimidine tract binding protein (PTB) is downregulated while nPTB is upregulated. Moreover, we observed an increase of SAFB, EWS and SAM68 at the onset of myogenic differentiation. Strikingly, these RNA binding proteins are reported to interact with transcription factors and to play a role in co-transcriptional AS. Thus, we are currently testing whether these splicing factors interact with myogenic transcription factors in a differentiation-dependent manner. The functional coupling between transcription and alternative splicing is being investigated within key regulated genes, such as the epsilon sarcoglycan (Sgce), which encodes a transmembrane protein component of the dystrophin-glycoprotein complex, linking the actin cytoskeleton to the extracellular matrix. Our studies aim at providing a chart of the splicing factors and the splicing events regulated during myogenesis and at elucidating their interplay with master regulators of muscle differentiation.

3.

THE PECULIAR SKELETAL MUSCLE APOPTOTIC BEHAVIOR

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Apoptosis plays an active role in maintaining skeletal muscle homeostasis (Ferreira et al. *Physiol Res* 2008, 57: 601–611). Its deregulation is involved in several skeletal muscle disorders such as myopathies, disuse and sarcopenia (Dupont-Versteegden, *World J Gastroenterol* 2006, 12:7463–7466). The aim of this work is to further highlight in vitro the apoptotic behavior of C2C12 myoblasts and myotubes. Cell death has been induced by etoposide, staurosporine and hydrogen peroxide and investigated by means of morphological and cytofluorimetric analyses. Myotubes appeared more resistant to apoptotic induction than myoblasts. In myoblasts treated with etoposide, nuclei with chromatin condensation were observed, in the presence of a diffuse DNA fragmentation, shown by TUNEL reaction. The latter appeared also in myotubes, where apoptotic and normal nuclei inside the same syncytium were revealed (D’Emilio et al. *Histol Histopathol* 2010, 25: 21-32). Staurosporine treated-myoblasts evidenced late apoptotic features and a high number of TUNEL-positive nuclei. Secondary necrosis appeared in myotubes, where myonuclei with cleaved DNA coexisted, again, with the normal ones. After H₂O₂ exposure, myotubes, differently from myoblasts, showed a poor sensitivity to cell death. Intriguingly, autophagic granules diffusely appeared in myotubes after each treatment. Moreover, mitochondria appeared better preserved than in myoblasts. Taken together, these findings demonstrate myotube resistance to apoptotic stimuli. Moreover, the presence of independent nuclear domains could explain a slower death of myotubes if compared to mononucleated cells. In addition, autophagy could preserve muscle cell integrity against chemical stimuli, making C2C12 cells, in particular myotubes, more resistant to apoptosis induction.

4.

RELAXIN AFFECTS SPONTANEOUS RHYTHMIC CONTRACTION AND VOLTAGE MEMBRANE POTENTIAL ON THE MOUSE COLON

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Relaxin has been reported to influence gastrointestinal motility in mice acting at either the neurotransmission or the smooth muscle level, depending on the gut segment considered. However, at present, nothing is known about the effects of relaxin on the electrophysiological properties of the gastrointestinal smooth muscle. In the present experiments the effects of relaxin on colonic motility in mice were further investigated and electrophysiological records in a single smooth muscle cell were also performed. For this combined approach, preparations from the proximal colon were mounted in organ baths for isometric recording of the mechanical activity, whereas membrane potential was recorded in current-clamp conditions by single microelectrode inserted in a smooth muscle cell. Colonic preparations exhibited spontaneous contractile activity consisting of rhythmic changes in isometric tension. Relaxin caused a decay of the basal tension, that persisted for the whole time of exposure, coupled by a stable and long-lasting increase in amplitude of the spontaneous contractions. Tetrodotoxin, only abolished the initial phase of the basal tension decay. Electrophysiological records showed rhythmic changes in the resting membrane potential. Relaxin induced the following changes: an early slow hyperpolarisation, followed by a slow spontaneous membrane potential recovery up to a slightly depolarisation and, finally, a late increase of the rhythmic rate of potential waves were observed with sometimes some spikes superimposed. These electrophysiological observations are in agreement with mechanical ones. The present results indicate that relaxin other than acting on the cholinergic neurotransmission, directly influences colonic smooth muscle properties.

5.

DIRECT LINEAGE REPROGRAMMING AS NOVEL STRATEGY TO GENERATE CARDIOMYOCYTES

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The direct lineage reprogramming is a promising strategy in regenerative medicine. Using a mix of exogenous transcription factors is possible to force a direct transdifferentiation to specific lineage without production of precursor cells. The aim of our study is to evaluate the feasibility to transdifferentiate canine skin fibroblasts into cardiomyocytes

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(iCM) from GRMD (golden retriever muscular dystrophy) and healthy dogs. For this purpose we used a mix of transcription factors (GATA4, TBX5, MEF2C), successfully used to transdifferentiate mouse fibroblasts into cardiomyocytes. Exogenous gene expression was obtained using three lentiviral vectors carrying the transcription factor genes and different resistance genes. A tracer gene of cardiac differentiation was also included for in vivo experiments (α MHC-GFP). It has been shown by Srivastava group that the same mix of transcription factors can drive transdifferentiation of mouse fibroblasts into cardiomyocyte. In our hand the expression of those transcription factors induced the expression of late cardiac markers in skin canine fibroblasts. Our experiments demonstrate a direct switch from fibroblast to iCM without any involvement of early cardiac genes. However, iCM were unable to contract spontaneously. This was probably due to an incomplete transdifferentiation process. In vivo experiments demonstrate that iCM can engraft in the heart of SCID/Beige newborn mice. However, further electrophysiological analyses are necessary to confirm ion channel functionality of transplanted iCM.

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6.

ADENOSINE-MEDIATED MODULATION OF THE nAChR-DEPENDENT $[Ca^{2+}]_i$ OSCILLATIONS IN DEVELOPING SKELETAL MUSCLE CELLS

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Adenosine receptors (ARs) are classified into different subtypes (A1, A2A, A2B and A3Rs) and are expressed in neurons and skeletal muscle cells. In the skeletal muscle ARs modulate the blood flow, the glucose uptake and the contractile force. In developing mouse myotubes, before innervation, the autocrine activation of embryonic nicotinic acetylcholine receptors (nAChRs) triggers spontaneous $[Ca^{2+}]_i$ oscillation and contractile activity. Taking into account that adenosine acts as a neuromodulator in central nervous system, here we investigated the possible functional interplay between ARs and embryonic nAChRs during myogenesis. Videomaging experiments revealed that the AR antagonist CGS 15943 (100 nM) blocked spontaneous $[Ca^{2+}]_i$ oscillations. Viceversa, the AR agonist NECA (100 μ M) induced an increase in the percentage of oscillating cells and in the frequency of Ca^{2+} events. In parallel, electrophysiological recordings performed in the cell-attached configuration indicated that CGS15943 reduced the nAChR mean open time, whereas NECA significantly increased it. In addition, electrophysiological recordings in oocytes injected with denervated skeletal muscle membranes showed that the activation of ARs reduced the nAChR-current rundown and slowed the nACh-current decay, while the AR inhibition elicited the opposite effect. These preliminary results suggest a modulatory effect of AR activity on the embryonic nAChR function in developing mouse myotubes. We hypothesize a new potential role for ARs in the regulation of nAChR-mediated events during skeletal muscle differentiation/regeneration.

