

LECTURE 1 Shahragim Tajbakhsh (Institut Pasteur-France)

Extrinsic and intrinsic regulation of muscle stem cells

The regulation of skeletal muscle stem cells during homeostasis and regeneration involves the interplay of multiple mechanisms. The mechanisms by which niche molecules and intrinsic factors regulate muscle stem cell quiescence and properties remain largely unknown. In a series of studies, we investigated Notch as a key mediator of muscle satellite cell stability and fate. Specifically, Notch mediates extrinsic (extracellular matrix) and intrinsic (microRNA) mechanisms to stabilise satellite cells within their niche. Interestingly, Notch/RBPJ-bound regulatory elements are located adjacent to specific collagen genes in adult muscle satellite cells. These molecules are linked to the ECM and constitute putative niche components. Notably, satellite cell-produced collagen V (COLV) is a critical component of the quiescent niche, as conditional deletion of *Col5a1* leads to anomalous cell cycle entry and differentiation of satellite cells. Strikingly, COLV specifically regulates quiescence through Calcitonin receptor mediated activity. In other studies, we have identified a microRNA pathway that is modulated by Notch, and it is required for stabilising muscle stem cells in their niche by regulating the migration status of the muscle stem cell. These observations lead us to propose a two-step mechanism for niche occupancy.



Prof. Shahragim Tajbakhsh obtained a Doctor of Philosophy degree in Biology from Carleton University, Canada (1988) working on the molecular biology of viruses. Following postdoctoral research at the Pasteur Institute he established an independent group in 2001 called "Stem Cells & Development" where he has been interested in how stem cells establish and regenerate organs and tissues, with a particular focus on skeletal muscle. The aim of the laboratory is to investigate stem cell properties during development and postnatally to understand how skeletal muscle is established, and how it regenerates during disease, and after injury. Areas of focus include quiescence, niche, self-renewal, symmetric and asymmetric cell divisions, ageing.

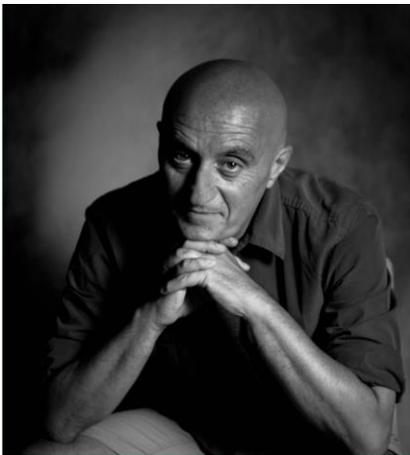
Prof. Tajbakhsh is an EMBO member, former Head of the Dept. of Developmental & Stem Cell Biology and co-Director of the "Laboratory of Excellence" Consortium, REVIVE, regrouping leading labs working on stem cells (2011-2022). He is member of 2 scientific councils for associations, several SABs and presides on editorial boards of 4 scientific journals. He has participated in a number of EU consortia (FP6, EuroStemCell; FP7, EuroSyStem, Optistem, NotchIT) and received several awards including the Chair of Excellence Louis Pasteur (Institut Pasteur, 2017) and the French Academy of Sciences/Fondation Generale de Santé, for achievements in stem cell research.

LECTURE 2 Paolo Sassone-Corsi (University of California Irvine-USA)

Common Threads: Metabolism, Epigenetics and the Circadian Clock

The circadian clock is responsible for biological timekeeping on a systemic level. The mammalian central pacemaker is localized in the hypothalamus, in a paired neuronal structure called the suprachiasmatic nucleus (SCN). The discovery that all tissues and virtually all cells contain an intrinsic circadian clock revolutionized the field, providing a conceptual framework towards the understanding of organismal homeostasis and physiological tissue-to-tissue communications (1). The circadian clock controls a remarkable array of physiological and metabolic functions through governing a significant portion of the genome. Furthermore, the clock drives cyclic chromatin remodeling associated to circadian transcription, including spatial nuclear organization (2). The circadian epigenome shares intimate links with cellular metabolic processes and has remarkable plasticity showing reprogramming during aging and in response to nutritional challenges (3, 4). We will present findings that reveal specific molecular connections between chromatin remodelers, metabolic pathways and the circadian clock.

1. Schibler, U. and Sassone-Corsi, P. (2002) A Web of Circadian Pacemakers. *Cell* 111, 919-922.
2. Aguilar-Arnal, L., Hakim, O., Patel, V. R., Baldi, P., Hager, G. L. and Sassone-Corsi, P. (2013) Cycles in spatial and temporal chromosomal organization driven by the circadian clock. *Nature Struct. Mol. Biol.* 20: 1206-13.
3. Asher G and Sassone-Corsi P. (2015) Time for Food: the intimate interplay between nutrition, metabolism and the circadian clock. *Cell* 161: 84-92.
4. Sato S, Solanas G, Peixoto FO, Bee L, Symeonidi A, Schmidt MS, Brenner C, Masri S, Benitah SA, Sassone-Corsi P. (2017) Circadian Reprogramming in the Liver Identifies Metabolic Pathways of Aging. *Cell* 170: 664-677



During the past three decades my research has focused on the molecular mechanisms of transcriptional regulation and chromatin remodeling, specifically in response to changes in signaling transduction and cellular metabolism. In the past twenty years we uncovered the specific role of transcriptional and epigenetic regulators in circadian clock function and deciphered how metabolic circuits intimately connect to the circadian system. Our studies have significantly impacted the fields of transcription, epigenetics, metabolism and endocrinology. Our expertise covers molecular, cellular, physiological and behavioral analysis of circadian rhythms in mammals, including genomics, metabolomics high-throughput profiling and Biocomputing. The high impact of our research is witnessed by the numerous high-profile publications, numerous invitations as plenary speaker at high-profile conferences and by an h-index of 123.

Positions: After PhD in Italy and post-doctoral studies in France and USA (1980-1988), PSC established his research group in Strasbourg, France, with the position of Directeur de Recherche (1989-2006). Moved to University of California, Irvine as Distinguished Professor and Chair of the Department of Pharmacology (2006-2011) and then as Director of the Center for Epigenetics and Metabolism (2011-present) and Donald Bren Professor (2011-present). PSC is also External Professor of the Max-Planck Institute (Germany).

Honors (partial list): EMBO Gold Medal (1994); Grand Prix Liliane Bettencourt, France (1997); Grand Prix Charles-Léopold Mayer of the Académie des Sciences, Paris (2003); Edwin B. Astwood Award, Endocrine Society, USA (2004); Ipsen Award in Endocrinology (2011); Transatlantic Medal of The Society of Endocrinology, UK (2012); Fellow of AAAS (2014). August and Marie Krogh Medal, Denmark (2015); Leonardo da Vinci Gold Medal, FMSI Federation, Italy (2016); Albert Hogan Memorial Award Lecture, University of Missouri (2017); UC Distinguished Faculty Award for Research (2018).

LECTURE 3 Carmine Settembre (TIGEM-Italy)

Developmental regulation of lysosome biogenesis shapes cellular identity and function

Lysosomes are catabolic organelles devoted to the degradation of intracellular proteins and organelles that are delivered to the lysosomes via autophagy. Lysosome biogenesis and autophagy are very dynamic processes, which are modulated in response to cues. My laboratory has recently identified the fibroblast growth factor (FGF) signalling as main regulator of post-natal activation of autophagy and lysosome biogenesis in chondrocytes of the cartilage during bone growth. This process is mediated by the FGFR3 and FGFR4 receptors through both transcriptional and post-translational mechanisms. The FGF-mediated induction of lysosomal catabolism is required to remodel the endoplasmic reticulum (ER) of chondrocytes through a process known as ER-phagy. Preliminary data indicate that ER-phagy promotes chondrocyte hypertrophic differentiation and collagen production. Our findings unveil an unexpected role of the lysosome/autophagy pathway in organismal development and growth.



My research has focused on the lysosome, autophagy and lysosomal storage disorders for the last 15 years. During my PhD (2004-2007) at the Telethon Institute of Genetics and Medicine I studied the role of autophagy in the pathogenesis of Lysosomal Storage Disorders (LSDs) and demonstrated that an impairment of autophagy accounts for part of the phenotypic manifestation LSDs, defining these disorders as ‘autophagic disorders’ (Settembre et al. 2008). Subsequently (in 2007) I moved to United States as visiting student and subsequently as post-doctoral fellow in the laboratory of Dr. Karsenty (Columbia University, NY) where I was involved in the study of the role of proteoglycans during skeletal development and growth (Settembre et al. 2008). In 2009, I moved to Houston at Baylor College of Medicine where I focused on the basic biological mechanisms regulating lysosome and autophagosome biogenesis and found that the formation of these organelles is co-regulated at the transcriptional level through the activity of a master transcription factor, TFEB (Settembre et al 2011). These studies also led me to identify a lysosome to nucleus signaling mechanism, through which the lysosome can control its biogenesis and function in response to environmental cues (Settembre et al. 2012). In 2013 I returned to Italy as assistant investigator at the Telethon Institute of Genetic and Medicine (TIGEM) and assistant professor at the Federico II University of Naples. IN 2016 I was selected as EMBO Young Investigator and my laboratory received the ERC starting grant. The main research interest of my lab is to study the role of lysosomal and autophagy pathways during bone growth and maintenance and develop novel therapeutic approaches for the treatment of skeletal abnormalities in genetic disorders (Cinque et al.2015, Bartolomeo et al. 2017, Forrester et al. submitted).

LECTURE 4 Nenad Bursac (Duke University-USA)

Muscle Mimicry in a Dish

Engineering of three-dimensional human skeletal muscle tissues is motivated by the need for improved physiological systems that would serve for modelling and studying of muscle diseases, pre-clinical drug development, and potential muscle regenerative therapies. In this talk, I will describe first-time engineering of contractile human engineered muscle tissues made of primary myogenic cells derived from muscle biopsies and myogenic progenitors derived from induced pluripotent stem cells by transient overexpression of satellite cell marker Pax7. Resulting bioengineered muscle microtissues ("myobundles") exhibit aligned architecture, multinucleated and striated myofibers, and a Pax7⁺ cell pool. They contract spontaneously and respond to electrical stimuli with robust calcium transients, twitch and tetanic contractions. During culture, myobundles maintain functional acetylcholine receptors and structurally and functionally mature, as evidenced by increased myofiber diameter, improved calcium handling and contractile strength, formation of triads, localization of dystrophin in sarcolemma, and enhanced expression of various maturation genes. In response to diversely acting drugs, myobundles undergo dose-dependent hypertrophy or toxic myopathy similar to clinical outcomes. In response to exercise-mimetic electrical stimulation, myobundles undergo significant hyperplastic and hypertrophic growth, enhanced force-generating capacity, and increased metabolic flux. When derived using cells from patients with congenital skeletal muscle diseases, myobundles exhibit expected pathological phenotypes. Upon implantation into immunocompromised mice for 3 weeks, the myobundles progressively vascularize and maintain functionality. Overall, biomimetic human myobundles provide an enabling platform for predictive drug and toxicology screening and development of novel therapeutics for degenerative muscle disorders.



Dr. Nenad Bursac is a Professor of Biomedical Engineering, Cell Biology, and Medicine at Duke University and one of the pioneers and leaders of the cardiac and skeletal muscle tissue engineering fields. In 1999, as a member of Dr. Robert Langer' group at MIT, he demonstrated the first engineering of functional heart tissues using mammalian cardiomyocytes. His postdoctoral research with Dr. Leslie Tung at Johns Hopkins University resulted in new methods to control architecture and function of 2- and 3-dimensional heart cell cultures. Currently, Dr. Bursac's research involves use of cell, tissue, and genetic engineering techniques and electrophysiological and biomechanical studies to advance fields of somatic and stem cell based therapies for heart and skeletal muscle disease.

For the last 20 years, Dr. Bursac's work has pushed the boundaries of the field by demonstrating a number of "firsts", including: the first use of bioreactors for functional cardiac tissue engineering; the first studies of electrophysiology and arrhythmias in engineered heart tissues; the first engineering of anisotropic cardiac tissue patch and methods to control patch anisotropy; the most functionally advanced mouse cardiac tissue patch; the first engineering of highly functional, large (40mmx40mm) heart tissues from human pluripotent stem cells; first engineering of functional human skeletal muscle tissues from primary and pluripotent stem cells; and first engineering of biosynthetic excitable cells and tissues for studies and treatment of cardiac arrhythmias and heart failure.

Dr. Bursac has authored more than 100 scientific manuscripts, presented over 120 invited talks, and has mentored more than 30 PhD students and postdoctoral and medical fellows. He co-directs Regeneration Next Initiative at Duke University. He is a recipient of the Stansell Family Distinguished Research Award, Mendel Center Award, and Stem Cell Innovation Award. In 2014, Dr. Bursac was the president of the North Carolina Tissue Engineering and Regenerative Medicine Society. Since 2015, Dr. Bursac has been a Fellow of American Institute for Medical and Biological Engineering and since 2018 a Fellow of Biomedical Engineering Society. Dr. Bursac has served on various NIH grant review panels and is a member of editorial boards of *Nature Scientific Reports* and *NPJ Regenerative Medicine*.

SESSION 1

SIGNALLING IN MUSCLE GROWTH, HOMEOSTASIS AND DISEASES

1.1 Role of muscle interstitial cells in neurogenic muscular atrophy

Luca Madaro¹, Daisy Proietti^{1,4}, Magda Passafaro^{1,3}, David Sala², Usue Etxaniz², Francesca Lugarini¹, Maria Vittoria Alfonsi¹, Chiara Nicoletti², Sole Gatto², Marco De Bardi¹, Ricardo Rojas-García⁵, Sara Marinelli⁶, Alessandra Sacco², Pier Lorenzo Puri^{1,2}

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During neurogenic muscle atrophy, the interruption of transmission of neurogenic signals to muscles, caused by loss of neuromuscular junction (NMJ) integrity, activates protein breakdown and reduces protein synthesis, leading to the loss of muscle mass and contractile activity. Homeostatic perturbations of skeletal muscles, such as acute or chronic injuries, activate a coordinate cellular response that includes infiltration of immune cells (i.e. macrophages) and expansion of a heterogeneous population of interstitial cells referred to as Fibro-Adipogenic Progenitors (FAPs) that instruct muscle satellite cells to eventually regenerate the injured myofibers. We report an unprecedented activation of FAPs in denervated muscles, in the absence of concomitant infiltration of macrophages and activation of satellite cells, that leads to a progressive accumulation of FAPs. Transcriptome analysis reveals a persistent activation of IL6/STAT3 signaling in FAPs from denervated muscle, which promotes myofiber atrophy. Pharmacological inhibition of IL6 and STAT3, as well as FAP-selective genetic ablation of STAT3, effectively countered denervation-mediated muscle atrophy. Similar results were obtained in a mouse model of Amyotrophic Lateral Sclerosis (ALS). This evidence indicates a previously unrecognized functional relationship between FAPs and neuromuscular integrity and reveals a novel function of FAPs in mediating muscle atrophy in response to denervation. The identification of FAPs with aberrant activation of IL6-STAT3 suggests potential new pharmacological interventions that selectively target aberrantly activated signaling in FAPs for the treatment of neurogenic muscular atrophies.

1.2 Are Toll like receptor and type I interferon key factors in autoimmune inflammatory myopathies: studies in an experimental murine model

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Type I interferon (IFN)-dependent proteins are upregulated in muscle and skin tissues of patients with idiopathic inflammatory myopathies (IIM).¹ Type I IFN induction might rely upon the activation of toll-like receptors (TLRs)² among which TLR-7/8 expression is upregulated in muscle tissue of IIM patients.³

Aim of this study was to investigate the role of activation of TLR-7/8 and type I IFN using an experimental model of the disease (EAM). To this end we induced autoimmune response in C57/Bl6 mice by injection of the amino-terminal portion of the murine auto-antigen Histon H1 t-RNA synthetase (HisRS). Disease activity was compared in the presence or absence of the TLR-7/8 agonist R848 in wild-type mice and in mice that failed to express the IFN $\alpha\beta$ receptor (IFN $\alpha\beta$ R null).

EAM induced by a single intramuscular immunisation with HisRS in absence of R848 spontaneously abated after 7–8 weeks. In contrast, the levels of anti-HisRS autoantibodies, endomysial/perimysial leukocyte infiltration and myofiber regeneration persisted until the end of the follow-up period (22 weeks after immunisation) in mice immunised with HisRS in the presence of the Toll like receptor agonist. Myofiber MHC class I molecules were also detectable in fibres of HisRS+R848 immunised mice but not mice that received HisRS alone. The induction of MHC class I likely licenses the spreading of inflammation to bystander muscles, since it is detectable in HisRS+R848 immunised mice only and precedes the immune response.