SESSION 2

SATELLITE CELLS AND MUSCLE REGENERATION

7.

miRNAs regulation in human satellite cells subjected to hypoxic environment

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Hypoxia is an aberrantly low oxygen content in cells, tissues or organs, generally due to the decrease in the atmosphere oxygen pressure. The normal composition of the air (78% N₂, 21% O₂ and 1% trace gases) can be slightly modified according to the altitude. The physiological adaptation of skeletal muscle tissues subjected to low oxygen condition can be studied directly in climbers. However, myogenic cell models can be generated to specifically test hypoxic conditions and evaluate transcriptome and microRNA dysregulations. MicroRNAs (miRNAs) are a class of non-coding regulatory RNAs of ~22 nucleotides in length. A large number of microRNAs is positively regulated in response to low oxygen. In

addition, specific miRNAs control the expression of myogenic factors, i.e. MyoD, Myogenin, Myf5, Pax3 and Pax7. In this study, we investigated the effects of hypoxia on myogenic-related miRNAs using a human satellite cell system. Muscle progenitors were isolated from Vastus Lateralis biopsies from different subjects, after the informed consent was obtained. We cultivated satellite cells in standard (21% O₂) and in hypoxic (3%O₂) condition, evaluating miRNA and myogenic marker expression during cell proliferation and differentiation. We focused on three microRNAs specifically expressed in muscle tissue, miR-1, miR-133b, and miR-206. Our results indicate that oxygen content regulates miRNA expression, and thus modulates satellite cell proliferation and differentiation. This phenomenon could explain, at least in part, the myogenic potential impairment observed in satellite cells isolated from climbers after prolonged exposure to extreme altitude (Mancinelli et al 2011).

8.

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 $\alpha 1$ 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.

9.

REGULATION OF SATELLITE CELL FUNCTION BY CYCLIN D3

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In regenerating adult skeletal muscle, the function of MyoD is critically required to coordinate cell cycle kinetics and terminal differentiation of myogenic progenitor cells. In previous studies we and others have shown that MyoD couples the onset of differentiation with cell-cycle arrest by inducing the cell-cycle inhibitors pRb and p21. However, MyoD also appears to induce the expression of factors deputed to promote cell division, such as cdc6 and cyclin D3. To start investigating the role of cyclin D3 in the control of muscle progenitor cell function, we used RNAi technology to knockdown cyclin D3 protein levels in C2 myoblasts, which resulted in reduced proliferation, premature expression of differentiation markers and impaired myotube formation. This suggested that cyclin D3 controls the balance between myoblast proliferation and differentiation. To assess the role played by cyclin D3 in skeletal muscle in vivo, we exploited cyclin D3 knockout mice. Cyclin D3-null tibialis muscles collected from 2-mo-old mice showed fewer satellite cells and a decrease in myofiber size when compared with wild-type controls. The analysis of primary myoblast cultures and of satellite cells activated in vivo following muscle injury revealed cyclin D3 is critically required for proliferative expansion of myogenic precursor cells. Finally, the analysis of satellite cells associated to single fibers indicated that cyclinD3-null myogenic progenitors have a higher propensity to differentiate and a reduced ability to generate undifferentiated reserve cells.

10.

THE TRANSCRIPTION FACTOR SNAI1 REGULATES MYOBLASTS PROLIFERATION AND NEO-VASCULARIZATION DURING MUSCLE REGENERATION.

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Snail is a zinc finger transcription factor involved in a broad spectrum of biological functions, such as cell differentiation, cell motility, cell cycle regulation, and apoptosis. In mouse embryo Snail transcript was observed in the myotome and sclerotome as well as in the limb bud primordium from 9.5 d.p.c. but the role that Snail plays in the muscle development is not fully understood. Here we show that Snail is strongly upregulated during muscle regeneration and it is expressed in proliferating C2C12 and primary mouse myoblasts. By microarray analysis, we identified 64 genes up-regulated and 139 down-regulated more than 3 fold in Snail knocked-down cells. Several down-regulated genes are involved in myoblasts proliferation, extracellular matrix formation and angiogenesis, suggesting a role of Snail in myoblasts expansion and neo-vascularization in early steps of muscle regeneration.

11.

APPROPRIATE LEVELS OF EXTRACELLULAR S100B PROTEIN IN INJURED MUSCLE ARE REQUIRED FOR CORRECT MUSCLE REGENERATION

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The Ca²⁺-regulated protein and damage-associated molecular pattern, S100B, is promptly released by muscle tissue upon acute injury and contributes to the expansion of muscle precursor cells during the early phase of muscle regeneration by binding to RAGE and bFGF/FGFR1 on the myoblast surface [1]. However, during the late phase of muscle regeneration extracellular S100B levels diminish concomitant with myoblast terminal differentiation, suggesting that appropriate phase-specific levels of extracellular S100B are required for correct muscle regeneration [2]. To assess this point, we induced muscle injury in the Tibialis anterior muscle of C57Bl/6 mice and subsequently inoculated the damaged muscle with non-immune IgG or an S100B neutralizing antibody during the first days of regeneration. We found that sequestering extracellular S100B up to post-injury day 3 resulted in impaired muscle regeneration characterized by a dramatic reduction of the number of infiltrated macrophages, which resulted prevalently in the M1 activation state compared with IgG-injected muscles, and a reduction of the number of proliferating and differentiating satellite cells. I.p. injection of anti-S100B antibody gave similar results to local injection. Similar experiments performed in RAGE^{-/-} mice suggested that the effects of S100B were RAGE-dependent. However, repeated i.m. injections of S100B during the regenerative process translated into a significant increase in the recruitment of overstaying macrophages and impaired regeneration, suggesting that S100B may act as a chemoattractant for macrophages, as supported by in vitro experiments. Because we found higher levels of released S100B in muscles of dystrophic (mdx) mice compared with WT mice, our data suggest that appropriate levels of extracellular S100B are required at specific phases of muscle regeneration for efficient regeneration, and that chronic release of S100B in muscular dystrophy might concur to the disease.

12.

COLLAGEN VI ENHANCES SATELLITE CELL SELF-RENEWAL AND MUSCLE REGENERATION

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Collagen VI (ColVI) is an extracellular matrix protein composed by three alpha-chains encoded by different genes and forming a microfilamentous network in different tissues. In skeletal muscles, ColVI deposition is mainly provided by interstitial muscular fibroblasts and the protein localizes in the endomysium surrounding muscle fibers. The critical role of ColVI in skeletal muscle is supported by the fact that mutations of COL6A1-COL6A3 genes cause various forms of inherited muscle disorders. Satellite cells (SCs) are the adult skeletal muscle stem cells and play essential roles in the

homeostasis and regeneration of skeletal muscles. SCs are located within a niche that includes myofibers and extracellular matrix. Currently, the function of specific extracellular matrix molecules in regulating SCs is unknown. Here, we investigated whether ColVI plays any role in regulating SC activity. ColVI was found to be a component of SC niche *in vivo* and the native protein was able to ameliorate the *in vitro* self-renewal of SCs. ColVI null (Col6a1^{-/-}) SCs displayed decreased *in vitro* self-renewal capability, a phenotype rescued by plating onto native ColVI. Moreover, lack of ColVI in Col6a1^{-/-} mice caused impaired muscle regeneration and reduced SC self-renewal capability after injury. When ColVI deposition was reinstated *in vivo* by grafting wild-type fibroblasts, the SC defects of Col6a1^{-/-} mice were rescued. These data indicate that ColVI plays a key role in the regulation of SC activity, and they open novel therapeutic venues for ColVI-related muscular dystrophies.

13.

MACROPHAGES AND MUSCLE REGENERATION: WHO DID YOU CALL A SCAVENGER?

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Muscle regeneration is a complex process that involves many different types of cells, both myogenic and non-myogenic. In particular, macrophages play a fundamental role: they phagocytose debris, release cytokines to regulate inflammatory response and stimulate satellite cells proliferation. *In vivo*, there are two main types of macrophages: M1 (pro-inflammatory) macrophages, that can stimulate proliferation of myoblasts, and M2 (anti-inflammatory) macrophages, which can stimulate the differentiation of myoblasts. Inflammation is known to play a major role in the pathophysiology of Duchenne Muscular Dystrophy. To study the effects of macrophage-released factors onto myogenic cells we use the murine macrophage cell line J774 to obtain a serum-free, conditioned medium (mMCM). We previously found that mMCM enhance the proliferation rate and the differentiation of rat and both normal and dystrophic human myoblasts. We are now trying to characterize its mechanism(s) of action in the murine model. We confirmed the pro-proliferative effect of mMCM on murine satellite cells but we found that mMCM did not have any negative effect on cell fusion, although it increased MyoD expression. We compared the effect of mMCM on satellite cells to that of macrophagic factors released by M1 and M2 human macrophages. M1 conditioned medium showed a pro-proliferative effect similar to mMCM, whereas M2 conditioned medium appeared to have an anti-proliferative effect. We also found that mMCM consistently has a clear anti-proliferative effect on primary mdx fibroblasts. A preliminary experiment on the effect of on satellite cell transplantation in dystrophic muscle was carried out, finding that apparently mMCM led to much better grafting. Finally, we investigated the effects of mMCM on macrophages polarization, using human monocytes from blood that were differentiated and then stimulated to acquire either M1 or M2 phenotype. Preliminary results showed that mMCM might push them towards the M2 anti-inflammatory phenotype.