Using mice deficient for the Type I IFN receptor (IFN $\alpha\beta$ R null) we demonstrated that IFN was necessary for the prolonged autoantibody response and for the spreading of the response. However, the immunization with HisRS+R848 elicits persistent local autoimmune disease in both WT and IFN $\alpha\beta$ receptor null mice suggesting that not all events downstream of TLR7/8 activation appear to be mediated via type I IFN.

This EAM model reproduces many characteristics of human IIM and may represent a tool for pre-clinical studies.

1. Wenzel J, Scheler M, Bieber T, Tuting T. Evidence for a role of type I interferons in the pathogenesis of dermatomyositis. *Br J Dermatol.* 2005;153:462–463. [author reply 463–464]

2. Drexlerand SK, Foxwell BM. The role of toll-like receptors in chronic inflammation. *Int J Biochem Cell Biol.* 2010;42:506–518.

3. A. Tournadre, V. Lenief, A. Eljaafari, and P. Miossec. Immature muscle precursors are a source of interferon-beta in myositis: role of Toll-like receptor 3 activation and contribution to HLA class I up-regulation. *Arthritis and rheumatism.* 64:533–541 (2012).

SESSION 1

SIGNALLING IN MUSCLE GROWTH, HOMEOSTASIS AND DISEASES

1.3 Myo-REG: a new web portal for exploring cell and signaling interactions in muscle regeneration

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Tissue homeostasis and regeneration is a complex process governed by the interplay between a multitude of cell populations. Following acute or chronic damage, these cell populations are activated, communicate via chemical mediators and make decisions about their fate through the activation or repression of internal signaling cascades. These are highly dynamic processes occurring with distinct temporal and spatial kinetics. The challenge toward a system level perspective of the regeneration process is the integration of this plethora of inter- and intra-cellular relationships. This information is presently dispersed in the scientific literature or in a variety of online resources. We have addressed this issue by focusing on the regeneration process of the muscle tissue governed by the crosstalk between satellite cells, fibro/adipogenic progenitors (FAPs), macrophages, etc. To meet this challenge, and to support the work of the myology community, we have developed a new portal, Myo-REG, that allows easy access to cell and molecular information in the context of muscle regeneration (myoreg.uniroma2.it). The information annotated in this portal is organized into two layers: the first represents cell-to-cell relationships, while the second describes the signaling interactions occurring inside each cell type.

Pathways annotation takes advantage of the information stored in SIGNOR1. Additional curation effort has been carried out to increase the coverage of molecular interactions related to the muscle regeneration process and to annotate cell-to-cell interactions. Myo-REG has a user-friendly interface, which allows users to explore, via an interactive model, cell interactions or to analyze in depth intracellular pathways. Each cell type is also annotated with the biomarkers that are commonly used to analyze or purify cells by cytofluorimetry. Moreover, users can perform a muscle-centered literature search and transform selected pathway into Boolean networks to be used for simple simulations.

1. Perfetto, L. et al. SIGNOR: A database of causal relationships between biological entities. *Nucleic Acids Res.* 44, D548–D554 (2016).

1.4 Dissecting the role of AMBRA1 in skeletal muscle

Lisa Gambarotto^{a*}, Martina Chrisam^{a*}, Silvia Castagnaro^a, Marianna Spizzotin^a, Francesca Nazio^{c,d}, Paolo Grumati^{a,b}, Paola Braghetta^a, Francesco Cecconi^{c,d,e} and Paolo Bonaldo^a

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Activating molecule in Beclin 1-regulated autophagy (AMBRA1) is a large intrinsically-disordered protein, implicated in the regulation of multiple cellular processes. Studies in mice with a randomly mutated *Ambra1* locus (*Ambra1*^{9^u/9^t}) showed that AMBRA1 deficiency is embryonic lethal, and that this gene is essential for the development of the central nervous system. Moreover, a recent work of our team suggested that AMBRA1 may also play a key role for muscle development in zebrafish and mouse. To dissect the roles of AMBRA1 in adult muscle, we generated skeletal muscle-specific *Ambra1* knockout mice (*Ambra1*^{fl/fl}::Mlc1f-Cre).

Interestingly, adult *Ambra1*^{fl/fl}::Mlc1f-Cre mice display lower body weight and reduced cross sectional area of the myofibers with respect to controls. Moreover, a high percentage of AMBRA1-deficient myofibers contain calsequestrin-positive aggregates. Ultrastructural analyses revealed a tubular arrangement of these structures and the presence of severely altered, swollen mitochondria in muscles from 6-month-old *Ambra1*^{fl/fl}::Mlc1f-Cre mice. To better clarify how AMBRA1 deficiency impacts on mitochondrial homeostasis, we isolated mitochondria from tibialis anterior and quadriceps muscles of aged (12-month-old) *Ambra1*^{fl/fl}::Mlc1f-Cre mice and analysed the derived protein extracts by western blotting. Surprisingly, preliminary studies in these samples revealed an increased presence of mitophagy mediators and autophagic markers in the mitochondrial fraction of AMBRA1-depleted muscles.

Altogether, our data suggest that AMBRA1 plays a role in the maintenance of skeletal muscle homeostasis. Although AMBRA1 is reported to act mainly in the initial steps of autophagy and mitophagy, the detected accumulation of pro-mitophagic and autophagosome markers in the mitochondrial fraction suggests that mitochondria are effectively targeted for degradation, but not properly cleared in AMBRA1-deficient myofibers. Further studies on *Ambra1*^{fl/fl}::Mlc1f-Cre mice will allow to elucidate more in deep the role of this adaptor protein in myofiber homeostasis.

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SIGNALLING IN MUSCLE GROWTH, HOMEOSTASIS AND DISEASES

1.5 Characterization of a novel embryonal rhabdomyosarcoma murine model**Enrico Pozzo^a**, Gabriele Sassi^a, Jason Yustein^b and Maurilio Sampaolesi^{a,c}^aDepartment of Development and Regeneration, KU Leuven, Belgium. ^bFaris D. Virani Ewing Sarcoma Center, Texas Children's Cancer Center and Dan L. Duncan Cancer Center, Department of Pediatrics, Hematology and Oncology, Baylor College of Medicine, Houston, TX, USA. ^cDepartment of Public Health, Experimental and Forensic Medicine, Division of Human Anatomy, University of Pavia, Italy

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in pediatric patients with an estimated overall event-free survival of 25-30% among patients with metastatic embryonal RMS. Currently used preclinical models fail to recreate the disease observed in the pediatric population due to the late onset of the disease in transgenic RMS murine models or the use of xenografts in immunocompromised mice. To this end, C57BL/6 mice were used to develop a novel transgenic murine model aimed at recreating the quick onset of metastatic embryonal RMS occurring in the pediatric population. From this murine model, 3 different primary cell lines were derived and characterized. These cell lines were able to engraft in the muscle of an immunocompetent syngenic mouse as well as induce distal metastasis. To further study the relationship between RMS and skeletal muscle niche, *in vitro* cocultures with mesoangioblasts (MABs) were set up. Although the fusion capacity of MABs in serum starvation conditions was unaltered, we observed poor differentiation propensity of RMS cells. However, by inducing the silencing of Met protein we observed an increase in myosin heavy chain-positive RMS cells during differentiation, which might correlate with the rescue of the myogenic phenotype in RMS. We are currently investigating the use of microRNAs to further favour the myogenic commitment and modulate the intrinsic myogenic propensity of RMS.

1.6 Musclin: a myokine induced by aerobic exercise useful to contrast muscle wasting during cancer**Andrea David Re Cecconi¹**, Michela Chiappa¹, Sara Previdi², Sergio Marchini², Luca Beltrame² and Rosanna Piccirillo¹¹Neuroscience Department, ²Oncology Department, IRCCS-Mario Negri Research Institute for Pharmacological Research, Milan, Italy

Physical activity extends life span of patients affected by certain types of cancer, also by contrasting the associated muscle wasting (i.e. cachexia). The most effective type of physical activity against muscle wasting during cancer seems to be aerobic exercise. So, we asked whether it promotes secretion of proteins by muscles (i.e. myokines) that may contrast cancer cachexia.

To mimic aerobic exercise, we infected C2C12 myotubes with PGC1 α -expressing adenoviruses, because PGC1 α is the main transcriptional coactivator involved in muscle adaptation during aerobic exercise. By microarray analysis, we identified a number of putatively secreted proteins inducible by PGC1 α that were further confirmed by Q-PCR. We measured by Q-PCR and WB their expression in Tibialis Anterior (TA) muscle of C26 bearing-mice, (i.e. cancer cachexia model) and their plasma levels by ELISA. To induce aerobic exercise adaptations, mice were run on treadmill for 5 consecutive days at a speed of 12 m/min and an uphill inclination of 15° for 45 min/day. Anaerobic exercise-like effects were obtained in plantaris muscle after 7 and 14 days from surgical removal of its synergist muscles (i.e. compensatory hypertrophy). We performed muscle *in vivo* electroporation of plasmids for musclin or its receptor (i.e. Npr3) and *in vitro* we evaluated protein synthesis/degradation of atrophying myotubes treated with supernatants from GFP or PGC1 α -overexpressing cells and Luciferase-based experiments.

Our microarray and Q-PCR analyses showed musclin as a PGC1 α -induced myokine. Conversely, its expression was unchanged in myotubes hypertrophying because of activated AKT (to mimic anaerobic exercise). Dexamethazone-treated myotubes or constitutively active (ca)FoxO3-expressing myotubes undergo atrophy as measured by increased rates of proteolysis and MuRF1 induction. Unlike GFP, musclin was able to contrast the dexamethazone-induced MuRF1 expression in Luciferase assays. Consistently, musclin-containing supernatants of PGC1 α expressing-myotubes restrained the caFoxO3-induced rates of long-lived protein degradation. Among other PGC1 α -induced myokines, we found only musclin strongly downregulated in cachectic muscles and plasma of C26-bearing mice even at times when their body weights were not lost yet. Of note, also its receptor Npr3 was downregulated in cachectic muscles. Thus, we electroporated Tibialis Anterior (TA) of C26-bearing mice with musclin-encoding plasmids and found musclin to preserve fiber area. Interestingly, five days of treadmill exercise was able to protect C26-bearing mice from muscle loss with no effect on tumor growth and to rescue the C26-induced downregulation of musclin (but not its receptor) in cachectic muscles and plasma. By contrast, musclin expression did not change in overloaded plantaris of mice, subjected to compensatory hypertrophy.

Musclin is a myokine induced specifically by PGC1 α , typically increased upon aerobic exercise and musclin overexpression is beneficial against muscle wasting during C26 growth or in atrophying myotubes. Overall, musclin could be a good drug option for cancer patients that cannot exercise and are at risk of developing cachexia.

SESSION 2

SATELLITE CELLS AND MUSCLE REGENERATION IN HEALTHY MUSCLE AND IN DISEASES

2.1 Pax3 modulates AhR-mediated resistance to Dioxin in muscle stem cells

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Inserm, IMRB U955, team 10, 94000, Créteil, France; Université Paris Est Créteil, Faculté de Médecine, 94000, Créteil, & Ecole Nationale Vétérinaire d'Alfort, 94700, Maisons-Alfort, France

We have identified a molecular link between the Aryl hydrocarbon Receptor (AhR) environmental stress pathway and *Pax3/Pax7* developmental genes during craniofacial development. Since *Pax3/7* are key regulators of muscle stem cells (muscle satellite cells), we investigated the cellular and molecular impact of chronic 2,3,7,8-Tetrachlorodibenzo-p-Dioxin (TCDD) exposure on skeletal muscle and satellite cells in the adult. We combined *in vivo* and *ex vivo* approaches, in order to analyse the impact of chronic exposure to TCDD in several muscles such as *tibialis anterior* and *biceps brachii*. We analysed the expression of *Ahr*, *Pax7* and *MyoD* in muscle stem cells and our study shows that TCDD is promoting MuSCs activation and differentiation through the TCDD receptor, *Ahr*. Upon TCDD exposure, skeletal muscle homeostasis is impaired. Strikingly, this activation is differentially impacting muscle stem cells according to *Pax3* or *Pax7* gene expression in muscle stem cells. While *Pax3* expressing stem cells are resistant to the effect of TCDD via induction of *Galr1* and induction of detoxification pathways, *Pax3*-negative muscle stem cells undergo activation and fuse or are lost by apoptosis. Our data therefore show that environmental stress mediated by *Ahr* signaling induced muscle stem cells activation resulting in a pervasive muscle stem cells contribution to uninjured adult muscle fibers. Importantly, this contribution is dependent on *Pax3/Pax7* muscle stem cells heterogeneity. In conclusion, exposure to pollution has deleterious effects on adult skeletal muscles linked to muscle stem cell heterogeneity.

2.2 Cripto modulates angiogenesis and EndMT by controlling the shaping of pro-healing macrophages in skeletal muscle regeneration

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Skeletal muscle regeneration requires the coordination of different cell types, such as muscle stem cells (satellite cells) and inflammatory cells. It is becoming clear that inflammation plays a key role during muscle repair and is driven by different types of macrophages (MPs), mainly represented by pro-inflammatory and pro-healing MPs, which increase sequentially during muscle repair process. The extrinsic control of macrophage identity and the crosstalk with other muscle populations are still poorly understood. Here we show a novel role of the developmental factor Cripto in this complex scenario. Upon acute injury, Cripto is re-expressed in the satellite cells and in a subset of pro-inflammatory and pro-healing MPs. Although the role of Cripto in the satellite cells has been partially elucidated, its function in the MP populations remains unclear. We found that Cripto progressively accumulates at the membrane of pro-healing MPs during muscle regeneration, suggesting a role for Cripto in the resolving phase. To address this issue, we have obtained and analysed a myeloid lineage-specific Cripto loss-of-function mouse model, which allow lineage tracing of Cripto LOF cells (*Tg:Ly2Cre::R26^{mTmG}::Cripto^{fl/fl}*). According to Cripto expression profile, we found that accumulation of pro-healing MPs was impaired in *Tg:Ly2Cre::R26^{mTmG}::Cripto^{fl/fl}* (Cripto My-LOF) mice, upon cardiotoxin (CTX) -induced acute injury. Of note, pro-healing MPs are necessary for efficient vascular remodelling and new evidences are recently emerging pointing to a key role in restricting the Endothelial-to-Mesenchymal Transition (EndMT) of endothelial progenitors. In line with this idea, time course analysis of capillary density and morphology showed impaired angiogenesis and increased EndMT in Cripto My-LOF skeletal muscle regeneration. Despite this phenotype, regeneration was not significantly affected. However, after a second round of CTX injury, a significant decrease of the cross sectional area was observed in Cripto My-LOF muscles, which persisted until day 30, indicating a failure in retaining a full regenerative capacity. To investigate this phenotype further, we evaluated the impact of Cripto My-LOF in the *mdx* model of Duchenne muscular dystrophy by using bone marrow transplantation. Strikingly, we found both a significant decrease of pro-healing MPs and a reduction of the cross sectional area in different muscles of *mdx* mice transplanted with Cripto My-LOF bone marrow. Notably, we found a trend towards an increase of fibrotic tissue in the diaphragm muscles, suggesting that the loss of Cripto in the infiltrating macrophages induces the worsening of the disease. Collectively, our findings shed light on a novel role of Cripto in modulating the inflammatory response both in acute injury and disease with a consequent impact on angiogenesis and muscle regeneration.

SESSION 2

SATELLITE CELLS AND MUSCLE REGENERATION IN HEALTHY MUSCLE AND IN DISEASES

2.3 High mobility group box 1 orchestrates regeneration in skeletal muscle

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Inflammation and tissue regeneration follow injury and consequently they are unavoidably entangled. High Mobility Group Box 1 (HMGB1) is a ubiquitous nuclear protein that is released by injured cells to serve a soluble message of tissue damage and to trigger "sterile" inflammation. We recently found that upon muscle injury, HMGB1 promotes either inflammation or regeneration by switching among mutually exclusive redox states. Of most direct utility, we identified a non-oxidizable mutant of HMGB1 as a promising drug candidate to promote muscle repair without exacerbating inflammation. HMGB1 appears to be a limiting factor in physiological conditions because *Hmgb1*^{+/-} mice show a marked delay in muscle repair and injection of exogenous HMGB1 accelerates muscle regeneration. Interestingly, HMGB1 acts on multiple cell types to promote muscle repair, in particular muscle stem cells and infiltrating cells. To identify the source(s) of HMGB1 during muscle repair, we took advantage of cell-specific HMGB1 knockout mice. Specifically, we evaluated muscle regeneration after acute injury in mice deficient for HMGB1 in muscle cells, endothelial cells or platelets. Our data indicate that HMGB1 derived from these different sources plays distinct roles in muscle regeneration, indicating that a timely and/or spatially regulated release of HMGB1 is required for optimal regeneration. Overall, our findings identified HMGB1 as a crucial mediator in muscle to orchestrate regeneration.

2.4 Nfix drives the phenotypical switch of macrophages for successful muscle regeneration

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Skeletal muscle regeneration requires specific interactions between macrophages (MPs) and myogenic cells at precise time windows. Nfix is a transcription factor expressed by several cell types and it has been shown as necessary to muscle regeneration in total Nfix KO mice. We observed that Nfix is expressed by MPs at the later stages of skeletal muscle regeneration. Specifically, Ly6C⁺ pro-inflammatory MPs exhibit the same level of Nfix while the percentage of Nfix⁺ Ly6C⁻ anti-inflammatory MPs always increases over the time during regeneration. Nfix silencing leads to a defect of anti-inflammatory phenotype acquisition and it maintains a pro-inflammatory phenotype upon M2 polarization. *In vitro*, functional experiments of proliferation and differentiation on WT myoblasts shown that M2 MPs lacking Nfix adopt WT M1 features. Since satellite cells also express Nfix, we generated *LysM*^{Cre}:*Nfix*^{fl/fl} mice which selectively lack of Nfix in MPs. After cardiotoxin injection, skeletal muscles of *LysM*^{Cre}:*Nfix*^{fl/fl} mice exhibit a delay of regeneration characterized by a persistence of necrotic and phagocytosed myofibers, an increase of MyoD⁺ cells proliferation and a later appearance of newly formed myofibers. Moreover, we observed that MPs failed to switch from pro- to anti-inflammatory phenotype *in vivo*. In literature, it has been demonstrated that a defect of MPs phagocytosis induces defect of phenotypical switch. Interestingly, we do not observe a modification of phagocytosis in MPs lacking Nfix suggesting that Nfix could be the key-link between the phagocytic process and the pro- to anti-inflammatory switch.

2.5 Decrypting the cell language: the secretome network during muscle regeneration

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Muscle regeneration is a finely orchestrated process with several cell types, participating in a complex cross talk which in turn modulates many cellular processes such as proliferation, migration, differentiation, apoptosis and fusion. In this regenerative context the different cell types communicate through the secretion of multiple molecules, cell-cell contacts and manipulation of the extra-cellular matrix.

In order to unravel the communication tangle of muscle regeneration, we profiled the secretome, cytokines and extracellular vesicles (EVs), at different time points during the regenerative process. Using a multiplex ELISA assay we analyzed the secretome of the mononuclear cell compartment isolated from injured muscle at different time points after cardiotoxin injury. We found that more than 90 cytokines and secreted molecules are significantly modulated during the regeneration process. We next performed an RNA-seq experiment on purified cell populations aimed at understanding which cell types produce each cytokine and which ones the specific receptor. With these data, we are building a global network in order to understand the time dependent connections between the different populations and to predict the role of uncharacterized molecules in this process. To obtain the vesicles from the entire regenerating tissue, we performed an *ex-vivo* culture of injured

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muscles. The purified EVs were analyzed using a flow cytometry approach to quantify and characterize the RNAs- and enzymes-containing EVs. Furthermore, we used the EVs to perturb the differentiation trajectories *in vitro* of different cell types involved in muscle regeneration.

These data supported by the *in silico* modeling provide a comprehensive communication network of muscle regeneration.

SESSION 3

SYNAPTIC TRANSMISSION AND E-C COUPLING IN HEALTHY AND DISEASED MUSCLE

3.1 Canonical Wnt and Hippo regulators ensure proper synaptic gene transcription and aggregation of acetylcholine receptors at the neuromuscular junctionDanyil Huraskin, **Jasna Friscic**, Nane Eiber, Said Hashemolhosseini

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Wnts regulate processes such as development and differentiation by canonical Wnt/beta-catenin dependent, and non-canonical signaling pathways. Another important pathway involved in the control of organ size, tissue regeneration and stem cell self-renewal is the Hippo pathway, with its signaling members YAP/Taz and transcription factors belonging to the Tead family. Recently, we elucidated the role of canonical Wnt activity in adult muscle fibers using an Axin2-lacZ reporter mouse model. In these mice, active canonical Wnt signaling is reflected by lacZ expression under control of the Axin2 promoter, which itself is a target gene and negative regulator of canonical Wnt signaling. Apart from other subcellular expression sites in muscle cells, we detected active canonical Wnt signaling at neuromuscular junctions. Interestingly, we showed for the first time that YAP/Taz/Tead1-mediated signaling accompanied canonical Wnt signaling in adult muscle fibers. Importantly, we now demonstrate that specific canonical Wnt and Hippo regulators ensure proper synaptic gene transcription and aggregation of acetylcholine receptors at the neuromuscular junction.

3.2 Binding of JPH1, a protein of the triadic membrane contact site of skeletal muscle cells, to CLIMP63, a microtubule-binding protein**Caterina Amato**, Maria Rosaria Catalo, Stefania Lorenzini, Daniela Rossi, Vincenzo Sorrentino

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In skeletal muscle, several proteins are organized at typical membrane contact sites between the plasma membrane and the sarcoplasmic reticulum, known as triads. Interactions between proteins at triads are essential to organize the macromolecular complex that mediates the mechanism of excitation-contraction coupling, which links nerve-induced depolarization with calcium release from sarcoplasmic reticulum, resulting in activation of contraction.

In order to identify novel interactions among triadic proteins, the enzyme-mediated labelling technique Proximity-dependent Biotin Identification (BioID2) was applied. In particular we were interested in identifying proteins interacting with JPH1, a protein responsible for the assembly of triads, where it acts as a molecular bridge between the T-tubule and the sarcoplasmic reticulum membranes.

To this aim, we generated and expressed a 3xmycBioID2-JPH1 fusion protein in HEK293T cells. Following cell treatment with biotin, biotinylated proteins were purified using streptavidin-conjugated magnetic beads and separated by SDS-PAGE. Western blot analysis revealed the presence of several biotinylated proteins, among which we identified CLIMP63. CLIMP63 is a microtubule binding protein expressed in all eukaryotic cells, which also plays a role as a molecular spacer in maintaining the shape of flat cisternae of the endoplasmic reticulum. Interestingly, in skeletal muscle CLIMP63 was shown to interact with Triadin, another protein of the triad that has been proposed to form a functional network with CLIMP63 in remodelling the cisternae of the sarcoplasmic reticulum.

We are currently working to verify the interaction between JPH1 and CLIMP63 in skeletal muscle to extend our knowledge on the assembly and maintenance of triadic junctions.

3.3 Polyglutamine-expanded androgen receptor causes primary toxicity to skeletal muscle in vivo**Caterina Marchioretta**^a, Marco Pirazzin^a, Mathilde Chivet^b, Bert Blaauw^c, Aram Megighian^a, Marco Sandri^{a,c}, Manuela Basso^d, Maria Pennuto^a

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Kennedy's disease, also known as spinal and bulbar muscular atrophy (SBMA), is a X-linked neuromuscular disorder affecting only males. SBMA is characterized by slowly progressive muscle weakness and atrophy, with degeneration of primary motor neurons in the brainstem and spinal cord. This disease is caused by expansions over 38 repeats of a trinucleotide CAG, encoding glutamine, in the first exon of the androgen receptor (AR) gene, resulting in an elongated polyQ tract in the translated protein. In order to clarify the molecular mechanisms underlying SBMA pathogenesis, our lab produced two transgenic mouse lines expressing human AR with either a non-pathogenic polyQ tract with 24 glutamine repeats (AR24Q) or the expanded polyQ tract with 100 glutamine residues (AR100Q). Our data demonstrated that overexpression of polyQ-expanded AR correlated with a decrease in survival, reduced muscle force, altered muscle metabolism and homeostasis and mitochondrial depolarization. Moreover, we observed a progressive deterioration of motor coordination only in AR100Q mice. In order to understand whether the specific alterations of motor function and muscle force in AR100Q result from defects in the motor unit, we analyzed motor neuron soma,

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SYNAPTIC TRANSMISSION AND E-C COUPLING IN HEALTHY AND DISEASED MUSCLE

axon, and neuromuscular junction (NMJ). Our results suggest that polyQ-expanded AR, in this mouse model, does not alter the motor neuron and nerves. Rather, polyQ-expanded AR formed 2% SDS-resistant microaggregates and inclusion bodies in the nuclei in muscle and not spinal cord and brainstem. Moreover, we found defects in the intrinsic capability of muscle to generate force associated with altered cytosolic calcium levels. Our results support the concept that polyQ-expanded AR is primarily toxic to muscle and it alters the excitation contraction coupling machinery.

3.4 CaV β 1: The missing link from voltage sensing to muscle mass homeostasis

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Muscle mass and fiber size undergo to rapid and significant changes according to environmental and pathological variations. Intrinsic muscle contractile activity, neurotransmission and neurotrophic factors are crucial components regulating the integrity of muscle mass. Alterations in the pattern of nerve-evoked electrical activity convey in a modulation of the signal, due to modification in the gene expression by switching on and off specific transcription factors. This excitation-transcription coupling is crucial for plastic adaptation and compensation after the loss of mass in mature muscle. When electrical activity is impaired, such as during neuromuscular diseases, massive muscle atrophy is observed.

Disuse atrophies are generally characterized by a later phase in which muscle wasting is stabilized and differentially expressed genes return to basal levels. This observation has suggested that molecular responses counteracting mass lost can take place. Few components of this compensatory response have been identified; in particular, a crucial role is played by the SMAD4 pathway. Nevertheless, it is still unknown which protein acts as first trigger of the atrophic-compensatory response after an electrical activity alteration. Proper candidates are molecules implicated in the muscle voltage sensing. Indeed, we focused our study on the beta subunit of the L-type of calcium channel CaV1.1. CaV β 1, the intracellular subunit of the channel, has the essential role in the adult skeletal muscle of targeting α 1S subunit to the membrane and of regulating its activity. There are published evidences showing that CaV β 1 can be both in membrane-anchored pool and in free cytoplasmic or nuclear pool. This property has been demonstrated as essential for the role of CaV β 1 as transcription factor in muscle precursor cells.

The present study shows that different splicing isoforms of CaV β 1 can be expressed in skeletal muscle, driving the compensatory response counteracting muscle mass loss after denervation. We demonstrate that CaV β 1 acts as a first modulator of SMAD4 signaling, conferring to the muscle its intrinsic ability to limit the atrophy due to electrical activity alterations. The elucidation of the role of CaV β 1 in pathological neuromuscular diseases will bring very new knowledge on muscle pathophysiology. Furthermore, we described the molecular mechanism regulated by CaV β 1 and identified a promising new therapeutic strategy against such as threatening conditions.

SESSION 4

GENETIC AND EPIGENETIC ALTERATIONS IN MUSCLE DYSTROPHIES AND MYOPATHIES

4.1 HDAC4 regulates skeletal muscle regeneration via soluble factors**Alessandra Renzini**, Nicoletta Marroncelli, Sergio Adamo and Viviana Moresi

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Skeletal muscle is endowed with a high regeneration potential, mainly due to the ability of muscle stem cells (satellite cells) to replicate and differentiate after an insult or in pathological conditions. Satellite cell behavior is tightly regulated by structural and biochemical cues from the niche, which regulate cell quiescence, self-renewal, proliferation or differentiation, by means of cell-autonomous interaction or paracrine signals (1, 2). Among epigenetic mechanisms, histone deacetylation has been proven to affect muscle regeneration. Indeed, non-specific (pan-) histone deacetylase (HDAC) inhibitors were found to improve muscle regeneration (3), and therefore are on clinical trial for the treatment of Duchenne Muscular Dystrophy (4). Conversely, deletion of HDAC4 in satellite cells was shown to inhibit satellite cell proliferation and differentiation, leading to compromised muscle regeneration (5, 6). In this study, we delineated the HDAC4 function in adult skeletal muscle, following injury, by using a tissue-specific HDAC4 null mouse line. We report that, in spite of HDAC4 functions in SCs, HDAC4 in skeletal muscle is important for a proper efficiency of muscle regeneration. Indeed, deletion of HDAC4 in skeletal muscle compromised muscle regeneration *in vivo*, by mediating soluble factors that influence muscle derived cell proliferation and differentiation. These findings shed further light on the multiple roles of HDAC4 in skeletal muscle regeneration that need to be considered when administering histone deacetylase inhibitors.

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4.2 Myoexosomes cargo triggers muscle regeneration and provides molecular cues for next-generation therapy in muscular dystrophy**Brambilla Andrea**, Villa Chiara, Meregalli Mirella, Marchetti Giulia, Torrente Yvan

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Muscular dystrophies are genetic neuromuscular disorders characterized by skeletal muscle degeneration and consequently enhanced fibrosis. Duchenne muscular dystrophy is the most common form where mutations on the dystrophin gene located on the X chromosome lead to the depletion of the functional protein. Considering the emerging role of extracellular vesicles in cell-to-cell communication, we hypothesized a beneficial effect on dystrophic muscle of exosomes derived from myogenic cells. This muscle improvement is mainly an enhancement of dystrophic muscle regeneration and it has obtained by the exosomes protein or/and non-coding RNA content. This hypothesis has been validated by the discovery in the muscle-derived exosomes of the full-length and the Dp71 dystrophin isoform and the miRNAs muscle-specific or, at least, involved in regeneration processes. These pro-myogenic effects have been tested *in vivo* on the dystrophic murine mouse model (mdx) and *in vitro* on the satellite cells derived from the mdx mouse. These observations highlight the myogenic signature of muscle-derived exosomes and develop new therapeutic strategies for the elucidation of the mechanisms involved in dystrophic muscle progression.

4.3 H3K9 methylation controls Fibro-Adipogenic Progenitors identity and skeletal muscle repair**Beatrice Biferalli^a**, Valeria Bianconi^a, Roberta Maggio^b, Tiziana Santini^c, Giovanna Peruzzi^c, **Chiara Mozzetta^a**^aDepartment of Biology and Biotechnology "C. Darwin", University Sapienza of Rome (Italy). ^bDepartment of Experimental Medicine, University Sapienza of Rome (Italy). ^cItalian Institute of Technology (IIT), Rome (Italy).

Fibro-Adipogenic Progenitors (FAPs) are crucial regulators of muscle homeostasis as they possess the intrinsic ability to either support muscle regeneration or to contribute to fibro-adipogenic degeneration of dystrophic muscles. Therefore, the elucidation of the molecular mechanisms controlling their phenotypical plasticity holds therapeutic potential.

Here we provide evidence that histone H3 lysine K9 methyltransferases (H3K9 KMTs), G9a, GLP and PRDM16, are key stabilizing epigenetic factors of FAPs-specific gene expression programs. Our data support a role for H3K9 KMTs in preserving FAPs identity by repressing alternative transcriptional programs through deposition of H3K9 di-methylation (H3K9me₂). Specifically, we show that PRDM16 controls G9a/GLP's genomic recruitment and H3K9me₂ deposition at muscle-specific loci. Of note, we found PRDM16, G9a and GLP enriched at the nuclear lamina of FAPs suggesting that they organize heterochromatin at the nuclear periphery to maintain the stable repression of genes encoding alternative developmental regulators. Accordingly, pharmacological inhibition or RNAi-mediated knock-down (KD) of H3K9 KMTs de-repress master myogenic genes in FAPs and induce the muscle differentiation program. These data are corroborated by transplants experiments showing that FAPs isolated from mice treated with G9a/GLP specific inhibitors, participate in myofibers formation in regenerating recipient mice. Together, our findings reveal a FAPs-specific epigenetic axis of therapeutic relevance since we demonstrate that *in vivo* inhibition of H3K9 methylation in dystrophic mice enhances skeletal muscle regeneration, inducing an increase in myofibers size and reduction of adipogenic and fibrotic scars.