SESSION 3

MUSCULAR DYSTROPHIES

14.

THE RAG2-IL2RB-DMD- MOUSE: A NOVEL MODEL TO ASSESS CELL- AND GENE-THERAPY STRATEGIES FOR MUSCULAR DYSTROPHIES.

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Cell transplantation is considered as a potential therapeutic approach to muscular dystrophies, however, it still requires deeper insights into the *in vivo* behaviour of transplanted human myogenic cells, for which an optimized model would be immunodeficient and dystrophic. This study focuses on the characterization of a new immunodeficient and dystrophic Rag2-Il2rb-Dmd- mouse model that we have recently developed. Methods We measured the whole body weight and the weight of different leg muscles. We measured the serum levels of creatine kinase (CK), and the regeneration process was quantified by immunohistochemical analysis of neonatal myosin heavy chain. We also performed physiology tests and assessed the feasibility of injecting human myoblasts.

This dystrophic model showed growth curves similar to mdx (total body and single muscles weight). In mdx mice, CK levels showed a significant decrease between 8 and 24/40 weeks of age, whereas Rag2-Il2rb-Dmd- showed an opposite trend. Rag2-Il2rb-Dmd- animals present a wider and delayed regeneration peak, which started between 10 and 16 weeks of age. Both dystrophic models presented similar decreases in the specific force. Transplantation of human myoblasts were successful in Rag2-Il2rb-Dmd- animals.

The muscular phenotype of this new mouse model clearly resembles that of the mdx model, and therefore constitutes a useful tool that can be used for assessing gene and cell therapy approaches, particularly using human myogenic stem cells.

15.

MUSCLE EXPRESSION OF PGC1 α AND RELATED SIGNALLING MOLECULES IN THE MDX MOUSE: IDENTIFICATION OF ALTERED PATHWAYS IN MECHANO-TRANSDUCTION AND OF NOVEL DRUG TARGETS.

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In Duchenne muscular dystrophy (DMD), the absence of dystrophin causes a complex pathogenetic cascade also involving sarcolemmal fragility and metabolic distress. Increasing evidences in mdx mouse model outline the potential benefit in reinforcing pathways of peroxisome proliferator-activated receptor (PPAR) γ coactivator 1 α (PGC1 α), a key modulator of oxidative metabolism. Accordingly, we found beneficial effects of resveratrol, a PGC1 α activator via sirtuin1 (Sirt1), in exercised mdx mice. To better understand the basal regulation of this mechano-sensitive pathway in dystrophic skeletal muscle we performed realtime-PCR experiments in gastrocnemius (GC) muscles of mdx mice at different ages. We found slightly higher levels of PGC1 α mRNA in mdx mice of 8 weeks of age vs. age-matched C57BL/10 (wt) mice. The increase in PGC1 α became highly significant at 16 weeks of age and was paralleled by an increased expression of Sirt1. Also, the expression of PPAR δ , a coregulator of the expression of β -oxidation enzymes, was significantly increased. The impact of the above alterations on metabolic phenotype has been evaluated on genes expression of myosin heavy chain (MHC) isoforms. A significant increase of slow-oxidative type I MHC and a decrease of fast-glycolytic IIb MHC were found in mdx GC muscle vs. wt ones. Then a modification of PGC1 α pathway, which likely triggers a protective slow-oxidative myofiber program, naturally occurs in the mdx mouse muscle and may modulate the outcome of physiological stimuli and of pharmacological strategies. Upstream mechanisms triggering the above changes and their functional outcome are currently under investigation (Supported by Duchenne Parent Project NL).

16.

INTRAPERITONEAL TRANSPLANTATION OF MICROENCAPSULATED SERTOLI CELLS COUNTERACTS MUSCLE INFLAMMATION AND RESCUES MUSCLE PERFORMANCE IN MDX MICE

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Duchenne muscular dystrophy (DMD), a progressive muscle degenerative disease associated with chronic inflammation, necrosis and fibrosis, is currently treated with antiinflammatory steroids, despite their limited efficacy and undesired side effects. Testicular Sertoli cells (SCs) have been successfully implanted to treat many experimental diseases due to their ability to secrete trophic, antiinflammatory and immunomodulatory molecules [1]. We transplanted microencapsulated SCs, within highly biocompatible microcapsules [2] into the peritoneal cavity of mdx mice, an animal model of DMD. Skeletal muscles from SC-treated mdx mice, compared with muscles from mock-treated mice displayed reduced necrosis, improved regeneration and depletion of infiltrated inflammatory cells, with a significant reduction of the number of macrophages already one week after transplantation. Serum IFN-gamma, a strong inducer of pro-inflammatory macrophages elevated in mdx mice was significantly reduced in SC-treated mice. Three weeks after transplantation SC-treated, but not mock-treated mdx mice showed recovery of muscle performance in treadmill running tests, and a comparable resistance to exercise-induced muscle damage to that of untreated wild-type mice. Unexpectedly, muscles from SC-treated mdx mice showed an increased expression of the dystrophin-related gene, utrophin (Utrn) which might contribute to the recovery of muscle homeostasis. Similarly to mdx mice, transplantation of SCs in an experimental model of autoimmune myositis resulted in the recovery of limb muscle mass

and dramatic reduction of necrosis in diaphragm. Our results suggest that i.p. transplantation of microencapsulated SCs might represent a powerful means to reduce muscle inflammation and create a more suitable microenvironment for muscle regeneration and the recovery of muscle performance in animals and, possibly patients affected from DMD or inflammatory myopathies.

[1] Mital et al. *Reproduction* 139(2010):495-504; [2] Luca et al. *Tissue Eng* 13(2007):641-8.

17.

OBSCURIN IS REQUIRED FOR ANKYRINB-DEPENDENT LOCALIZATION OF DYSTROPHIN AND SARCOLEMMA INTEGRITY IN SKELETAL MUSCLE FIBERS

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Obscurin is a large myofibrillar protein that contains several interacting modules, one of which mediates binding to muscle-specific ankyrins. Interaction between obscurin and the muscle-specific ankyrin sAnk1.5 has been found to regulate the organization of the sarcoplasmic reticulum in striated muscles. Additional muscle-specific ankyrin isoforms, namely ankB and ankG, localized at the sub-sarcolemma level have been shown to contribute to the organization of dystrophin and β -dystroglycan at costameres. Accordingly, we investigated whether obscurin might be involved in the sub-sarcolemma localization of ankB and ankG, and eventually in the assembly of dystrophin and β -dystroglycan at costameres in skeletal muscle fibers. We found that in mice deficient for obscurin, ankB was displaced from its localization at the M-band, while localization of ankG at Z-disk was not affected. In obscurin KO mice, localization at costameres of dystrophin, but not of β -dystroglycan, was altered and the sub-sarcolemma microtubule cytoskeleton was disrupted. In addition, these mutant mice displayed marked sarcolemmal fragility and reduced muscle strength. Altogether, these results support a model where obscurin, by targeting ankB at the M-band, contributes to the organization of sub-sarcolemma microtubules, localization of dystrophin at costameres and to maintenance of sarcolemmal integrity. These results add a new twist to obscurin function due to interaction with proteins of the extra-sarcomeric cytoskeleton. Accordingly, obscurin appears to represent a multifunctional anchoring protein that on one hand establishes interactions with sarcomeric proteins and on the other hand enables complex formation with extra-sarcomeric proteins, like the muscle-specific ankyrin isoforms, that help to connect the sarcomeres with the SR and with the sub-sarcolemmal cytoskeleton.