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GENETIC AND EPIGENETIC ALTERATIONS IN MUSCLE DYSTROPHIES AND MYOPATHIES

4.4 Therapeutic activity of modified U1 core spliceosomal particles in Spinal Muscular AtrophyIrving Donadon^a, Erica Bussani^a, Danilo Licastrò^b, Federico Riccardi^a, **Franco Pagani^a**^aHuman Molecular Genetics International Centre for Genetic Engineering and Biotechnology, Trieste, Italy ^bCBM, Trieste, Italy

RNA splicing mutations represent a significant amount of disease causing defects. Our group is interested in engineering components the spliceosome to bypass splicing defects. In the last years, we have developed a novel therapeutic strategy to correct splicing defects based on modified U1 snRNAs (Exon Specific U1, ExSpeU1). ExSpeU1s are small RNA molecules that bind by complementarity to intronic sequences downstream the 5' splice site and correct exon skipping caused by different splicing mutations. We have evaluated the therapeutic activity of ExSpeU1s in Spinal Muscular Atrophy. In a severe Spinal Muscular Atrophy mouse, ExSpeU1, either expressed by transgenesis or by AAV vectors rescued the underlying aberrant SMN2 splicing defect, increase the SMN protein and extensively extends life span. The rescued adult mice showed normal morphology of the neuromuscular junction and normal number of motoneurons. Off target analysis by RNA-SEQ on a human model cell line showed a limited number of differentially expressed genes and splicing events caused by the ExSpeU1 expression. Our findings indicate that precise engineering of the U1 core spliceosomal RNA particle has therapeutic potential in SMA and in pathologies associated with exon-skipping mutations.

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4.5 Genetic deletion and pharmacologic targeting of GLUD1 breaks glutamine competition between macrophage and satellite cells improving muscle regenerationMin Shang^a, Federica Cappellesso^a, Ricardo Amorim^a, Jens Serneels^a, Mario di Matteo^a, **Emanuele Berardi^b**, Massimiliano Mazzone^a^aVIB Vesalius Research Center, Katholieke Universiteit Leuven. ^bDepartment of Kinesiology, Katholieke Universiteit Leuven

Activation of satellite cells sustains muscle regeneration upon injury. During this process, macrophages infiltrate the injured tissue and participate to its repair by different means. However, if and how macrophages influence satellite cell activation is poorly studied. Here, we show that macrophage metabolism strongly affects skeletal muscle regeneration by impinging on the composition of the extracellular milieu. In particular, we argue that knockout of glutamate dehydrogenase (GLUD1) in macrophages prevents glutamine anaplerosis and thus utilization of this amino acid by macrophages. It follows that glutamate is then rerouted towards glutamine synthesis and secretion, favored by the induction of glutamine synthetase (GS) in GLUD1-deficient macrophages. Glutamine release by macrophages sustains satellite cell activation, proliferation and differentiation, altogether accelerating muscle regeneration and functional recovery. As a consequence, genetic targeting of GLUD1 in macrophages or pharmacologic inhibition of this enzyme ameliorates acute and chronic muscle necrosis upon cardiotoxin-induced injury, ischemic damages, and strongly prevents age-related muscle loss. These results demonstrate that glutamine competition between macrophages and satellite cells restricts muscle regeneration upon injury.

4.6 The voice of patients and their families: Parent Project Onlus

Gloria Antonini

Parent Project onlus is an association of patients and parents of children affected by Duchenne and Becker Muscular Dystrophies (DMD and BMD), considered the most common among rare diseases and for which there is still no cure. The association is committed to funding research and disseminating the multidisciplinary approach that has so far enabled doubling the patients' life expectancy and improving their quality of life. Research support is the key objective of the Parent Project Association. The Scientific Office manages all activities related to the support of research and the dissemination of scientific information to patients, families and the outside world. The office also manages the Italian DMD/BMD patient registry, an integral part of the Global Registry established by the Treat-NMD, a network of excellence whose objective is the coordination and harmonization of research in the field of neuromuscular diseases. Like every year, Parent Project will organize, on the 16th and 17th of February 2019 in Rome, the International Conference on Duchenne and Becker Muscular Dystrophy where medical-scientific updates and dedicated spaces to families will be combined in a balanced way.

SESSION 5 METABOLIC ALTERATIONS AND MUSCLE DISEASES

5.1 Mitochondrial adaptation in parvalbumin knockout muscle fibres

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In skeletal muscle, mitochondrial Ca²⁺ uptake plays important roles in organ homeostasis. In order to understand the importance of mitochondria in cytosolic Ca²⁺ buffering, we explored the effects of removing parvalbumin (PV), the most important cytosolic Ca²⁺ buffer. By using a PV knockout (KO) mouse model, we are investigating whether the absence of PV induces a compensatory mechanism on the expression and function of the mitochondrial Ca²⁺ uptake machinery (MCU complex). The data collected so far confirm that the absence of PV induces an increase of mitochondrial Ca²⁺ uptake accompanied by a profound adaptation of the expression of the MCU complex components. Furthermore, cytosolic Ca²⁺ transients in PV KO muscle fibres show that the time to reach the peak upon stimulation and the time to half relaxation are prolonged in PV KO muscle fibres in comparison with WT animals, given to a major fatigue resistance and ability to sustain muscle activity for prolonged period of PV KO mice. Intriguingly since PV is one of the most downregulated atrogenes, the genes modulated during several types of atrophy, and that mitochondrial Ca²⁺ controls skeletal muscle tropism, we decided to study the role of PV on muscle trophism through denervation experiments on PV KO mice. Denervation atrophy was triggered by sciatic nerve section and fibres size was evaluated. When PV is absent, loss of muscle mass was reduced compared to innervated control fibres demonstrating that PV can partially protect muscles from denervation-induced atrophy. Silencing experiments gave coherent results. Tibialis anterior muscles of WT were transfected *in vivo* with plasmids encoding either shLuc or shPV. Fibres positive for shPV are significantly larger than fibres expressing the shLuc control plasmid. In conclusion, our results indicate that PV plays an important role in spatiotemporal control of cytosolic Ca²⁺ responses on mitochondrial Ca²⁺ uptake and have a profound impact on skeletal muscle trophism.

5.2 Loss of Mitochondrial Calcium Uniporter rewires skeletal muscle metabolism and substrate preference

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Skeletal muscle mitochondria readily accumulate Ca²⁺ in response to SR store-releasing stimuli thanks to the activity of the Mitochondrial Calcium Uniporter (MCU), the highly selective channel responsible for mitochondrial Ca²⁺ uptake. MCU positively regulates myofiber size in physiological conditions, and counteracts pathological loss of muscle mass. Here, we show that skeletal muscle-specific MCU deletion inhibits myofiber mitochondrial Ca²⁺ uptake, impairs muscle force and exercise performance, and determines a slow-to fast switch in MHCs expression. Mitochondrial Ca²⁺ uptake is required for effective glucose oxidation, as demonstrated by the fact that in muscle-specific MCU^{-/-} myofibers oxidative metabolism is impaired and glycolysis rate is increased. Although defective, mitochondrial activity is partially sustained by increased fatty acid (FA) oxidation. In MCU^{-/-} myofibers, PDP2 overexpression drastically reduces FA-dependency, demonstrating that decreased PDH activity is the main trigger of the metabolic rewiring of MCU^{-/-} muscles. Accordingly, PDK4 overexpression in MCU^{fl/fl} myofibers is sufficient to increase FA-dependent respiration. Finally, as a result of the muscle-specific MCU deletion, a systemic catabolic response impinging on both liver and adipose tissue metabolism occurs.

5.3 Diet-based metabolic reprogramming impacts on the differentiation potential of muscle progenitor cells and ameliorates the *mdx* dystrophic phenotype

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Duchenne muscular dystrophy (DMD) is a lethal muscle disease characterized by tissue wasting and coupled to systemic metabolic disorders. The resulting defects in mitochondrial functionality cause an unbalance between impaired oxidative phosphorylation and increased glycolysis in the muscle fiber. We have recently demonstrated that satellite cells (MuSCs) and fibro/adipogenic progenitors (FAPs) from a dystrophic muscle are also characterized by a “dystrophic metabolic signature”. We have also observed that such metabolic defects impact on the differentiation potential of FAPs and MuSCs.

Prompted by these findings, we set about designing a diet regimen aimed at restoring a “wild type metabolic state” in dystrophic progenitor cells and asking whether such a diet could have any effect on the severity of

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the dystrophic phenotype. To this end we fed *mdx* mice on a short-term high fat diet (HFD) to force a metabolic reprogramming based on fatty acid utilization rather than glycolysis.

Unbiased mass spectrometry-based proteomics demonstrated that diet-treated dystrophic FAPs and MuSCs revert the dystrophic metabolic signature, resulting in the upregulation of enzymes involved in the TCA cycle and oxidative phosphorylation. Remarkably, such metabolic reprogramming paralleled a noticeable amelioration of the dystrophic phenotype. Namely we observed a recovery from the muscle atrophy observed in young *mdx* mice and a reduced fibrosis in the diaphragm. In addition by mapping the proteomic profiles on a literature-derived signaling network, we observed that the HFD significantly rewires the FAP proteome. In particular, in the WNT pathway we noticed that β -catenin expression, a key adipogenic regulator which is down regulated in *mdx* FAPs, was restored back to wild type levels in mice fed with the high fat diet, a result that is compatible with a tighter control of adipogenesis.

Consistently, we demonstrated that pharmacological up-regulation of β -catenin, through the inhibition of GSK3 β , protects muscles from glycerol-induced fatty degeneration, which is mainly caused by aberrant FAP adipogenesis.

These results offer a proof of principle for a novel therapeutic approach for muscular dystrophies based on metabolic reprogramming of stem and progenitor cells of the muscle niche.

5.4 Metabolic changes associated with muscle expression of SOD1G93A

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Amyotrophic lateral sclerosis (ALS) is a severe neurodegenerative disorder, characterized by motor neurons death, muscle atrophy and paralysis. ALS is classified into sporadic or familial forms and among the familial cases approximately 20% are caused by dominant mutations in the gene coding for superoxide dismutase (SOD1) protein. ALS is a multi-systemic and multifactorial disease that affects whole body physiology and induces severe metabolic changes in different tissues, including skeletal muscle. However, whether alterations in the plasticity and metabolism of muscle fibers are the result of motor neuron degeneration or alternatively occur independently of it remain to be elucidated. To answer this, we used a mouse model (MLC/SOD1G93A) overexpressing the SOD1 mutant gene selectively in skeletal muscle. We observed an alteration of muscle glucose metabolism associated with the induction of Phosphofructokinases and Pyruvate dehydrogenase kinase 4 expression, which led to the inhibition of Pyruvate conversion into Acetyl-CoA. Moreover, we demonstrated that the MLC/SOD1G93A transgene was associated with a preferential use of lipid energy fuel by muscle fibers. We provided evidences that muscle metabolic alterations occurred before disease symptoms and independently of motor neuron degeneration, indicating that skeletal muscle is likely an important therapeutic target in ALS.

5.5 Muscle-specific Plin2 downregulation affects ectopic lipid metabolism and myofiber size

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Aging is characterized by a decline in muscle mass and quality leading to sarcopenia. A role for ectopic accumulation of lipid metabolites in sarcopenia was recently proposed. Fat accumulates within lipid droplets surrounded by proteins, such as Plin2. Perilipin2 (Plin2) belongs to a family of five highly conserved proteins, which are known for their role in lipid storage. Recent data suggest that the biological role of Plin2 is much more complex than previously thought. Plin2 appears to have an important function in cell metabolism and is involved in several human pathologies, including liver steatosis and Type II diabetes. In humans high level of Plin2 is associated with loss of muscle strength, however its role in skeletal muscle is still unclear.

We performed muscle-specific in vivo experiments of Plin2 downregulation (shPlin2) in mice and analysed the effects on myofiber size and lipid composition.

We found that Plin2 downregulation induces a 30% increase of myofiber cross sectional area (CSA), independently of mTOR. Moreover, shPlin2 leads to an alteration of intracellular lipid content, including a decrease of triglycerides and ceramides, and a modulation of genes involved in lipid synthesis. Plin2 downregulation also protects muscles from denervation-induced atrophy.

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Our data indicate that Plin2 could be a key factor that controls muscle mass by regulating intracellular lipid content and could be considered as a therapeutic target to counteract sarcopenia.

5.6 Defective glycosylation of IGF-1Ea prohormone and IGF-1 secretion in fibroblasts from congenital disorders of glycosylation

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Congenital disorders of glycosylation (CDG) are rare genetic diseases in which glycosylation pathways are defective. Postnatal growth failure is common in children with CDG and impairment of the insulin-like growth factor-1 (IGF-1) cascade is suggested as the cause of failure to thrive in these patients [1]. In particular, decreased levels of IGF-1, the binding protein IGFBP-3 and acid-labile subunit (ALS) have been described in patients with CDG [2]. Immunoblot analysis showed incomplete glycosylation of ALS and IGFBP-3 and impaired ternary complex formation in CDG [2-3], while the reasons behind the low IGF-1 level found in CDG patients remain unknown. We recently demonstrated that intracellular IGF-1 is mainly expressed as prohormone (proIGF-1Ea) containing an intrinsically disordered region, i.e. the Ea-peptide, which is heavily N-glycosylated [4]. In this study, we tested the hypothesis that CDG caused a defect of Ea-peptide glycosylation which is directly associated with decreased proIGF-1Ea production and IGF-1 secretion.

We transiently transfected fibroblasts from control subjects and different CDG subtypes (ALG3, ALG8, GMPPB and PGAP2) with the plasmid construct containing sequence encoding proIGF-1Ea; protein lysates were subjected to SDS-PAGE and immunoblotted with an anti-IGF-1 antibody. The supernatants of transfected fibroblasts were also used to treat the MCF-7 cells to evaluate their effects on IGF-1 receptor (IGF-1R) phosphorylation. Finally, fibroblasts were treated with 2-Deoxyglucose (2-DG) (0,5 g/L) to evaluate the effects of this glucose and mannose analogue on proIGF-1Ea production.

Immunoblot analysis showed that fibroblasts from ALG3, ALG8 and GMPPB patients showed a defect of proIGF-1Ea glycosylation compared to controls and PGAP2 fibroblasts. Cell culture media from IGF-1Ea-transfected ALG3 fibroblasts also showed a significant decrease of IGF-1R phosphorylation, suggesting IGF-1-secretion deficit in this CDG subtype. Treatment with 2-DG increased the proIGF-1Ea level both in CDG and controls fibroblasts, leading to an accumulation of an under-glycosylated form which was still able to fold and secreted by the cells.

In conclusion, these results show that proper proIGF-1Ea glycosylation and IGF-1 secretion is impaired in fibroblasts from CDG patients. Thus, the correction of proIGF-1Ea glycosylation defects (e.g. by 2-DG), may represent a novel therapeutic approach to increase local and systemic IGF-1 production in CDG patients.

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SESSION 6

MUSCLE FIBROSIS, SARCOPENIA AND CACHEXIA

6.1 Nature and role of interstitial non myogenic cells in human fibrotic muscles

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Fibrosis is one of the common pathological outcomes of many chronic diseases, and the main complication in many muscular dystrophies (MD). MD represent an heterogeneous group of disorders characterized by weakness and/or progressive degeneration process of skeletal muscle with a wide clinical presentation and severity. Among MD, Duchenne muscular dystrophy (DMD) is a fatal genetic disorder with an early onset, caused by mutations in the dystrophin gene. Oculopharyngeal muscular dystrophy (OPMD) is an autosomal dominant inherited, slow progressing, late onset degenerative muscle disorder caused by a short triplet expansion in the PABPN1 gene. MD, whether they involve repeated cycles of degeneration and regeneration such as in DMD or not such as OPMD, result inexorably in skeletal muscle atrophy inevitably associated with fibrosis. Fibrosis represents the final consequence of the muscle degeneration process, as a result of reactive and/or reparative processes. These processes involve mechanical, humoral and cellular factors. Fibrotic muscular substitution is attributed to excess deposition of extracellular matrix components (ECM).

Fibroblasts are known to be involved in several biological processes such as wound healing, inflammation, and angiogenesis. However, their causal implication in dystrophic muscle progression and fibrosis remain still poorly characterized. Here, we characterized interstitial non myogenic cells (CD56-) isolated from control and affected fibrotic muscles of OPMD and DMD patients. Proliferation capacity, adipogenic/osteogenic differentiation, lifespan studies, co-culture experiments, FACS analysis and high-dimensional single-cell analysis have been performed. Xenotransplantation experiments to decipher the influence of CD56- cells during muscle regeneration *in vivo* were also performed. We demonstrate that human CD56- cells from fibrotic muscles are different compared with those from control muscles, showing a strikingly high proliferative capacity, an effect on fusion index *in vitro* and an exacerbated secretion *in vivo*.

6.2 The effect of muscle activity on tumor cell growth and survival

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Exercise is now recommended in multimodal therapies for cancer patients. The beneficial effects of exercise span from the rescue of muscle homeostasis to the control of inflammation, ultimately resulting in increased survival of tumor bearing animals and patients. A modern vision of the skeletal muscle includes the idea that this highly vascularized organ poses an important paracrine and endocrine activity, which is exercise-dependent. Secreted muscle factors (myokines) affect multiple target tissues. Only recently, a pioneer study showed that the tumor itself can be targeted by myokine-directed NK cells and its growth blunted following muscle stimulation by exercise. Whilst suggested by other studies, it is not clear if muscle cells per se possess an antitumoral activity, nor its dependence by mechanical stimulation. This project aims to demonstrate *in vitro* that (a) muscle cells secrete factors with anti-tumor activity (by either stopping cell proliferation or inducing cell death) and (b) mechanical stimulation of muscle cells affects their secretome, possibly enriching it in antitumoral factors; in addition, an initial characterization of the products released by muscle cells is proposed, with the aim to start identifying the biochemical nature of these antitumoral factors.

6.3 Altered iron metabolism promotes cancer cachexia

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Cachexia is a multi-organ wasting syndrome characterized by irreversible skeletal muscle atrophy that dramatically increases both the morbidity and mortality in various diseases. Despite its high prevalence in cancer patients, knowledge regarding the mechanism of cancer-induced cachexia remains very scarce.

In this study, we first show that iron deprivation by several means (knockdown of transferrin receptor-TfR, responsible for the cellular uptake of iron, and selective iron chelators) induces myotube atrophy *in vitro*. Moreover, cachectic mice bearing C26-colon cancer feature striking alterations in key regulators of iron metabolism, notably an overexpression of ferroportin (iron exporter), a downregulation of TfR and an altered ferritin (main storage site of iron) recycling. Consistently, we found a decreased iron loading in skeletal muscle and in spleen along with a strong increase in serum levels, indicating that iron homeostasis is altered at a systemic level in cachectic mice. Intriguingly, normalizing iron levels *in vitro* counteracted cancer-induced

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myotubes atrophy.

Overall, we evidenced that iron metabolism is strongly altered in cachectic mice, resulting in extensive export of iron from skeletal muscle to the bloodstream, while *in vitro* data prove that iron availability directly influences muscle mass. Currently, we are investigating the effects of restoring iron homeostasis on muscle wasting in tumor-bearing mice, as well as the pathways underlying iron-dependent regulation of muscle mass

6.4 Targeting mitochondria with SS-31 in experimental cancer and chemotherapy-induced cachexia

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Cancer cachexia is a debilitating muscle wasting condition defined also as an energy-wasting syndrome (1). Mitochondria play a central role, being the main energy source. Indeed, mitochondrial alterations and low intracellular ATP have been found in the skeletal muscle of cachectic animals (2).

The present study aimed at evaluating the effects of a mitochondrial-targeted compound (SS-31) on muscle wasting in C26-bearing mice either under unrestricted tumor growth (C26; 14 days of tumor growth) or treated with chemotherapy (oxaliplatin+5-fluorouracil; C26 OXFU; 28 days of tumor growth).

Animals with unrestricted tumor growth exhibited a reduction of muscle mass, muscle strength and food intake. OXFU administration was able to double the lifespan of C26 mice, although resulted in exacerbated muscle wasting. SS-31 administration counteracted body weight and food intake loss in C26 mice and, while borderline significantly protected from muscle wasting. Regarding mitochondrial markers, both C26 and C26 OXFU animals exhibited a reduction of PGC-1 α , Cytochrome c and SDH protein levels with no effect of SS-31 administration. In C26 OXFU mice, also the SDH total activity and ATP content were reduced. In C26 mice SS-31 increased SDH activity and ATP content. Mitochondrial alterations found in C26 mice also associated with a strong reduction in protein synthesis, that was improved by SS-31 treatment.

In conclusion, targeting mitochondria with SS-31 exerts some beneficial effects on C26-bearing mice with unrestricted tumor growth, partially protecting from body weight and food intake loss, muscle wasting and counteracting the impairment of muscle oxidative capacity leading to energy wasting. Further investigation and treatment optimization is required in order to demonstrate a potential effectiveness of SS-31 in the more severe chronic model (C26 OXFU mice).

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6.5 Role of ghrelin peptides in aging

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Sarcopenia is a multifactorial syndrome defined as the irreversible loss of skeletal muscle mass and functionality occurring during aging. Muscle atrophy and impaired regeneration are the main features of this syndrome but the underlying mechanisms and etiology remain poorly defined. Acylated and unacylated ghrelin (AG and UnAG, respectively) are circulating peptide hormones derived by the *Ghrl* gene and mainly produced by the stomach. AG, through its canonical receptor Ghsr-1a, induces a strong release of growth hormone and stimulates appetite and food intake. Although UnAG does not activate this receptor, it shares with AG several common biological activities. In particular, on skeletal muscle, both peptides counteract atrophy and promote myoblast differentiation through a yet unknown receptor. Moreover, UnAG enhances skeletal muscle regeneration after injury, stimulates satellite cell functions, and ameliorates the dystrophic phenotype in mdx mice. The modulation of AG/UnAG plasmatic levels and of their signaling during aging suggest that the AG/UnAG system may be a possible regulator of sarcopenia establishment. We are studying the role of AG/UnAG in sarcopenia prevention by means of transgenic mice characterized by high levels of circulating UnAG and *Ghrl*^{-/-} mice that lack all *Ghrl*-derived peptides.

We observed that lack of ghrelin gene has a relevant impact on muscle performance in aged mice (12-month-old animals), while higher UnAG circulating levels apparently do not impact significantly on muscle. Conversely, ghrelin peptides are able to regulate age-related metabolic adaptation.

These findings suggest indeed a potential role of AG/UnAG system in sarcopenia onset.

(Work supported by Fondazione Cariplo)

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SESSION 7

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7.1 Engineering skeletal muscle tissue with innovative 3D bioprinting approaches**Marco Costantini^{a,b}**, Jan Guzowski^a, Wojciech Świążkowski^b and Cesare Gargioli^c^aInstitute of Physical Chemistry, Polish Academy of Sciences, Warsaw, Poland. ^bFaculty of Materials Science and Engineering, Warsaw University of Technology, Poland. ^cDepartment of Biology, University of Rome Tor Vergata, Rome, Italy

Within the human body, skeletal muscle (SM) tissue is responsible for a multitude of fundamental functions. All voluntary movements that characterize our lives, from a single step to a simple smile, would not be possible without this highly specialized tissue.

In the last two decades, driven by the limited regenerative capacity of SM tissue, researchers have developed numerous approaches to restore/repair it. However, the obtained results are still far from being satisfactory. Here, we present a new strategy for the fabrication of artificial myo-structures with functional morphologies based on an innovative 3D bioprinting approach. This system is based on a microfluidic printing head (MPH) coupled with a co-axial nozzle extruding system that allows high-resolution 3D multi-cellular bioprinting of hydrogel fibers ($\varnothing \approx 100 \mu\text{m}$) and high cell viability. To promote myoblast differentiation, we formulated a tailored bioink with a photocurable semi-synthetic biopolymer, namely PEGylated fibrinogen, and we encapsulate cells (C2C12 or human pericyte) in 3D scaffolds composed of unidirectionally aligned hydrogel fibers. The 3D fabricated samples were tested both *in vitro* and *in vivo* to evaluate their capability of supporting myogenesis and sarcomerogenesis. The results showed that after 21 days of culture *in vitro*, myoblasts properly spread and fused forming highly aligned long-range multinucleated myotubes, with abundant and functional expression of myosin heavy chain (MHC) and laminin (LAM). Besides, the 3D biofabricated constructs when grafted *in vivo* led to a substantial improvement if compared to bulk-hydrogels (used as control) of muscle-like architectural organization with the formation of tightly-packed, highly parallel and completely striated myotube fibers.

7.2 A human neuromuscular junction model system: an organ-on-a-chip approach.**Ersilia Fornetti^a**, Cesare Gargioli^a, Victorio Pozo Devoto^b, Alberto Rainer^c, Giancarlo Forte^b and Stefano Cannata^a^aDepartment of Biology, University "Tor Vergata", Rome, Italy. ^bCenter for Translational Medicine, FNUSA ICRC, Brno, CZ. ^cTissue Engineering Unit, University "Campus Bio-Medico", Rome, Italy.

The formation of the neuromuscular junction (NMJ) at the interphase between motoneurons and skeletal muscle, is a complex multistep process involving a variety of signaling molecules and pathways. A derangement in NMJ integrity and signaling can be caused by both neurodegenerative diseases and muscular pathologies. *In vitro* modeling of this complex structure could represent a powerful tool to help unravel the mechanisms leading to its degeneration and repair. Nonetheless, to date, no reliable and predictive *in vitro* human models of NMJ in physiological and pathological conditions exist. It is possible to obtain human motoneurons out of induced Pluripotent Stem Cells and human skeletal muscle from perivascular muscle progenitors, namely Pericytes, can be isolated from muscle biopsies. Additionally, the microfluidic technology, unlike mass co-cultures, allows spatial and temporal control over microenvironments by manipulating either one or the other cell population independently. Our preliminary results demonstrate that it is possible to successfully co-culture human skeletal muscle differentiated from Pericytes with iPSCs-derived motoneurons. Hence, exploiting an organ-on-a-chip approach, we propose a set up for a novel human NMJ model system to investigate the occurrence of NMJ mismatches in disease. While being designed as a reliable platform to investigate the molecular actors of NMJ processes, the setup is versatile enough to host patient-specific cells and perform functional and molecular analysis.

7.3 New biomimetic scaffolds for the expansion of functional adult satellite cells and the generation of mature myofibers ex vivo**Francesca Gattazzo^{a,b}**, Béatrice Laurent^{a,b}, Naïm Jalal^c, Mustapha Zidi^c, Frédéric Relaix^{a,b}, Hélène Rouard^{a,b}, Nathalie Didier^{a,b}^aUnité d'Ingénierie et de Thérapie Cellulaire, EFS Ile de France, Créteil, France ^bEquipe 10 Biologie du Système Neuromusculaire, INSERM IMRB U955, Faculté de médecine, Créteil, France. ^cBioingénierie, Tissus et Neuroplasticité (BIOTN) - EA 7377, Faculté de médecine, Créteil, France

The adult muscle stem cells, termed satellite cells (SC), possess a remarkable regenerative capability and represent the source of election for cell therapy strategies for skeletal muscle repair. Despite their great potential, the therapeutic use of SC in clinic is still limited by their poor number and by the absence of methods to efficiently amplify them *in vitro*. Once isolated from the specialized microenvironment where they reside *in vivo*, called the niche, SC spontaneously differentiate, losing their engraftment and their self-renewal potential. To overcome this limitation and in order to expand functional adult SC in a manner compatible with clinical application, we developed a natural hydrogel containing growth factors and cytokines of human origin with tunable mechanical properties. We compared the behaviour of adult SC purified from young and aged mouse muscles when expanded on an hydrogel mimicking the mechanical properties of skeletal muscle fibers (~12

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kPa) vs standard culture conditions (i.e. on gelatin coated-dish). Using this hydrogel we could significantly improve the total number of cells obtained upon passages, preserve the expression of Pax7, while limiting the Myogenin induction. Moreover, we could partially restore the proliferation rate of aged SC. When engrafted *in vivo*, young SC expanded on hydrogel could repopulate SC niche and contribute to muscle regeneration demonstrating the preservation of their myogenic potential. In addition, we have preliminary evidence that this method of amplification could be extended to human SC. Altogether, these promising results support the possibility of using our hydrogel for the production of functional myogenic cells for clinical application. Interestingly, by using the same hydrogel but with different rigidity, we could favor the differentiation of SC and the generation of mature unidirectionally oriented myofibers. This system might provide a powerful tool for the modelisation of neuromuscular disorders and for pharmacological screening.

7.4 Fibrosis rescue improves cardiac function in dystrophin-deficient mice and Duchenne patient-specific cardiomyocytes by immunoproteasome modulation

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Patients affected by Duchenne Muscular Dystrophy (DMD) develops a progressive dilated cardiomyopathy (CM) characterized by inflammatory cell infiltration, necrosis and cardiac fibrosis. Standard treatments consider the use of β -blockers and angiotensin-converting enzyme inhibitors that are symptomatic and unspecific towards DMD disease. Medications that target the cardiac fibrosis are in early stages of development. Here, we demonstrated immunoproteasome (IP) dysregulation in affected heart of mdx (murine animal model of DMD) and cardiomyocytes derived from induced pluripotent stem cells (iPSCs) of DMD patients. More interestingly, IP inhibition ameliorates cardiomyopathy of mdx mouse and reduces the development of cardiac fibrosis. Our finding of a cardioprotective function of IP expression suggests its modulation among novel treatments to be tested in future clinical trials to rescue dilated CM of DMD.

POSTER SESSION:
ALWAYS ON DISPLAY

POSTER SESSIONS

ABSTRACTS

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P.01 The role of vitamin D binding protein (VDBP) in cancer cachexia

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Vitamin D binding protein (VDBP), also known as *Group-specific component* (Gc-globulin), is a multifunctional serum glycoprotein synthesized by hepatocytes that belongs to the albumin gene family. Besides the binding and transport of vitamin D metabolites in blood, VDBP has other activities, including binding and clearance of monomeric G-actin released from dead cells and acting as a chemotactic cofactor for C5a.

Proteomic analysis shows that VDBP is upregulated in patients with early-stage breast cancer, ovarian cancer, oral squamous cell carcinoma, diabetes mellitus, multiple sclerosis, and COPD, all susceptible to progressive muscle loss or cachexia, raising the hypothesis that VDBP might play a role in muscle wasting.

We therefore explored the direct action of VDBP in skeletal muscle and we found that VDBP indeed induces atrophy in C2C12 myotubes. The finding that VDBP expression increases in tumor-bearing mice undergoing cachexia further supports the notion that VDBP could play a role in the onset/progression of cancer-associated muscle wasting.

P.02 Silencing Nfix rescues Muscular Dystrophy by delaying muscle regeneration

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Muscular Dystrophies are severe disorders due to mutations in structural genes that cause skeletal muscle wasting compromising patient mobility and respiratory functions. Although previous works suggested enhancing regeneration and muscle mass as therapeutic strategies, these led to no long-term benefits in humans. Here we propose a conceptually new idea based on making a dystrophic muscle slower in regeneration and more oxidative, by silencing of the transcription factor *Nfix*. In different forms of Muscular Dystrophy, lack of *Nfix* rescues histopathological and functional hallmarks of dystrophic muscle. More importantly, silencing *Nfix* in post-natal dystrophic mice, when the first signs of the disease already occurred, rescues the pathology. On the contrary, *Nfix* overexpression in dystrophic muscles pushes regeneration and markedly exacerbates the pathology. We therefore provide evidence that current strategies are based on a misconception and offer a proof of principle for a novel therapeutic approach.

P.03 Identification of a novel TFEB-exercise dependent gene

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Physical training is a major challenge for the organism, involving almost all organs and apparatus; not least, exercise drives a series of systemic health benefits through the improvement of muscle performances. Although during the past some of the molecular actors have been identified, several mechanisms underpinning exercise biology still remain elusive.

In last years we have been focused in elucidating the role of the transcription factor EB, a calcineurin-activated TF responsible for the control of metabolic flexibility during exercise in a PGC1 α independent manner. We reported that TFEB translocates into myonuclei during physical activity following calcium transients and regulates glucose uptake and glycogen content by controlling the expression of glucose transporters, glycolytic enzymes and pathways related to glucose homeostasis. In addition, TFEB induces the expression of genes involved in mitochondrial biogenesis, fatty acid oxidation and oxidative phosphorylation. These coordinated actions optimize mitochondrial substrate utilization, thus enhancing ATP production and exercise capacity. Based on these evidences, we recognized TFEB as a critical mediator of beneficial effects of exercise on metabolism. From this point, we started to identify new putative exercise-induced metabolic regulators by crossing exercise-related gene expression profile data; interestingly, we identified a new gene with unknown function belonging to the Riken cDNA collection that we called Exe-Riken. Exe-Riken encodes for a 125 amino-acids protein, conserved among placental mammals; moreover, the human homolog gene shares more than 70% identity in sequence with the murine one. Bioinformatic predictions evidenced the presence of a Nuclear Localisation Signal (NLS) and a Nuclear Export Signal (NES), suggesting the possibility that Exe-Riken acts as a metabolic nuclear co-regulator. Microarray data show that muscular expression is very low compared to brown-adipose tissue or intestine; indeed, muscular transcript is hardly detectible in resting conditions, even if it is more than 50 fold induced in different models of TFEB activation.

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Concluding, more gain and loss of function data are needed to uncover the role of Exe-Riken gene; nevertheless these evidences support the idea that Exe-Riken is a novel exercise-related metabolic regulator.