18.

CHARACTERIZATION OF THE PKC θ DEPENDENT IMMUNE RESPONSE IN MUSCULAR DYSTROPHY

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Inflammation is an essential feature in the onset and progression of muscular dystrophy. Moreover, gene- and cell-based therapeutic approaches have been shown to possibly elicit T-cell immunity in patients with Duchenne's muscular dystrophy. We recently observed that lack of PKC θ in mdx, greatly improved muscle maintenance, regeneration and strength, preventing massive inflammation and wasting. Although PKC θ is expressed in both immune cells and skeletal muscle, the observed phenotype was primarily due to lack of PKC θ in BM-derived cells. However, PKC θ is currently proposed as a target to selectively manipulate T cell functions, promoting the establishment of a regulatory and immunotolerant phenotype. We are using the bi-genetic mouse model mdx/ θ ^{-/-} we generated to dissect the role of T-cell subclasses in DMD, as well as to validate targeting PKC θ as a valuable approach to counteract disease progression. We are characterizing morphology, immune cell population composition, inflammatory pathways activity, cytokine/chemokines release in mdx/ θ ^{-/-} as compared to mdx mice, at different ages. Moreover, to investigate the T-cell dependent contribution to the pathogenesis of the disease, we transplanted WT and PKC θ ^{-/-} spleen T-cells into the immunodeficient scid/mdx mice. Preliminary results demonstrated that PKC θ expressing T cells are highly and

specifically recruited into dystrophic muscle, worsening the muscle phenotype. Moreover, we observed that lack of PKC θ improved T cell differentiation into the Treg phenotype in vitro. Taken together our results suggest that T-cells are primarily involved in the pathogenesis of DMD, and that targeting PKC θ may favour the establishment of an immunotolerant environment, improving muscle maintenance.

19.

EXPLOITING VASOPRESSIN SIGNALING IN MUSCULAR ATROPHY AND DYSTROPHIES

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Arginine-Vasopressin (AVP) is a neurohypophyseal hormone able to induce differentiation in myogenic cell lines and primary satellite cells. V1aR is the only AVP receptor expressed in skeletal muscle. By interacting with V1aR, AVP activates phospholipases C and D, increases cytosolic Ca²⁺ concentrations and regulates cAMP levels. The AVP-dependent increase in cytosolic calcium activates CaMK and calcineurin pathways resulting in the formation of multifactor complexes on the promoter of muscle specific genes. Our previous data demonstrate that V1aR expression is modulated during muscle regeneration and the stimulation of AVP signaling strongly enhances regeneration of injured muscles. 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 inhibiting inflammation. The 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 calcineurin-IL-4 pathway and by the inhibition of atrophic genes expression mediated by FOXO phosphorylation via Akt-dependant pathway. By all the above we are 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.

SESSION 4

E-C COUPLING IN HEALTH AND DISEASES

20.

THE NEURONAL GROWTH ASSOCIATED PROTEIN 43 IS EXPRESSED IN SKELETAL MUSCLE FIBERS CLOSE TO MITOCHONDRIA AND CALCIUM RELEASE UNITS

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The neuronal Growth Associated Protein 43 (GAP43), is involved in mechanisms controlling pathfinding and branching of neurons during development and regeneration. For many years this protein was classified as neuron-specific, but recent evidences suggest that GAP43 is expressed not only in neurons, but also in glial cells and in muscles from patients affected by various myopathies. We have investigated the expression and subcellular localization of GAP43 in mouse satellite cells, myotubes, and adult muscle (extensor digitorum longus or EDL) using Western blotting, immuno-fluorescence in confocal microscopy and electron microscopy (EM). Our results indicated that GAP43 is indeed expressed in both myoblasts and differentiating myotubes, and its cellular localization changes dramatically during maturation: in myoblasts the localization appeared to be mostly nuclear, whereas with differentiation the protein started to display a sarcomeric-like pattern. In adult fibers, GAP43 expression was evident with the protein labeling forming (in longitudinal views) a double cross striation reminiscent of the staining pattern of other organelles, such as calcium release units (CRUs) and mitochondria. Experiments done in EDL muscles fixed at different sarcomere lengths using confocal microscopy and EM, revealed the localization, from the sarcomere Z-line, of GAP43 positive foci, falling between that of CRUs and of mitochondria. Preliminary results from grip test, revealed gender dependent differences on force development on GAP43 heterozygous mice. These data lead the way to further investigation about the possible physiological and structural roles of GAP43 protein in adult fiber function and disease.

21.

POST-NATAL DEVELOPMENT OF SARCOPLASMIC RETICULUM IN FAST-TWITCH SKELETAL MUSCLES: A PIVOTAL ROLE FOR CALSEQUESTRIN1.

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Transition in molecular composition of fast-twitch skeletal muscles takes place to accommodate specific functional requests. The Sarcoplasmic Reticulum (SR), in early stages of development, expresses a variety of slow/cardiac/isoforms. In EDL (extensor digitorum longus), slow isoforms are down-regulated whereas fast isoforms increase in relative density in parallel to maturation of E-C coupling machinery. In fact Calsequestrin (CASQ1), the main Ca²⁺ binding protein of SR in adult fast-twitch muscles the SR-ER Ca²⁺ ATPase (SERCA1) and the Ca²⁺ channel (RYR1), accumulate during post-natal development. The aim of this study is to compare EDL from WT and CASQ1-null mice, in the first month of development. Expression of main structural and functional SR proteins was studied by western blotting and RT-PCR in conjunction with Ca²⁺ release properties. The main findings in 1-month-old CASQ1-null mice were: i) down-regulation of CASQ2, Sarcalumenin and SERCA1 since post-natal day 16; ii) normal expression of DHPR and tendency of RYR1 to accumulate despite mRNA down-regulation; iii) activation of the Unfolded protein response (UPR) via the XBP1 pathway. Taken together these results suggest a pivotal role for CASQ1 in orchestrating SR maturation of fast-twitch muscles during post-natal development and indicate that regulation of Ca²⁺ signals might be the possible mechanism.

22.

STRUCTURAL ADAPTATION OF THE EC-COUPLING APPARATUS IN CALSEQUESTRIN-1 KNOCKOUT MICE: ANY ROLE IN THEIR SUSCEPTIBILITY TO MALIGNANT HYPERTERMIA (MH) AND ENVIRONMENTAL HEAT STROKE (EHS)?

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Calsequestrin (CASQ) is the major sarcoplasmic reticulum (SR) Ca²⁺ binding protein of skeletal fibers, expressed in two different isoforms (CASQ 1 and 2) at different developmental stages. Ablation of CASQ1 in mice is not lethal, but results in increased susceptibility of CASQ1-null mice to trigger lethal episodes when exposed to volatile anesthetics or heat, hyper-metabolic disorders known as malignant hyperthermia (MH) and environmental heat stroke (EHS). What causes MH/EHS susceptibility of CASQ1-null mice is still under investigation. Here, we analyzed the post-natal maturation of the sarcotubular system in CASQ1-null EDL fibers and its possible implication in the hyper-metabolic syndrome. Western-blot analysis indicates that CASQ2, abundantly expressed at birth, is down-regulated post-natally, just as in wild type (WT) EDL, leaving the majority of knockout fibers without any CASQ. Structural post-natal maturation of the EC coupling units in knockout fibers is characterized by a) an incomplete reorganization of the T-tubule from longitudinal to transversally oriented, as it would normally occur in WT muscle, and b) formation of multi-layered junctions, characteristic of adult CASQ1-null EDL fibers, a change that starts after 1 month of age. Interestingly, mice at early stages of development (around 1 month of age), when the number of multi-layered junctions is limited, show lower susceptibility to heat stress than adults (~20% vs. ~80% of lethality) and a lower internal temperature (reduced hyperthermia). These findings suggest that the structural modifications of the EC coupling apparatus occurring during post-natal maturation may play a role in the MH/EHS susceptibility of CASQ1-null male mice.