P.04 Over-expression of mIGF-1 in skeletal muscle attenuates the effects of sarcopenic obesity

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Sarcopenia is the progressive aging-related loss of skeletal muscle mass and function occurring during aging. It is the result of multiple factors, including changes in the metabolic state or in the neuromuscular system, inflammatory pathway activation and altered production and tissue responsiveness of trophic factors. In particular changes in hormonal level, including decrease of insulin-like growth factor (IGF-1), contribute to sarcopenic condition. The process of age-related muscle loss combined with increased body fat is defined sarcopenic obesity and is associated with decline of muscle strength and function. To evaluate whether the over-expression of mIGF-1 (a muscle-specific IGF-1 isoform) is able to interfere with the mechanisms of sarcopenic obesity, we induced this condition in wt and mIGF-1 mice feeding them with a high-fat diet (36% energy by fat) for three months and comparing them with mice fed with a control diet (5,7% fat). mIGF-1 mice showed resistance to HFD-induced obesity displaying a body-weight gain significantly lower than that of wt mice and a decreased fat accumulation along with a less severely impaired glucose tolerance. These observations correlated with a reduced intramuscular lipid content, a less pronounced muscle atrophy and a lower inflammation, suggesting a protective role of mIGF-1 against sarcopenic obesity.

P.05 THE ROLE OF RAPTOR IN ADULT SKELETAL MUSCLE

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Mammalian target of rapamycin (mTOR) plays a central role in cell growth. mTOR assembles into two distinct multiprotein complexes, namely the rapamycin-sensitive complex mTORC1 and the rapamycin-insensitive complex mTORC2. One of the key members of the mTORC1 complex is a 150kDa protein called Raptor, which has been shown to be able to recruit mTOR substrates S6K1 and 4EBP1 on mTORC1. Mice lacking Raptor only in skeletal muscle from birth show a pronounced myopathy leading to a premature death. However, treating adult mice with the specific mTORC1 inhibitor rapamycin does not lead to a myopathic phenotype, and even improves muscle physiology in aged mice. Here we want to examine the role of Raptor and mTORC1 using a new CreER-inducible transgenic mouse in which we can delete Raptor in muscles of adult mice (Raptor k.o.). Activation of Cre by treatment with tamoxifen leads to a rapid loss of Raptor transcript and protein levels. Also the phosphorylation levels of ribosomal protein S6, a known mTORC1 target, are strongly reduced in Raptor ko mice. One month after Raptor deletion, muscle weight and basic histology are unchanged. A longer deletion of Raptor, however, leads to a myopathic phenotype with central-core structures and a high number of small and large muscle fibers.

P.06 Identification of novel molecular targets to manipulate satellite cell function

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Skeletal muscle ability to repair depends on the function of Satellite Cells (SCs) to proliferate, differentiate, and eventually repair the damage. At the same time, a fraction of SCs undergoes self-renewal, to preserve the stem cell pool. Satellite cell function decline in certain pathological conditions, such as Duchenne Muscular Dystrophy (DMD). Satellite cell exhaustion and defects in their ability to self-renew have been suggested to contribute to the impairment in regeneration in DMD. Our previous works demonstrated that lack or inhibition of Protein kinase C θ (PKC θ), in a mouse model of Duchenne Muscular Dystrophy (mdx mouse), modulates the quality of immune cell infiltration, leading to a reduction in muscle damage. In parallel, we have observed an improvement in muscle regenerative ability, even at a late stage of the disease, suggesting a positive effect also on the satellite cell compartment.

PKC θ influences many aspects of muscle growth and homeostasis, however the effects on muscle stem cells remain unknown. In this study we show that PKC θ is expressed in SCs. Total genetic knockout of PKC θ , or its pharmacological inhibition, have no effect on quiescent satellite cell number in healthy muscle. Instead, the ability of SCs to repopulate their niche after injury is significantly increased in absence of PKC θ . *In vitro* experiments on isolated myofibers, and the analysis of Notch signalling, suggested that self-renewal is increased in absence of PKC θ . These results indicate that PKC θ may be a promising target to enhance SC function in pathological contexts.

POSTER SESSION:
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P.07 Histone 3 Lysine 9 methyltransferases G9a and GLP as potential pharmacological targets in skeletal muscle regeneration and Duchenne Muscular Dystrophy

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Histone Lysine Methyltransferases (KMTs) are epigenetic modifiers that dynamically control gene expression during stem cell differentiation. Among the different KMTs, EHMT2/G9a and EHMT1/GLP, responsible of mono- and di-methylation of Lysine 9 of histone H3 (H3K9), are of particular relevance in myogenesis as they prevent premature differentiation of myogenic precursors repressing the expression of muscle-specific genes. We hypothesize that modulation of their activity might exert beneficial effects on muscle regeneration *in vivo* by promoting the myogenic expression program thus enhancing regenerative capacity of myogenic progenitors in dystrophic muscles. Duchenne Muscular Dystrophy (DMD) is the most severe form of dystrophy that leads to progressive muscle weakness because fat and fibrotic scars gradually replace functional muscle. Pharmacological therapies for DMD should aim to counteract this fibro-adipogenic degeneration and to promote the compensatory regeneration to slow down progression of pathology.

By using murine models of injury-induced regeneration and fatty degeneration, we show that *in vivo* inhibition of G9a/GLP-mediated H3K9me2 efficiently promotes muscle regeneration and blocks fat infiltration. This is accompanied by an accelerated myogenic capacity of muscle stem cells (MuSCs) and an impaired adipogenic differentiation of fibro-adipogenic progenitors (FAPs). Moreover, *in vivo* delivery of G9a/GLP inhibitors in mdx mice (the murine model for DMD) induces an increase in myofibers' size and concomitantly reduces adipogenic and fibrotic infiltrations.

Taken together, our results demonstrate a pro-regenerative effect of G9a/GLP specific inhibitors *in vivo*, providing proof of concept that pharmacological treatment with KMTs inhibitors might be further developed as a potential therapeutic approach to counteract DMD progression.

P.08 Tyrosol delays dexamethasone-induced skeletal muscle wasting

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Muscle atrophy occurs under various catabolic conditions caused by hormone imbalances, severe injury, sepsis, cancer and aging¹. Muscle atrophy causes loss of muscle mass, resulting in muscle weakness, fatigue, and exacerbates complications. Many studies have shown that dexamethasone treatment promotes the degradation of proteins related to muscle mass². Dexamethasone is a synthetic glucocorticoid and a well-known anti-inflammatory drug, but, on the other hand it has been shown to induce oxidative stress. Increasing evidence has demonstrated that prolonged exposure to a high dosage of Dexamethasone may lead to an increase in reactive oxygen species production that directly resulted in mitochondrial dysfunction, by increasing mitochondrial permeability. Thus, it was found that some antioxidants from natural sources such as Tyrosol, a virgin oil flavonoids³, can effectively scavenge free radicals, thus reducing or eliminating oxidative stress.

Here, Tyrosol action has been investigated in C2C12 cells exposed to dexamethasone, by means of morpho-functional approaches. Dexamethasone-treated cells show a diffuse damage and, in particular, a reduced fiber size, if compared to control condition. In fact, if long and confluent myotubes can be observed in control samples, those exposed to dexamethasone appear as immature, smaller syncytia. Differently from control cells, treated-myotubes show condensed myonuclei, sometimes bleb presence and mitochondria alterations; these latter are characterized by disorganized cristae and loss of mitochondrial membrane potential. Tyrosol administration before glucocorticoid treatment prevents muscle wasting and improves mitochondrial morphology and functions. Therefore, these preliminary findings demonstrated, by an ultrastructural point of view, that Tyrosol can effectively attenuate Dexamethasone-induced skeletal muscle damage, and these data encourage the use of this natural antioxidant in preventing glucocorticoid-induced muscle atrophy.

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P.09 HMGB1 as a novel target in Duchenne Muscular Dystrophy

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High Mobility Group Box 1 (HMGB1) is a nuclear protein that acts as a chromatin chaperone to modulate gene transcription and DNA repair. Beside its daily job, HMGB1 can be released by stressed or dead cells to serve a soluble message of tissue damage and to trigger "sterile" inflammation. We recently demonstrated that extracellular HMGB1 has a dual role upon tissue injury, by switching among diverse redox states, with first oxidized HMGB1 that stimulates inflammation and later reduced HMGB1 that promotes tissue regeneration. Despite the numerous studies that reported the importance of HMGB1 in muscle biology and its overexpression in muscular dystrophies, little is known about the role of HMGB1 in muscle-wasting disorders. By exploiting *mdx* mice, a model of Duchenne Muscular Dystrophy (DMD), we are investigating if HMGB1 contributes to the dystrophic phenotype. We found that HMGB1 undergoes oxidation in *mdx* muscles, suggesting that it might have a role in sustaining chronic inflammation in DMD. To better evaluate both the source and the role of HMGB1 in DMD, we generated *mdx* mice deficient for HMGB1 in muscle cells (*mdx-Hmgb1^{-/-}* mice). As expected, the HMGB1 level was low in muscles of *mdx-Hmgb1^{-/-}* mice and most importantly, the expression of oxidized HMGB1 was negligent as observed in healthy muscles. From treadmill analyses, we observed that our knock-out mouse model showed improved performances compared to *mdx* control mice, confirming that HMGB1 plays a deleterious role in the dystrophic phenotype. Moreover, we found an overexpression of myogenic genes and a decreased expression of inflammatory genes in the *mdx-Hmgb1^{-/-}* mice, suggesting that the lack of HMGB1 affects both myogenic cells and inflammatory response in *mdx* mice.

In conclusion, our results identified HMGB1 as a novel target in DMD by demonstrating that its pro-inflammatory isoform contributes to the exacerbation of the dystrophic phenotype. Our final goal is to develop HMGB1-directed strategies as innovative treatments for DMD.

P.10 Group I Paks support muscle regeneration and counteract cancer-associated muscle atrophy

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Skeletal muscle is characterized by an efficient regeneration potential that is often impaired during myopathies. Understanding the molecular players involved in muscle homeostasis and regeneration could help to find new therapies against muscle degenerative disorders. Previous studies revealed that the Ser/Thr kinase p21 protein-activated kinase 1 (Pak1) was specifically reduced in the atrophying gastrocnemius of Yoshida hepatoma-bearing rats. So, we evaluated the role of group I Paks during cancer-related atrophy and muscle regeneration.

We examined Pak1 expression levels in the mouse Tibialis Anterior muscles during cancer cachexia induced by grafting colon adenocarcinoma C26 cells and *in vitro* by dexamethasone treatment. We investigated whether the overexpression of Pak1 counteracts muscle wasting in C26-bearing mice and *in vitro* also during interleukin-6 (IL6)-induced or dexamethasone-induced C2C12 atrophy. Moreover, we analysed the involvement of group I Paks on myogenic differentiation *in vivo* and *in vitro* using the group I chemical inhibitor IPA-3.

We found that Pak1 expression levels are reduced during cancer-induced cachexia in the Tibialis Anterior muscles of colon adenocarcinoma C26-bearing mice and *in vitro* during dexamethasone-induced myotube atrophy. Electroporation of muscles of C26-bearing mice with PAK1-expressing plasmids preserves fiber size in cachectic muscles by restraining the expression of atrogen-1 and MuRF1 and possibly by inducing myogenin expression. Consistently, the overexpression of PAK1 reduces the dexamethasone-induced expression of MuRF1 in myotubes and increases the phospho-FOXO3/FOXO3 ratio. Interestingly, the ectopic expression of PAK1 counteracts atrophy *in vitro* by restraining the IL6-Stat3 signalling pathway measured in luciferase-based assays and by reducing rates of protein degradation in atrophying myotubes exposed to IL6. On the other hand, we observed that the inhibition of group I Paks has no effect on myotube atrophy *in vitro* and is associated with impaired muscle regeneration *in vivo* and *in vitro*. In fact, we found that mice treated with the group I inhibitor IPA-3 display a delayed recovery from cardiotoxin-induced muscle injury. This is consistent with IPA-3 impairing *in vitro* myogenin expression and myotube formation in vessel-associated myogenic progenitors, C2C12 myoblasts, and satellite cells.

Our data provide novel evidence that is consistent with group I Paks playing a central role in the regulation of muscle homeostasis, atrophy and myogenesis.

POSTER SESSION:
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P.11 Involvement of a RAGE/p38 MAPK/myogenin axis in cancer cachexia

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Cachexia is a highly debilitating multifactorial syndrome affecting more than 50% of patients with advanced cancer, characterized by severe muscle wasting leading to pronounced weight loss, impaired quality of life, reduced response to anti-cancer therapy, and premature death. Inflammatory cytokines, such as TNF α are the main atrophy-inducing factors in cachexia causing excess catabolism of myofibrillary proteins through activation of the ubiquitin-proteasome systems (UPS)¹.

An unexpected connection between the muscle-specific transcription factor, myogenin, and the induction of atrogenes expression in different atrophying conditions (including TNF α -induced atrophy) has been reported^{2,3}. However, the receptor able to upregulate myogenin expression in atrophying conditions has not been identified yet. We demonstrated that an appropriate recruitment of RAGE (receptor for advanced glycation-end products) by its ligands, S100B and HMGB1, concurs to skeletal muscle development and restoration of muscle homeostasis in physiological conditions and upon acute muscle injury⁴. Here, we investigated whether RAGE might up-regulate the expression of myogenin via p38 MAPK pathway in atrophying conditions, as in the case of myoblasts, and lead to activation of the catabolic program. We found that: i) Lewis lung carcinoma (LLC)-bearing mice re-express RAGE in myofibers and myogenin in myonuclei; ii) muscles of LLC-bearing RAGE-null (*Ager*^{-/-}) mice show reduced loss of mass and reduced *Fbxo32* (atrogin1), *Trim63* (MuRF1) and myogenin expression compared with LLC-bearing WT mice; iii) the upregulation of *Ager* in atrophying C2C12 myotubes precedes the increase in *Myog* (myogenin), *Fbxo32* and *Trim63* levels; iv) RAGE signaling is involved in the mechanism through which TNF α induces atrophy *in vitro* (i.e., upregulation of myogenin via activation of the catabolic kinase, p38 MAPK); and v) high doses of S100B, as found in the serum of cachectic mice, induce up-regulation of RAGE and myogenin expression with concomitant activation of p38 MAPK and induction of the UPS in myotubes and in muscle tissue. Thus, increased expression/activity of the RAGE/ p38 MAPK/myogenin axis in muscle tissue appears to concur to cancer cachexia.

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P. 12 Characterization of biomechanical signals on the functional remodeling of X-MET

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Tissue Engineering represents a valid alternative to cell therapy in skeletal muscle regeneration. The X-MET is an engineering vascularized skeletal muscle tissue able to recapitulate the complex morphological properties, the architecture and the function of skeletal muscle. The X-MET shows biomechanical properties similar to muscles, is able to contract spontaneously as well as to respond to electrical stimulation. In particular, the force-frequency relation is similar to that provided for soleus muscle. Furthermore, we are working on the characterization of its capacity to develop mechanical power. Based on these features, X-MET can be considered a useful experimental tool for *in vitro* studies to characterize the molecular mechanisms involved in the maintenance of the muscle phenotype, to define the molecular signature of the pathophysiological changes, to test the drugs and, therefore, to limit the use of live animals. In addition, an alternative aim of our study is to define the functional plasticity of X-MET subjected to mechanical stimuli. Interestingly, preliminary evidences suggest that different mechanical tensions induce a functional remodeling of X-MET toward a non-skeletal muscle phenotype. Thus, X-MET represents an ideal 3D *in vitro* model for basic research, drug screening/discovery and regenerative medicine, advancing the understanding of pathogenesis and the development of therapies for muscle diseases.

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P.13 CD26/DPP4 Expression in Muscle Biopsies of Patients Affected by Idiopathic Inflammatory Myopathies

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T lymphocytes play a role in the pathogenesis of dermatomyositis (DM) and polymyositis (PM), diseases characterized by skeletal muscle immune-mediated damage¹. PM and DM are rare disorders, whose diagnosis and treatment are still highly unsatisfactory. Activated T cells are the predominant inflammatory infiltrates in muscle biopsies of patients affected by DM or PM and the lack of T regulatory cells has been implicated in the persistence of muscle damage. T-cell activation antigen CD26/DPP4 is an intrinsic membrane glycoprotein and a serine exopeptidase that has been involved in the activation of T lymphocytes and amplification of inflammatory cytokines production. The enzymatically active CD26/DPP4 is selectively expressed by activated T cells (in particular T helper type 17 -Th17- cells) and has been described as a negative selection marker for human regulatory T cells (T_{regs})²⁻⁴. The aim of this study has been to evaluate the expression of CD26/DPP4 in muscle biopsies of patients affected by DM/PM and to correlate its level of expression with clinical and histological features. We have evaluated muscle biopsies of 6 DM and 6 PM patients, selected on the base of high inflammatory activity and low chronicity. 6 healthy controls have also been evaluated. Preliminary histochemical analysis of CD26 distribution revealed that the molecule is predominantly expressed in muscle sections from DM patients. It is only partially associated to T cell membranes, while it is mainly distributed in its soluble form in the extracellular matrix that surrounds both necrotic and regenerating myofibers as well as infiltrating leukocytes. With regard to clinical and histological features, we found that CD26 expression is associated with the typical DM skin involvement and that its levels positively correlate with the presence of perivascular inflammatory infiltrates in the biopsies analyzed. Our data suggest that CD26 may represent a suitable marker for the differential diagnosis between DM and PM, and a potential novel target for selective immune-therapies.