23.

OXIDATIVE STRESS UNDERLIES SUDDEN DEATH IN CALSEQUESTRIN-1 KNOCKOUT (CASQ1-NULL) MICE SUFFERING OF MALIGNANT HYPERTERMIA (MH) AND ENVIRONMENTAL HEAT STROKE (EHS)

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Mice lacking CASQ1 - a protein that modulates Ca²⁺-release channels or ryanodine receptors 1 (RYR1) - suffers of MH and EHS, life-threatening disorders triggered respectively by volatile anaesthetics and high temperature. It has been proposed that, during MH/EHS crisis, excessive oxidative stress leads to increases RYR1 Ca²⁺ leak, over-contraction and rhabdomyolysis of skeletal fibers. We treated CASQ1-null mice for 2 months with N-acetylcysteine (NAC, a potent anti-oxidant) administered in drinking water (1% w/v). NAC treatment resulted in significant protection of CASQ1-null mice from lethal episodes induced by halothane (2%, 1h at 32°C) and heat (41°C, 1h): respectively, the rate of mortality was 78.6 vs 25.0% and 85.7 vs 30.0% in controls vs NAC-treated animals. This protection is likely the results of several factors: a) reduction of oxidative stress, as shown by a decrease of mitochondrial superoxide flashes (mSOF) frequency (P<0.05); b) decreased internal temperature during heat-stress protocol (from 42.1 to 40.8 °C); and c) lower number of fibers undergoing rhabdomyolysis (from 37.6 to 11.6 %). Furthermore, NAC treatment results also in a significant increase of the threshold for caffeine-induced contraction (in-vitro contraction test). These results suggest that a) increased oxidative stress may exacerbates the RYR1 instability that underlies MH/EHS episodes and b) anti-oxidants drugs should be considered for the prevention/prevention of MH and over-heating skeletal muscle disorders.

SESSION 5

MYOPATHIES AND NEUROMUSCULAR DISEASES

24.

CATTLE CONGENITAL PSEUDOMYOTONIA: AN ANIMAL MODEL FOR INVESTIGATING HUMAN BRODY DISEASE

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An inherited muscle disorder defined as “congenital pseudomyotonia” has been described in two important Italian cattle breeds and, as a single case, in a cross-breed calf in the Netherlands. Clinically the disorder is characterized by an exercise-induced muscle contraction. Cattle pseudomyotonia has been well characterized at both genetic and biochemical levels. By DNA sequencing of affected calves, we have provided evidence of mutations in ATP2A1 gene coding for sarco(endo)plasmic reticulum Ca²⁺-ATPase, isoform1 (SERCA1). Moreover we have demonstrated that cattle pathological muscles are characterized by a selective reduction in the level of expression of SERCA1. On the basis of symptoms and of genetic and biochemical confirmations, cattle pseudomyotonia has been defined as the true counterpart of human Brody disease, a rare inherited disorder of skeletal muscle due to a SERCA1 deficiency, resulting from a defect of ATP2A1 gene. Although for both Brody disease and pseudomyotonia the selective reduction in the expression levels of SERCA1, has been indicated as causative of the disease, the pathophysiological mechanism underlying this deficiency has not yet been clarified. Recently, we have presented the crystal structure of bovine SERCA1. This result together with data on the possible role of the Quality Control System, ubiquitin-proteasome, in the reduction of expression levels of the mutated SERCA1, demonstrate that a single mutation is sufficient to perturb the three dimensional structure of SERCA1 protein and induce a genetic disease. This study reflects the enormous potential of domestic animals to gain further insights into human medicine.

25.

THE ADAPTATIONS OF MICE SKELETAL MUSCLE TO SHORT TERM STEROID ADMINISTRATION

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To understand the molecular mechanisms underlying steroid myopathy we studied skeletal muscle adaptations to dexamethasone (DEX) administration (5 mg Kg⁻¹ day⁻¹) in adult mice at different times (3 and 7 days). We analyzed: creatine kinase (CK) serum levels; absolute and specific force development and fatigue index in extensor digitorum longus (EDL) and soleus (S) isolated intact muscles; MHC isoform composition; muscle fibres cross-sectional area (CSA); master controllers of muscle protein breakdown (MPB) (MuRF-1, atrogin-1); master controllers of autophagy

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(Beclin-1, p62) 7); master controller of metabolism (PGC-1 α), and citrate synthase (CS) and ATP synthase expression. All determinations, but those performed in intact S and EDL muscle were done in vastus lateralis muscle. Significant fibers atrophy was observed at 7 days DEX (14%) only; no significant change of the functional parameters and no MHC isoforms transition were observed both at 3 and 7 days DEX, a significant up-regulation of MPB (MuRF-1) was found at 7 days DEX while no changes were observed in autophagy system (p62, Beclin1) both at 3 and 7 days DEX. Moreover, a significant increase of CS and ATP synthase expression were observed at 3 days DEX only, without any change in PGC-1 α expression. Our preliminary data suggest that in the early phases of myopathy, atrophy is supported by the activation of the ubiquitin-proteasome system and that it is not related to a mitochondrial dysfunction.

26.

AKT PATHWAY IN SBMA SKELETAL MUSCLE

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Spinal and bulbar muscular atrophy (SBMA), also known as Kennedy's disease, is a rare inherited neuromuscular disease characterized by the loss of lower motor neurons in the brainstem and spinal cord and skeletal muscle weakness and atrophy. The disease is caused by expansion of a polyglutamine tract (polyQ) in the gene coding for androgen receptor (AR). SBMA fully affects only males. Females even if homozygous for the mutation have mild disease manifestations. The sex-dependent nature of SBMA is due to the fact that polyQ-AR is converted to a toxic species by binding to its natural ligand, testosterone. SBMA has long been considered a pure motor neuron disease. According to this model, polyQ-AR acts in motor neurons to cause their death, resulting in denervation-induced atrophy of skeletal muscles and loss of motor function. However, there is clinical and experimental evidence suggesting a primary role for skeletal muscle in disease induction and progression. We have previously shown that post-translational modifications influence the neurotoxicity of polyQ-AR. In particular, we have previously shown that Akt-mediated phosphorylation of polyQ-AR protects against toxicity by promoting protein degradation through the proteasome. In order to describe how Akt signaling is regulated in vivo, we performed biochemical analysis to measure the level of activity of Akt and several of its downstream targets, such as GSK-3 β , mTOR, p70S6K and 4E-BP1 in a knock-in mouse model of SBMA. Our data suggest that Akt signaling pathway is altered during SBMA progression.

27.

ROLE OF THE CELL'S QUALITY CONTROL SYSTEM IN THE PATHOGENESIS OF LGMD-2D AND BRODY DISEASE.

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Unfolded Protein Diseases (UPDs) are genetic disorders in which gene mutations result in unfolded or misfolded proteins that either aggregate or are prematurely disposed of by the Quality Control System (QCS), although, often, their function is only partially impaired. Type 2D Limb Girdle Muscular Dystrophy (LGMD-2D) is caused by mutations in α -sarcoglycan, a protein that, together with β -, γ - and δ -sarcoglycan, forms a structural and signaling complex in the sarcolemma of striated muscle cells. LGMD-2D can be considered an UPD as α -sarcoglycan mutants, being misfolded, are intercepted by QCS and degraded by the ubiquitin-proteasome system. Brody's disease (BD) is characterized by exercise-induced impairment of muscle relaxation due to the lack of the Ca²⁺-pump SERCA1 from the sarcoplasmic reticulum membrane. Here we show that SERCA1 mutants are ubiquitinated and dismantled by the proteasome, suggesting that also BD is an UPD. Unfortunately, to date no effective therapies are available to treat LGMD-2D or BD. Therefore, a primary challenge is the identification of potential pharmacological targets. In this perspective, we demonstrate that proteasomal inhibition rescues both the expression and proper localization of α -sarcoglycan mutants as well as the expression of mutated SERCA1 and its Ca²⁺-pumping activity. Moreover, we also show that these mutants can be quantitatively and qualitatively rescued by using small molecules that force protein folding. All together these data open the way to new pharmacological strategies, for LGMD-2D and BD, expected "to cure" the mutated proteins either by promoting their folding or preventing their disposal.

28.

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

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The Neutral Lipid Storage Diseases with Myopathy (NLSDM) is a newly recognized disease caused by massive accumulation of triglycerides in the muscles due to mutations in the ATGL. The pathogenic mechanisms underlying muscle damage in NLSDM is completely unknown. Taking advantage of ATGL knockout (ATGL^{-/-}) mouse model (kindly provided by Dr. Zechner) we have investigated at histological, functional, and molecular level the potential pathogenic events associated with NLSDM. Histological analysis, performed on different muscle types (tibialis anterior, soleus, edl, diaphragm) of 2.5 months old mice, revealed a reduction in the cross sectional area of the single myofibres in ATGL^{-/-} edl and tibialis anterior muscle, compared to WT. This was associated with the presence of several myofibres with central nuclei, indicating either the activation of a compensatory, but defective, regenerative mechanism or alteration in the maturation process. To further address this point, we have analyzed the expression markers of the myogenic program such as desmin (proliferation) and myogenin (commitment), which expression normally decrease at the stage of mature muscle. RTqPCR and western blot analysis revealed a significant increase in gene and protein expression of both desmin and myogenin in ATGL^{-/-} diaphragm muscle compared to WT. Moreover we have analyzed, by RTqPCR, the expression of the subunit gamma of acetylcholine receptor that is undetectable or low in innervated adult active muscle. We noted an up regulation of the expression of this subunit in ATGL^{-/-} diaphragm muscle compared to WT, indicating probably an alteration of the neuromuscular junction. Functional analysis revealed a decrease in the specific and maximum force of EDL muscle of ATGL^{-/-} mice compared to WT; instead we have not noted difference both in the specific and maximum force in soleus muscle. This observation correlates with the histological analysis.

29.

CHARACTERIZATION OF hCLC-1 CHLORIDE CHANNEL MUTATIONS ASSOCIATED WITH MYOTONIA CONGENITA

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Myotonia congenita (MC) is an inherited muscle disease characterized by muscle stiffness during forceful movement, due to sarcolemma electrical instability. MC stems from loss-of-function mutations in the CIC-1 chloride channel gene. Three CIC-1 mutations (G190S, A531V and F167L) individuated in MC patients were introduced in recombinant hCIC-1 and functionally characterized in HEK cells using whole-cell patch-clamp. A531V greatly decreased chloride current density but the open probability voltage-dependence (Po) and deactivation kinetics were similar to WT. Instead, G190S determined a remarkable positive shift of Po. Although the underlying mechanisms were different, the dramatic reduction of A531V and G190S chloride currents fully explain their role in determining MC. Conversely, F167L did not affect chloride current density, kinetics, and voltage dependence. We thus characterized the compound mutations found in patients carrying F167L, namely G355R, G284R, Y686X, P558S and R105C. The G284R, G355R and Y686X mutations did not generate any chloride current, suggesting that they constitute the main contributor to myotonia in heterozygous patients. Whereas R105C currents were very similar to WT, P558S profoundly affected current kinetics and voltage dependence. Nevertheless, both mutations reduced chloride current density, confirming their guilty role in MC. Co-expression experiments would allow to study the interaction between compound mutations. Supported by Telethon-Italy (grant GGP10101) and AFM-France (grant 15020).

30.

IN VITRO ANALYSIS OF DIAPHRAGM NEUROJUNCTION FUNCTIONALITY

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In the last years, new studies showed the relevance of skeletal muscle within the pathogenesis of the ALS [1], giving to the neuromuscular junction (NMJ) a pivotal role in the communication between muscle and nerve. Within this contest, we developed an experimental set-up to evaluate the mechanical functionality of the NMJ in murine animal models. The experimental set-up, together with the developed software, allowed for the evaluation of the NMJ functionality through the comparison between muscle response following membrane stimulation and response following nerve stimulation. In particular, the electrical pulses that reach the membrane of the muscle through the solution by-pass the biochemical signaling that runs along the nerve; on the contrary, when electrical pulses are delivered directly on the nerve, to simulate neuron activity, the nerve transmission pathway is activated. The comparison of muscle response to specific stimulation protocol can give an in-depth information on the functionality on the NMJ, and possible discrepancies in the responses can give the rise to further morphological or biochemical studies on the NMJ. The set-up and the software have been developed with reference to the diaphragm muscle, both for the importance that it owns in this pathology, and for the specimen shape that allows to keep a piece of tissue within its associated nerve (phrenic nerve) more easily. With this set-up, in addition to the classic parameters that can be measured with ex vivo mechanical measurements [2], NMJ functionality can be evaluated computing new proposed parameters, as the “neurotransmission failure” or the “intra-train fatigue” [3,4].

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31.

POST-NATAL MOLECULAR AND MORPHOLOGICAL ADAPTIVE CHANGES IN A MOUSE MODEL OF RECESSIVE CPVT

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The sarcoplasmic reticulum (SR) is responsible for calcium cycling in heart. Cardiac calsequestrin (CASQ2) contributes to calcium homeostasis by virtue of its low-affinity/high-capacity calcium binding properties, maintains SR architecture and regulates excitation-contraction-coupling, especially or exclusively upon β -adrenergic stimulation. Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an inherited arrhythmogenic disease associated with cardiac arrest in children or young adults. The recessive CPVT variants are due to mutations in either the CASQ2 gene or the triadin gene.

Since the content of CASQ2-R33Q was drastically reduced in heart of adult CASQ2^{R33Q/R33Q} mice, the post-natal development was studied from day 2 to week 8. The main findings are: a) 80% reduction of CASQ2-R33Q is an early event in the post-natal development and is accompanied by a drastic reduction of triadin; b) GRP78 levels are elevated as evidence of ER stress and UPR; c) no signs of either apoptosis or autophagy are detected; on the contrary, pro-survival signals are activated in 2-week-old hearts, as inferred by increase of Bcl-2 and by activation of Akt and ERK1/2 pathways; d) the transcription factor Egr-1 is up-regulated as well as Egr-1-dependent calcium-regulating proteins, e.g., STIM1; e) morphological changes of jSR and of SR-T tubule junction are evident as post-natal day 2; f) yet another model of recessive CPVT, the CASQ2^{-/-} mouse, does not display the same adaptive pattern.

Expression of CASQ2-R33Q influences molecular and ultra-structural heart development; post-natal, adaptive changes appear to be pro-survival, capable of ensuring a new equilibrium to be hampered only under emotional stress or β -adrenergic stimulations.

SESSION 6

MUSCLE ATROPHY AND AGING

32.

TARGETING INTEGRIN SIGNALLING PREVENTS STARVATION-INDUCED AUTOPHAGY VIA JNK/p38 MEDIATED REGULATION OF miR-21

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Cell proliferation, migration and survival are controlled by cytokines including growth factors and by components of the extracellular matrix. Integrin receptors functionally link extra-cellular matrix components, such as collagen or laminin to intracellular signalling molecules such as Focal Adhesion Kinase (FAK) or Src. Lack of extra-cellular components leads to different muscle pathologies such as Congenital muscular dystrophy or Bethlem myopathy. Col6A^{-/-} mice, as model of the Bethlem disease, are characterized by a block of the autophagic machinery as a primary source of muscle damage. We investigated the involvement of integrins signalling in the control of autophagy in muscle cells. In-vitro studies, revealed that pharmacological inhibition of FAK (using F14 inhibitor), prevented autophagy during nutrient deprivation in C2C12 cells. In particular, F14 prevented starvation-induced inactivation of AKT/Foxo axis, while it strongly induced p38 and JNK activities. Those MAPKs in turn activate the expression of miR-21; indeed, pharmacological inhibition of both p38 and JNK prevented miR-21 up-regulation while inducing AKT dephosphorylation. These results demonstrate that FAK inhibition during starvation induces the activation of the p38/JNK-miR-21 axis, which in turn maintains AKT activity preventing autophagy. Mir-21 may thus represent a key player in the outside-in integrin signalling, mediating p38/JNK and AKT pathways cross-talk.