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P.14 Opposing effects of 25-hydroxy- and 1 α ,25-dihydroxy-vitamin D₃ on pro-cachectic cytokine- and cancer conditioned medium-induced atrophy in C2C12 myotubes

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Loss of skeletal muscle is one of the main features of cancer cachexia. Vitamin D₃ (VD) deficiency is associated with impairment of muscle mass and performance and is highly prevalent in cachectic patients; therefore, VD supplementation has been proposed to counteract cancer cachexia-associated muscle loss. However, in both cachectic cancer patients and tumor-bearing animals, VD supplementation led to disappointing results, urging the need for a better understanding of VD activity on skeletal muscle. We hence explored the direct biological effects and mechanisms of action of 1,25-dihydroxy-VD and its precursor 25-hydroxy-VD on atrophying myotubes. Cancer-associated muscle wasting was mimicked *in vitro* by treating C2C12 myotubes with cancer cell conditioned medium, a combination of TNF- α and IFN γ , or IL-6 pro-cachectic cytokines. We demonstrated that only 25-hydroxy-VD was able to protect from atrophy by activating Akt signaling and protein synthesis, while 1,25-dihydroxy-VD had an atrophic activity per se, inducing the expression of muscle-specific E3 ubiquitin ligases and affecting the autophagic flux. Moreover, the contrasting activities of these VD metabolites on C2C12 myotubes depend on a differential induction of VD-24-hydroxylase and transformation of VD metabolites in pro-atrophic 24-hydroxylated products.

Altogether these data might explain the lack of efficacy of VD treatment *in vivo* for the protection of muscle mass in cancer.

POSTER SESSION:
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P.15 The effects of microgravity on human skeletal muscle regeneration

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During long spaceflights, the microgravity condition is able to induce muscle atrophy. This process could also influence the functionality of the satellite cells (SCs), adult muscle stem cells, responsible for both growth and regeneration of skeletal muscle in adult life following injury or stress. Therefore, we studied the differentiated SCs functionality after real microgravity onboard the International Space Station (ISS) in astronaut in order to better understand atrophy mechanisms that involved skeletal muscle, and if satellite cells are affected in this process.

To that end, we performed gene expression evaluation with array cards (Human Microarray 60K) and muscle-specific miRNAs analysis in SCs obtained from *Vastus Lateralis* skeletal muscle needle-biopsies of astronaut and controls subjects on ground. Noteworthy, we discovered a dysregulation of specific genes involved in atrophy, ubiquitin-proteasome and apoptosis pathways related to hypertrophy and atrophy in myotubes exposed to microgravity on ISS compared to myotubes on ground. Furthermore, we found an up-regulation of miR-1, miR-206, miR-133a and miR-133b in myotubes cultured on ground compared to ISS. Interestingly, by exposing both control subjects and astronaut SCs to simulated microgravity (-0,001 g), we observed that the gene expression of oxidative and mitochondrial markers are down-regulated in myotubes subjected to simulated microgravity compared to on ground condition, confirming mostly the data obtained with the arrays. These findings suggest that the enhanced atrophy process is linked to a microRNA dysregulation together to oxidative and mitochondrial genes involvement. Therefore, thanks to this study will be possible to intervene through specific identified targets by the myomiR and gene modulation, in order to counteract the atrophy process induced by microgravity.

P.16 2-Deoxyglucose interferes with IGF-1Ea prohormone glycosylation and IGF-1 secretion

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Insulin-like growth factor-1 (IGF-1) is a polypeptide growth factor with essential roles in normal body growth and development. IGF-1 is synthesised as a prohormone (proIGF-1) requiring enzymatic activity to yield the mature IGF-1. The proIGF-1Ea is the predominant isoform produced in normal tissues and contains an intrinsically disordered region, i.e. the Ea-peptide, which is heavily N-glycosylated [1].

The study aims to investigate the effect of 2-Deoxyglucose (2-DG), a glucose and mannose analogue with anticancer and anti neurodegenerative properties, on IGF-1 glycosylation. Moreover, the effects of sugars (mannose and galactose) and amino sugar (glucosamine) on glycan sugar biosynthesis of proIGF-1Ea was also evaluated.

ProIGF-1Ea-transfected HEK293 cells were grown in low glucose DMEM media (1g/L glucose) and treated with different concentration (ranging from 0,125 to 1g/l) of 2-DG, mannose, galactose and glucosamine. Cells and supernatant medium were harvested 24 hours post-transfection. Total cell lysates were subjected to SDS-PAGE and immunoblotted with an anti-IGF-1 antibody. The supernatants of transfected cells were also used to treat the MCF-7 cells to evaluate their effects on IGF-1 receptor (IGF-1R) phosphorylation.

2-DG interferes with proIGF-1Ea glycosylation preventing the formation of the normal, highly glycosylated proIGF-1Ea of ~17kDa. 2-DG also determined a concomitant dose-dependent increase of a ~12 kDa protein that probably represents an aberrant under-glycosylated proIGF-1Ea form. Interestingly, this protein was still stable and efficiently secreted. Accordingly, despite the loss of highly glycosylated proIGF-1Ea after the 2-DG treatment, the condition media of these cells fully activated the IGF-1R of MCF-7 cells. Both mannose and galactose treatment increased proIGF-1Ea production however, the proIGF-1Ea glycosylation was incomplete with mannose, determining the appearance of a ~14 kDa band instead of ~17kDa. On the contrary, the production of glycosylated proIGF-1Ea was decreased after glucosamine treatment and its molecular mass was markedly decreased at the higher dose of glucosamine.

Taken together, our results indicate that sugars or sugars analogues interfere with N-glycosylation of proIGF-1Ea, and thereby modulates IGF-1 downstream signaling. These effects suggest that modulation of proIGF-1Ea glycosylation may represent a future diagnostic and therapeutic tool to regulate IGF-1 production. Further studies are needed to identify the composition of proIGF-1Ea underglycosylated appearing after 2-DG, mannose and glucosamine treatment.

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POSTER SESSION:
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P.17 The HDAC inhibitor givinostat dampens the atrophy program induced by TNF-related cytokines in human skeletal myotubes *in vitro*

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Muscle atrophy is a severe pathologic process induced by multiple factors that leads to weakness and loss of muscle mass. Muscle atrophy is therefore associated with a number of conditions ranging from temporal disuse to severe genetic pathologies such as Duchenne muscular dystrophy (DMD). In normal muscles, atrophy can be reversed because the inflammatory and fibrotic processes are transitory and a specialized type of cells, the satellite cells, can regenerate damaged muscles. In DMD patients, inflammation and fibrosis are persistent and the satellite cells have a reduced regenerative capacity, as a consequence, atrophy leads to irreversible muscle loss.

New pharmacological treatments are needed to counter the abnormal atrophy caused in the context of the genetic mutations causing DMD.

Histone deacetylase (HDAC) and histone acetyltransferase (HAT) catalyse the reactions that maintain the homeostatic level of lysine acetylation in thousands of cellular proteins. In muscle cells of DMD patients, this equilibrium is altered with the prevalence of deacetylase activity. The inflammatory pathways leading to atrophy activated in DMD muscles are regulated by acetylation, and HDAC inhibitors (HDACi) are candidate molecules to dampen this response induced by various cytokines.

Givinostat is a pan-HDAC inhibitor currently in phase III clinical trial for the treatment of DMD. Immunohistochemistry analysis of patients' biopsies demonstrated that treatment with givinostat led to a reduced fatty replacement, necrosis, fibrosis and an increase of the cross sectional area of the myofibers. This results show the effectiveness of an epigenetic treatment in the context of a genetic disease.

To further characterize the molecular mechanism of action of givinostat on atrophy induction in muscle, we used *in vitro* differentiated human skeletal myotubes stimulated with two cytokines of the TNF superfamily, TNF- α and TWEAK.

We found that givinostat countered the upregulation of the key atrogene TRIM63 induced by both cytokines at transcriptional and post transcriptional level. Muscle cells treated with the two cytokines, activated the expression of HDAC4, involved in muscle atrophy. Furthermore HDAC6 has been recently described as an atrogene that interact with Atrogin-1 multiprotein complex and givinostat could effectively inhibit HDAC6 activity in muscle cells. The gene expression of myosin heavy chain I and II that are targeted to degradation during atrophy, was strongly downregulated by both cytokines and upregulated in the presence of givinostat. Since both cytokines activate the NF- κ B pathway, we investigated the effect of givinostat on p65 phosphorylation and we found that givinostat could effectively reduce it. In agreement with this result, ongoing experiments suggest that p65 nuclear localization is also inhibited in the presence of givinostat.

Taken together, these data indicate that givinostat counters the atrophy program induced by the two potent atrophy-inducing cytokines in normal skeletal muscle cells and suggest that a similar mechanism may take place in DMD muscle cells.

P.18 Identification of a novel FoxO-dependent regulator of muscle mass

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Muscle atrophy results from transcriptional adaptations occurring during aging (sarcopenia) and also in diseases such as cancer (cachexia), AIDS, denervation, sepsis, heart failure, diabetes, etc. Excessive muscle loss ultimately aggravates diseases and increases morbidity and mortality. An exacerbated activation of FoxO family members leads to increased protein breakdown and muscle wasting. Microarray analysis during fasting showed that FoxOs are required for the induction of several atrophy-related genes (atrogenes) that have important role in the autophagy-lysosome pathway, ubiquitin-proteasome pathway, unfolded protein response, mitochondrial function, etc.. [1] However, the activation of the already identified atrogenes cannot sustain all the protein breakdown during atrophy. Indeed, the discovery of new unknown players involved in muscle protein degradation is now of potential interest.

We identified several new FoxOs-dependent genes, here called RIKENs, whose functions were until now unrevealed. We showed that in particular one gene that we called RIKEN1 is up-regulated in catabolic condition such as fasting, disuse and cancer cachexia mouse models. This was also confirmed in cancer cachexia patient samples. Colocalization experiments *in vitro* and *in vivo* showed a Riken1GFP-LC3cherry interaction in the autophagosomes suggesting a potential role in the autophagy-lysosome pathway. Site-directed mutagenesis in the predicted LIR motifs of RIKEN1 allowed us to determine which of these motifs are involved in the interaction with LC3. Importantly, Knocking-down RIKEN1 protected from fasting-induced

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atrophy. Moreover, the knocking-down of RIKEN1 decreased LC3II puncta in FDB isolated fibers suggesting that RIKEN1 may have a role in the formation of autophagosomes.

Our findings will contribute to the identification and the characterization of a completely new FoxO-dependent mediator of muscle mass loss that plays a role in autophagy-lysosome system in order to develop new therapeutic approaches against muscle wasting.

P.19 The mass cytometry application to unravel the skeletal muscle physiological and pathological state

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The skeletal muscle has a robust, finely orchestrated and sophisticated capacity to regenerate. The regeneration process is governed by the interplay between different populations of resident mononuclear cells, which directly or indirectly contribute to restoring tissue homeostasis.

Pathological conditions, such as muscular dystrophies, ageing or cancer (as rhabdomyosarcoma) lead to an alteration in the regeneration capacity, which reflects in a variation of the relative abundance and the functional integrity of muscle mononuclear populations. Describing how the profile of mononuclear cell populations changes in the different conditions is essential to elucidate the physiological processes that govern muscle regeneration. In addition, this information could help to comprehend how the pathological states originate and evolve in time.

We have used mass cytometry, a high content single cell technology to monitor the kinetic of changes of the mononuclear cell populations in the muscle. By this approach, we have confronted muscles from wild type with and from a mouse model of Duchenne muscular dystrophy (*mdx*) which undergoes chronic damage and regeneration cycles. In both systems, we have monitored how the profile of mononuclear cell populations changes with ageing. In addition, in both systems we have also studied the kinetic of regeneration after an acute damage caused by injection of a cardiotoxin (CTX). Finally, we investigated how the mononuclear population profile varies in cancer condition in order to identify the cell type that initiate the tumor. To this end, we have compared the cell populations of healthy muscles with that of a rhabdomyosarcoma, a common type of soft tissue sarcoma in children and adolescents, under 20 years of age. To achieve our objective, we adopt the $KRAS^{LSL;SG12D::Trp53^{fl/fl}}$ conditional mouse model in which the undifferentiated pleomorphic myosarcomas are induced in a spatio-temporal controlled manner by using an adenovirus vector expressing the CRE recombinase. In this mouse model, by inducing chromosomal rearrangements it is possible to achieve constitutive activation of the oncogene KRAS along with the inactivation of the Trp53 tumor suppressor gene. Hence exploiting this inducible cancer model, we were able to analyze tumor progression and composition, unravelling differences in terms of initiating cell population.

P.20 mTOR is required for maintaining myofiber integrity and muscle force

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Skeletal muscle is a very plastic tissue, rapidly changing its contractile properties and size under various pathological conditions or during changes in muscle recruitment. Muscle size during postnatal growth is a combination of increased protein synthesis and the incorporation of new nuclei from proliferating satellite cells. In adult muscle, however, muscle size is exclusively the result of a balance between protein synthesis and protein degradation. One of the major regulators of protein synthesis is the kinase mTOR. In order to determine the role of mTOR in adult skeletal muscle, we eliminated mTOR specifically from adult skeletal muscle fibers. Preliminary results show that loss of mTOR leads to a loss of sarcomere integrity and fiber contractility, showing the importance of maintaining a minimal amount of mTOR signaling active also during adult muscle homeostasis.

P.21 FAP heterogeneity: important functional feature or experimental noise?

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In a complex organism no pair of cells is identical and single cell technologies have challenged biological models based on ensemble analysis of quasi-homogeneous populations. The question is whether the observed cellular heterogeneity has any functional impact rather than being only a result of experimental noise. Fibro/adipogenic progenitors (FAPs) are Sca1⁺ population residing in the interstitium of the skeletal muscle. In the *mdx* mouse, an animal model of Duchenne Muscular Dystrophy (DMD), FAPs are

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one of the populations responsible for the development of ectopic tissues, such as intramuscular adipose tissue (IMAT) and fibrotic tissue. To characterise the immunophenotype of FAPs we performed a mass cytometry analysis and revealed a variety of sub-populations characterized by heterogeneity in the expression of 12 antigens. The numerosity of some of these sub-populations is dynamic and varies in different experimental models, for instance wild type versus *mdx* mice. Most striking is the identification of two sub-populations expressing correlated levels of Sca1 and CD34. Muscles of *mdx* mice are characterised by approximately the same amount of both sub-populations. We investigated further this heterogeneity by sorting the two sub-populations. In addition to the “macro-heterogeneity” that distinguishes the whole Sca1 compartment, we observe an intrinsic heterogeneity (micro-heterogeneity) characterized by a spread in the expression levels of Sca1 that is higher than the heterogeneity that may derive from experimental noise. Preliminary results indicate that FAP population heterogeneity parallels a heterogeneity in the differentiation potential. The future challenge is to understand the functional implication of these two levels of heterogeneity in the development of myopathies.

P.22 Pericyte derived-IGF-1 is required during muscle recovery after acute injury.

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Satellite cells, defined as adult muscle stem cells, are both located beneath myofiber basement membranes and closely associated with capillary endothelial cells. We previously observed that 90% of capillaries were associated with pericytes in adult mouse and human muscle. We also have shown during post-natal growth that, by promoting post-natal myogenesis through Insulin like-growth factor 1 and stem cell quiescence through Angiopoietin-1, pericytes play an important role in the microvascular niche of satellite cells.

Consistently, here we show in mice models of deletion of microvascular IGF-1 that following injury a prolonged lack of IGF-1 modifies the behavior of satellite cells and that regeneration is delayed, associated with an important inflammation and myofibers hypotrophy probably due to a delayed fusion of cycling precursors when pericyte-derived IGF-1 production is impaired. In conclusion, pericytes also exert paracrine effects on adjacent myogenic cells during muscle repair in adulthood in order to restore a functional muscle.