33.

THE P97/VCP ATPASE IS CRITICAL IN MUSCLE ATROPHY AND FOR THE ACCELERATED DEGRADATION OF MOST MUSCLE PROTEINS

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The p97/VCP ATPase complex facilitates the extraction and degradation of ubiquitinated proteins from larger structures. We therefore studied if p97 participates to the rapid degradation of myofibrillar proteins during muscle atrophy. Electroporation of a dominant negative p97 (DNp97), but not the WT, into mouse muscle reduced fiber atrophy caused by denervation and food deprivation. DNp97 (acting as a substrate-trap) became associated with specific myofibrillar proteins and its cofactors, Ufd1 and p47, and caused accumulation of ubiquitinated components of thin and thick filaments, which suggests a role for p97 in extracting ubiquitinated proteins from myofibrils during atrophy. DNp97 expression in myotubes reduced overall proteolysis by proteasomes and lysosomes and blocked the accelerated proteolysis induced by FoxO3, which is essential for atrophy. Expression of p97, Ufd1 and p47 increases following denervation, at times when myofibrils are rapidly degraded. Surprisingly, p97 inhibition, though toxic to most cells, caused rapid growth of myotubes (without enhancing protein synthesis) and hypertrophy of adult muscles. Thus, p97 restrains post-natal muscle growth, and during atrophy, is essential for the accelerated degradation of most muscle proteins.

34.

INTRACELLULAR SIGNALING IN ER-STRESS INDUCED AUTOPHAGY IN SKELETAL MUSCLE CELLS.

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Skeletal muscle remodelling in response to muscle disuse and unloading is known to be associated to the so-called ER-stress, which in turn activates autophagy and contributes to muscle atrophy. Different molecules are involved in ER-stress induced autophagy, among which PKC θ has been recently described. In this study we dissected both in vitro and in vivo ER-stress induced autophagy pathways in muscle. We demonstrated that PKC θ is strongly activated in cultured

myoblasts during ER-stress induced by different stimuli, such as TG or TN treatment, and it localizes into LC3 positive autophagic dots. No AKT de-phosphorylation, nor Foxo3A or GSK3 β activation were observed in these conditions. Moreover, PKC θ inhibition and/or lack in myoblasts prevented ER-stress-induced LC3 activation and autophagic dots formation, while its activation resulted in LC3 activation in the absence of ER-stress. In vivo, lack of PKC θ prevented both fasting-and immobilization-induced autophagy and muscle atrophy, irrespective of Akt pathway inhibition. Taken together these results demonstrate that PKC θ works as an ER-stress sensor in skeletal muscle, required for autophagy activation, and can be proposed as a novel molecular target to maintain muscle homeostasis in response to external stimuli, such as disuse and unloading, still allowing intracellular clearance.

35.

STUDY OF THE ROLE OF THE TRANSCRIPTION FACTOR FoxO1 IN SKELETAL MUSCLE DURING MUSCLE ATROPHY

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Skeletal muscle atrophy is a debilitating consequence of fasting, denervation, cancer and other systemic diseases. These different conditions have in common an increased activation of protein degradation systems. FoxO transcription factors are key regulators that play a critical role in the atrophy program being sufficient and necessary for the expression of genes belonging to both proteolytic pathways, the ubiquitin-proteasome and the autophagy-lysosome systems. In skeletal muscle, the FoxO family is comprised of three isoforms: FoxO1, FoxO3 and FoxO4. To investigate the role of FoxO1 in skeletal muscle, we generated two muscle specific knock-out mice: a myosin light chain 1 (MLC1)-driven constitutive animal and tamoxifen-inducible mice. Knock-out animals are fully viable, fertile and phenotypically normal and indistinguishable in appearance from wild type litter mates. In this study, we want to understand which are the atrophy-related genes specifically under the control of FoxO1 during denervation. Indeed, only a subset of the atrophy-related genes are under FoxO1 control. Importantly, FoxO1 loss is not sufficient to protect from muscle atrophy after 14 days of denervation. The study of the intracellular signaling pathways indicate that the FoxO1 is required for the IGF-PI3K AKT pathway but not for mTOR signaling. In conclusion, these findings are valuable in order to develop new methods for the prevention of muscle wasting in many systemic diseases and to promote recovery.

36.

STUDY OF THE ROLE OF MITOCHONDRIAL PROTEIN OPA1 IN SKELETAL MUSCLE

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Mitochondria are dynamic organelles which adapt their morphology by fusion and fission events to the bioenergetic requirements of the cell. Skeletal muscle is a tissue with high energy demand and mitochondrial plasticity plays a key role on its homeostasis. Indeed, alterations in mitochondrial morphology, distribution and function are common features in atrophying muscles. Moreover, dysregulation of mitochondrial dynamics affects the signalling pathways that regulate muscle mass. Mitochondrial fission is largely controlled by Drp1, whereas mitochondrial fusion mainly depends on the activity of the mitofusins (MFN1 and MFN2) and OPA1. To investigate the in vivo role of the mitochondrial fusion protein OPA1, we have generated a skeletal muscle-specific Opa1 knock-out mice. Interestingly, OPA1 ablation in muscles is lethal at post-natal day 8. OPA1 deficient mice exhibit a progressive decrease in body weight compared with wild-type animals. Muscle biogenesis in OPA1 knock-out mice is not compromised but they display a profound atrophy. At ultra-structural level OPA1-deleted muscles maintain sarcomere organization but show mitochondria alterations and appearance of lipid droplets. These preliminary results, strongly suggest the important contribution of Opa1 in the control of the homeostasis of skeletal muscle and open a new set of candidate targets for developing new drugs against muscle loss.

37.

GENE EXPRESSION MODULATION OF THIOREDOXIN REDUCTASES DURING SKELETAL MUSCLE AGEING

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TRX system is one of the most important redox-regulating antioxidant complex with significant quenching capability against induced oxidative stress. This antioxidant system contains two major forms of Thioredoxin, Trx1, mainly cytosolic (the TXN gene product) and mitochondrial Trx2 (the TXN2 gene product) as well as a NADPH-dependent thioredoxin reductase (TrxR), which reduces the oxidized form of thioredoxin and other protein or non-protein substrates. Thioredoxin reductases are encoded by three separate genes: TXNRD1 encodes the cytosolic TrxR1; TXNRD2 encodes the mitochondrial TrxR2 and TXNRD3 encodes TrxR3, predominantly expressed in testis. Each of them gives rise to several different splice variants. Despite increasing evidence suggests the involvement of the TRX system in several human diseases and pathological conditions, very little is known about its role in the regulation of skeletal muscle adaptation and contractile activity during ageing. Recently, we showed that glutathione redox couples in skeletal muscle are more susceptible to oxidation than Trxs and that, although Trx proteins were up-regulated in skeletal muscle of aged mice, they do not modulate the redox-regulated adaptation to contractile activity that fails during ageing (Dimauro et al., 2012). Starting from these results, we hypothesize that exogenous ROS and the ageing process would differently modulate the TrxR transcripts and isoforms, thus regulating the adaptive process in these systems. In order to verify this hypothesis, we investigated the expression profile of TrxR in skeletal muscle tissue and cells. Our preliminary data document the differential expression of TrxR isoforms both at mRNA and protein level in C2C12 myotubes. The exposure of these cells to H₂O₂ determined an increase of the four mRNA TrxR1 isoforms analyzed, while TrxR2 isoforms were unaffected. Although further studies are needed to define the role played by TrxR co- and post-transcriptional regulation upon oxidative stress and their biological significance, a detailed elucidation of these mechanisms in both normal and ageing process may provide new insights into ageing mechanisms and contribute to the development of novel treatments for human diseases.

38.

PLIN2 EXPRESSION IN HUMAN SKELETAL MUSCLE IS ASSOCIATED WITH INACTIVITY AND AGING

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Inter-fibre muscle fat infiltration is a phenomenon associated with sarcopenia and/or physical inactivity. Much less is known about lipid deposition within muscle fibres and its possible consequences for muscle function. Intracellular lipid droplets are associated with Perilipins (Plins), proteins involved in lipid storage and mobilization. We investigated the intra-fibre expression of muscle-specific Plin2 in *vastus lateralis* of subjects of different age, either healthy or affected by mobility-limiting pathologies (hip osteoarthritis), compared with the expression of adipose tissue-specific Plin1. Plin2 expression is present in both young and old subjects, increases with age and is particularly evident in patients with decreased limb mobility, where it correlates with muscle quality. Moreover, in patients a direct correlation was found with the expression of the main Plin2 transcriptional regulator PPAR- γ and with a dramatic increase in p53 phosphorylation, a possible sign of muscle waste. These last changes were absent in healthy young and in active and sedentary old subjects, where an unexpected activation of NF- κ B was observed. Thus, beside inter-fibre fat infiltration, a lipid deposition occurs *within* the muscle fibres as a consequence of inactivity without NF- κ B activation, and appears to be exacerbated by age, and correlates with muscle weakness and loss of muscle quality.

SESSION 7

CACHEXIA AND MUSCLE WASTING

39.

MIGRATING TUMOR CELLS AND MUSCLE WASTING

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Cachexia is a syndrome characterized by progressive weight loss with depletion of lean body mass and muscle, which occurs in the presence of underlying illness. It is reported in patients with several chronic diseases, including cancer. The prevalence of cachexia among patients affected by different diseases varies accordingly to the nature of the specific chronic illness. Cachexia syndrome directly accounts for the death of 20% of cancer patients. Selective skeletal muscle wasting is a main pathological feature exhibited in cancer, and reflects an imbalance between protein synthesis and catabolism. Several studies indicate four main pathways as responsible for skeletal muscle catabolism: the ubiquitin–proteasome pathway (UPP), protease-mediated degradation, autophagocytosis and inflammation. To date these pathways has been deeply investigated, suggesting that the regulation of these cellular processes take place mainly within the myofiber niche¹. Recently, we and other laboratories obtained several data showing that human tumor cells can be fused with myoblasts into myotubes and myofibers. In this study we aim to deeply investigate the possible contribution of migrating tumor cells to cancer-induced cachexia. In particular, we used both in vitro cell system and xenograft animal models obtained by subcutaneous injection of human gastrointestinal tumor cells in nude mice. We provided evidences for a novel mechanism by which migrating cancer cells are directly involved in muscle atrophy.

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40.

CAVEOLINS AND CAVINS IN MUSCLE-DERIVED TUMOURS

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Rhabdomyosarcomas (RMS) are muscle-derived tumours with a significant incidence in children. RMS cells exhibit a widespread pattern of muscle-specific markers, the detection of which may be useful for discriminating the tumour grading in order to optimize the timing and doses of chemotherapy. By analyzing human RMS specimens and in vitro cell lines, we determined the time window of expression of Caveolins (Cav-1, -2 and -3) and some Cavins family members (i.e. PTRF/Cavin-1 and MURC/Cavin-4). In detail, the simultaneous expression of Cav-1, Cav-2 and PTRF/Cavin-1 defined a signature predictive of immature cells, whereas the expression of Cav-3 and MURC/Cavin-4 was restricted to more differentiated cells. In addition, in immature cells Cav-1 was tyrosine-phosphorylated in a Src-dependent manner, resulting in increased cell proliferation and migration. As a result, the subcutaneous injections into nude mice of RMS cells overexpressing the wild-type Cav-1 form resulted in a significant tumour growth, which was instead prevented by the over-expression of a non-phosphorylatable form of Cav-1. Overall, it can be concluded that Caveolins and Cavins can be useful to identify the degree of cellular differentiation in RMS and that phosphorylation of Cav-1, in particular, plays a key role in tumour aggressiveness.

41.

IMPAIRMENT OF MYOGENIC MARKERS IN HUMAN MUSCLE WASTING

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The present study investigated the relationship between prolonged muscle immobilization and gene expression of myogenic factors such as MyoG, MyoD, Myf5 and Pax7. For comparison, the same factors were analyzed in obese subjects, reported to be sarcopenic, and in breast cancer patients, rarely showing muscle atrophy. Three muscle biopsies (vastus lateralis) were obtained at different time points by volunteers recruited for a period of bed-rest (day -1, 7 and 33 of bed-rest). Abdominal muscle biopsies were taken during surgery in patients affected by: benign disease (controls), breast cancer, obesity. Gene expression of MyoG, MyoD, Myf5 and Pax7 was assessed by qRT-PCR. Pax7 and Myf5 expression at days 7 and 33 of bed-rest tends to reduction vs day -1. MyoD levels decrease of 75% and 50% after 7 and 33 days, respectively, and MyoG tends to increase. In breast cancer patients the expression of MyoD, Pax7 and Myf5 decreases significantly of about 40% vs controls. In obese patients MyoD expression decreases of about 50% vs controls, Myf5 increases significantly while Pax7 tends to increase. These results show that bed-rest is associated with a tendency to down-regulation of Pax7 and Myf5 mRNA levels. This could indicate a reduced satellite cell population, possibly resulting from enhanced differentiation, as suggested by MyoG levels. By contrast, obesity appears associated with maintenance of the satellite cell population, as suggested by the increased levels of both Myf5 and Pax7. Finally, the pattern observed in breast cancer patients could indicate an impairment of myogenesis.

42.

AUTOPHAGIC DEGRADATION CONTRIBUTES TO MUSCLE WASTING IN CANCER CACHEXIA

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Muscle protein wasting in cancer cachexia is a critical problem. The underlying mechanisms are still unclear, although the ubiquitin-proteasome system has been involved in the degradation of bulk myofibrillar proteins. The present work has been aimed to investigate if also autophagic degradation plays a role in the onset of muscle depletion in cancer-bearing animals as well as in glucocorticoid-induced atrophy. The results show that autophagy is induced in muscle in three different models of cancer cachexia as well as in glucocorticoid-treated mice. These results demonstrate that autophagy contributes to the complicated network that leads to muscle atrophy. In this regard, particularly intriguing is the observation that in both cancer hosts and TNF α -treated C2C12 myotubes insulin can only partially blunt autophagy induction. This would suggest that autophagy is triggered through mechanisms that cannot be circumvented by using classical up-stream modulators, prompting to identify more effective approaches to target this proteolytic system.

43.

MECHANISMS UNDERLYING EXERCISE-MEDIATED RESCUE OF CACHEXIA

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Cachexia is a syndrome associated with many diseases, characterized by severe muscle atrophy and weakness, accounting for poor prognosis and worsening patients quality of life. Recent studies showed that physical activity after cancer diagnosis ameliorates the prognosis, although the underlying mechanisms are still poorly understood. With the aim to characterize the pathways involved in exercise-mediated rescue of cachexia, we investigate the effects of spontaneous physical activity (wheel running) in colon carcinoma (C26)-bearing mice. All major diagnostic criteria for cachexia are reversed by exercise, including rescue of body weight, muscle atrophy and fatigue, ultimately leading to increased survival. These data suggest a potential role for the use of exercise mimetics to counteract cachexia. Indeed, C26-bearing mice treated with AICAR, a well-known exercise-mimetic, significantly lost less body and muscle weight than C26-bearing vehicle-treated mice. In order to assess whether muscle contraction plays a role in the exercise-mediated rescue of cachexia, we denervated one limb of (C26)-bearing mice and assessed muscle mass following spontaneous running. Interestingly, exercise exerts positive effects on (C26)-bearing mice muscle mass independently from denervation, suggesting cross-education induced by exercise on the denervated muscle. Our data suggest that exercise counteracts muscle wasting through a systemic effect and that exercise mimetics can be used to ameliorate cachexia.