P.23 Ly6c-hi inflammatory monocytes accumulate in mdx mouse spleen and contribute to dystrophic muscle pathology

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Inflammatory monocytes known as Ly6c-hi monocytes are among the first innate immune cells to be recruited to the site of tissue injury. Once inside the tissue, they give rise to inflammatory macrophages. We recently described the kinetics of the early inflammatory cell infiltrate in lower limb muscle in the *mdx* mouse model of muscular dystrophy, and showed that Ly6c-hi monocytes begin to infiltrate *mdx* muscle from the earliest stages of disease, namely the pre-necrotic stage at 2 weeks of age, reach a peak during the necrotic phase at 4 weeks and gradually decline by 12 weeks of age when anti-inflammatory macrophages begin to predominate. Recent studies have identified the spleen as an important reservoir of Ly6c-hi monocytes and splenic monocytes were found to contribute to myocardial damage following ischemia, and to disease progression in ALS. However, the contribution of splenic monocytes to dystrophic muscle inflammation remains to be elucidated. In this study we show that Ly6c-hi monocytes accumulate in the spleen of *mdx* mice over the course of the disease. Removal of the spleen at 2 weeks of age led to a significant reduction in the number of infiltrating Ly6c-hi monocytes in lower limb muscle at 4 weeks. This reduction was accompanied by lower levels of the monocyte specific chemokine CCL2, and reduced muscle necrosis compared to control mice. Interestingly, the number of Ly6c-hi monocytes in the bone marrow increased following splenectomy, suggesting an attempt to compensate for the absence of the splenic reservoir. We also examined the contribution of splenic Ly6c-hi monocytes to acutely injured WT muscle (cardiotoxin (CTX) induced injury). Similar to our findings in chronic muscle injury in *mdx* mice, acute CTX injury was associated with a steady accumulation of Ly6c-hi monocytes in the spleen. An initial drop in the number of Ly6c-hi monocytes in the spleen at 3 hours following CTX muscle injury suggested an early deployment of the existing steady state reservoir. This was followed by a sustained increase in their number in the spleen up to 24h following CTX injury to replenish the monocyte pool. Splenectomy altered the early kinetics of Ly6c-hi recruitment to CTX injured muscle and was associated with changes in the muscle cell infiltrate at day 10 following injury suggesting that splenic Ly6c-hi monocytes are qualitatively different from those recruited from the bone marrow. Collectively, these results suggest that splenic Ly6c-hi monocytes play an important role in both dystrophic and acutely injured muscle. By elucidating the origin, phenotype, and functional characteristics of

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inflammatory monocytes in dystrophic muscle we hope to gain an important insight into the pathogenesis of inflammation in muscular dystrophy and uncover novel therapeutic targets.

P.24 Immune cells of *mdx* mice fed with a high fat diet secrete high levels of IGF-1 that promote satellite cells differentiation

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The muscle fiber structural fragility in Duchenne Muscular Dystrophy (DMD) patients is coupled to mitochondrial metabolic dysfunctions that contribute to predispose to muscle wasting. Such metabolic perturbations result in reduced mitochondrial functionality and increased glycolytic flux. Starting from the premise that the metabolic dysfunctions may contribute to the pathology we aimed at restoring mitochondrial oxidative phosphorylation by feeding *mdx* dystrophic mice with a fat-rich chow (HFD). We observed that the HFD regimen exerts beneficial effects on the dystrophic phenotype as bespoken by an increase in the fiber cross sectional area of *tibialis anterior* (TAs) and a reduced diaphragm fibrosis. We next asked whether the immune compartment could be a sensor of the metabolic rewiring and promote a beneficial effect on the muscle regeneration process. To this end we purified satellite cells from *mdx* mice fed with a "standard" diet and cultivated them with a medium conditioned by CD45⁺ cells isolated from TAs of *mdx* mice. Only the CD45⁺ isolated from mice fed on the HFD were able to produce soluble factors that stimulated SCs to synthesize myogenin and to fuse to form elongated myotubes. To identify the factor(s) responsible for the observed pro-myogenic effect we profiled 200 cytokines synthesized by CD45⁺ cells to identify the ones that were regulated by the diet. The finding that IGF-1 was one of the few cytokines whose production was positively modulated by the HFD pointed to this factor as the main mediator of the positive effect on muscle regeneration.

P.25 Approaches to delay the progression of Muscular Dystrophy

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Muscular Dystrophies (MDs) are severe genetic disorders mainly due to mutations in structural proteins, causing contraction-induced damages. Previous attempts to treat these diseases raised from the idea that accelerating muscle growth and regeneration would exert beneficial effects. We recently demonstrated that slowing down the degeneration-regeneration cycles and switching muscle fibers towards a slow-twitching phenotype by silencing the transcription factor Nfix leads to a morphological and functional amelioration of the dystrophic phenotype.

On the basis of the identification of the molecular pathways regulating Nfix expression, we are now developing a pharmacological approach to inhibit Nfix in MDs.

At the same time, another strategy considered is the use of antioxidants to protect myofibers from oxidative stress generated by muscular contraction. Different evidences are indeed establishing the importance of dietary anthocyanins for preventive and ameliorative strategies against chronic diseases. We therefore tested the therapeutic benefit of a cyanidin-enriched diet in the progression of the MD. Interestingly, dystrophic mice fed with a cyanidin-enriched diet show a morphological and functional amelioration of the dystrophic phenotype through mechanisms that involve both cell survival and anti-inflammatory pathways. All these evidence strongly demonstrate that promising therapeutic and supporting strategies to slow down the disease progression in dystrophic patients is to reduce, the oxidative stress, muscle regeneration and muscle contraction achievable through different strategies.

P.26 The interference with IL-6 trans-signaling modulates secondary mechanisms of dystrophic muscle

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Duchenne muscular dystrophy (DMD) is a genetic X-linked disease caused by mutations in dystrophin gene. The primary defect is the absence of dystrophin protein leading to a cascade of pathological events including muscle fiber degeneration, fibrosis and necrosis. Among factors involved in the pathogenesis of muscular dystrophy the extent of chronic inflammatory response and oxidative stress might be responsible for the appearance and progression of pathological changes in dystrophic muscles. To date inflammation is considered the principal determinant of degenerative processes in dystrophic muscle and the glucocorticoids

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are the only available anti-inflammatory treatment for DMD. However these aspecific molecules present long-term side effects, hence the needs of identifying and studying factors that can reduce both inflammation and oxidative damage slowing down the progression of pathology. A potential candidate related to inflammation is Interleukin 6 (IL-6), a pleiotropic cytokine with pro and anti-inflammatory effects, depending on the signaling that it will activate: IL-6 classic signaling has regenerative and anti-inflammatory effects whereas IL-6 trans-signaling, principally mediated by the IL-6 receptor alpha (IL6R), has pro-inflammatory actions. Particularly IL-6 is highly expressed in DMD patients and in mdx mouse model and it is involved in the transition from an acute to chronic inflammation. In addition we recently demonstrated that IL-6 over-expression in mdx mice (mdx/IL6 mouse) is sufficient to induce the exacerbation of dystrophic phenotype, closely approximating the disease progression in DMD human patients. Based on these evidences we generated a new mouse model in which IL-6 trans-signaling is inhibited by the genetic ablation of IL6R, (mdxIL6R^{-/-}), to verify whether the modulation of IL-6 trans-signaling could ameliorate the dystrophic phenotype. In particular we analysed key players involved in secondary mechanisms of the pathology leading to muscle wasting. In the present study we performed analysis of relevant markers of the myogenic program, inflammatory response and redox status at different ages and stages of pathology in muscles of mdxIL6R^{-/-} and mdx mice. Furthermore to evaluate whether the interference with IL-6 trans-signaling could influence the robustness of dystrophic muscles we analysed myofiber necrosis and muscle functionality at the acute phase of the pathology.

P.27 Intracellular calcium dyshomeostasis in GAP-43-knockout cardiomyocytes

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The neuronal Growth Associated Protein 43 (GAP-43) has been largely studied as neuronal specific molecule involved in many processes in the nervous system.

We have previously found that GAP-43 is also expressed in skeletal muscle where it localizes nearby the calcium release units and, interacting with calmodulin, indirectly modulates the activities of dihydropyridine and ryanodine Ca²⁺ channels (Guarnieri *et al*, PLoS One8:e53267, 2013; Caprara *et al*, Front Physiol. 7:493, 2016).

The aim of this study is to define the role of GAP-43 in intracellular Ca²⁺ homeostasis in cardiomyocytes (CM). For this purpose, intracellular Ca²⁺ levels were analyzed in CM isolated from heart of neonatal wild type (WT) and GAP-43 knockout (KO) C57/BL6 mice. Both WT- and KO-CM were cultured for 7 days after isolation and then their spontaneous intracellular Ca²⁺ oscillations were monitored using fluorescence video-imaging techniques and the fluorescent Ca²⁺ indicator, Fluo-4. The fluorescence images were acquired at 1 frame/150 ms with a 12 bit digital EMCCD camera. The temporal analysis was calculated as the mean fluorescence intensity signal in a selected single cell, as f/f_0 , where f is the fluorescence emission of a single loaded cell that was acquired during the time lapse, and f_0 is the mean fluorescence intensity of the same cell calculated from images acquired during the first 3 s. Using specific software, we analyzed the *frequency* (number of peaks/min), *time to peak* and *maximum peak value* of intracellular Ca²⁺ oscillations (Clampfit 10.2, Molecular Devices, Canada) and investigated the presence of *irregularities* in the Ca²⁺ oscillation kinetics. These irregularities were identified by detecting the presence of *low peaks* (presenting an amplitude at least lower than the 10% of the preceding Ca²⁺ peak amplitude), *bursts of Ca²⁺ fluctuations* that did not regain the baseline, and *irregular phases* with an irregular rhythm in the oscillation frequency (Anomaly Explorer software tool, Penttinen *et al*, PLoS One10:e0135806, 2015).

The data are expressed as means±standard error of the mean, and were compared using Students' t-tests. Both WT- and KO-CM presented spontaneous Ca²⁺ oscillations., KO-CM compared to WT-CM showed spontaneous Ca²⁺ oscillations with a higher frequency (83.9±4.2 vs 41.3±4.6 peaks/min, p<0.001), longer time to peak (19970±922.9 vs 10500±471.7ms, p<0.001) and higher maximum peaks (1.31±0.01 vs 1.16±0.19 f/f_0 , p<0.001). In addition, the KO-CM compared to WT-CM showed also a greater percentage of cells with irregularities (91%, n= 41/45 vs 70%, n=35/50 cells), a higher number of low peaks (1,88±0.35 vs 0.32±0.14 low peaks/cell, p<0.001), a higher number of irregular burst fluctuations (6.24±0.79 vs 2.83±0.55 irregular bursts/cell, p<0.01) and a higher number of irregular phases (3.76±0.53 vs 1.56±0.37 irregular phases/cell, p<0.01).

In conclusion, these preliminary results strongly suggest that the presence of GAP-43 is required in the modulation of intracellular Ca²⁺ handling in cardiomyocytes and, consequently, it could play an important role in the functional processes of the cardiac muscle.

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P.28 A molecular toolbox to modulate muscle progenitor cell differentiation.

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The molecular pathways controlling muscle stem cells differentiation trajectories are influenced by inputs and stimuli from different cell types in the muscle niche. The availability of a comprehensive small-molecule toolbox to manipulate the cell fate decisions in physiological and pathological conditions, would be of enormous interest for both basic and translational purposes. To this end we set out to develop cell-based assays suitable for high content screenings of drugs that would perturb the differentiation potential of fibro/adipogenic progenitors (FAPs), satellite cells and mesoangioblasts. A total of approximately 5000 drugs were screened in search of compounds that would promote or inhibit adipogenesis, fibrogenesis myogenesis or osteogenesis. Approximately 50 compounds showed some activity in at least one of these assays. We were interested in a strategy to identify compounds with a similar phenotypic outcome, for instance inhibition of adipogenesis, attained by different mechanisms. To this end we compared transcriptomics and proteomic profiles after drug perturbation and principal component analysis revealed that drugs perturbing adipogenic differentiation of FAPs from mdx mice act by clearly different mechanisms. We will report characterization of the mechanism of action of selected drugs perturbing adipogenesis, fibrogenesis and osteogenesis. Our study highlights that the stem cell fate is determined by an intricate network of stimuli and regulatory mechanisms involving pro-anabolic pathways, gene expression regulation and stress responsive pathways. A thorough understanding of these mechanisms and the ability to rationally modulate them will offer new strategies to treat many pathological conditions by combinatorial approaches.

P.29 Could PIN1 be a target to delay the ageing process in skeletal muscle?

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Pin1 is a Peptidyl Prolyl cis/trans isomerase (PPIase), involved in post-phosphorylation control of protein function.

Pin1 function is mostly implicated in cell proliferation. Here we propose to investigate its potential function in muscle remodelling, a process that takes place in post-mitotic myofibers. To study the consequences of Pin1 loss on skeletal muscle we used Pin1 KO and WT mice at different ages (2 months-, 4 months- and 18 months-old). Analysis of Myosin Heavy Chain isoforms composition showed a significant shift towards slower MHC isoforms in Soleus and Tibialis of Pin1 KO mice (at 4 and 18 months of age) versus age-matched control animals. These findings were confirmed by immunostaining of muscle cryo-sections with a monoclonal antibody directed against the slow MHC1 isoform. The slow fiber types conversion was associated with higher level of PGC1 α transcript. Muscles from Pin1 KO mice showed lower cross sectional area (CSA) of muscle fibers than sex-matched WT mice, at 2 and 4 months but not at 18 months of age.

Furthermore, 18 months old WT mice showed a reduction of average myofiber CSA compared to the CSA of sex-matched younger mice (4 months- old) [gastrocnemius 1591 vs 1387 μm^2 ; tibialis 1633 vs 1429 μm^2 ; EDL 1460 vs 1371 μm^2 ; biceps 1793 vs 1564 μm^2 , respectively]. In the case of Pin1 KO mice no similar age-dependent CSA decline was found, suggesting a protective role of Pin1 loss against muscle fiber atrophy in aged mice. At the molecular level, 18 months old Pin1 KO mice showed lower age-dependent alterations of genes encoding key molecules involved in muscle mass homeostasis, respect to age-matched WT mice: lower myostatin induction, lower LC3 content, higher expression of the protein synthetic signaling proteins p70 ribosomal S6 kinase and of its phosphorylated form. In addition, a lower alteration of factors involved in neuromuscular junction instability/denervation (RUNX1, myogenin, GADD45a) was found. The in vivo four limbs hang test showed higher muscle performance and motor coordination in 18 months Pin1 KO mice respectful to the WT counterpart. Overall these preliminary results suggest that depletion of Pin1 correlates with a slow fiber conversion of adult muscles and with a protection against age-dependent muscle fiber atrophy.

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P.30 Sphingosine kinase 1 and 2: their role in skeletal muscle cell phenotype

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Sphingolipids represent a class of bioactive molecules capable of modulating many crucial biological processes. Sphingosine kinase functions through the phosphorylation of sphingosine to sphingosine 1-phosphate (S1P) in order to keep the balance in the sphingoid molecules involved in the destiny of many cell types, such as the pro-apoptotic factor, ceramide and the pro-survival factor, S1P.

There are two isoforms of sphingosine kinase, sphingosine kinase 1 (SphK1) and sphingosine kinase 2 (SphK2), that share high sequence similarity, but distinct distribution, regulation and function. They are expressed in all tissues including skeletal muscle. In particular, our group and others have shown that S1P formed via SphK1 is able to act as trophic and morphogenic factor in skeletal muscle cells (1,2).

Here, we compare the role of the two SphK isoforms in the regulation of myogenic cell degeneration. Particular attention will be focus on the correlation between the intracellular localization and functions of both isoforms in normal cells and impaired cell metabolic conditions (atrophy, autophagy).

The findings provide evidence for a distinct role for SphK1 and SphK2, in the control of myotube phenotype, thereby representing new potential distinct targets for obtaining beneficial effects in several physiological and pathological conditions.

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P.31 Steroid myopathy: understanding the pathogenesis

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Steroid-myopathy is a well-known consequence of glucocorticoids excess in the human body. The mechanism that links glucocorticoids use to muscle atrophy is not fully understood. It has been shown that the processes underlying steroid myopathy in humans can develop just few days after glucocorticoid administration highlighting the possibility to identify the primary pathogenic phenomena studying the very early stages of the phenomenon. The aim of the project is to study the adaptations of intracellular molecular pathways induced by a single dexamethasone (DEX) administration in healthy subjects. The study was conducted on 18 volunteers, with ages comprised between 20 and 30 years. For each subject, one baseline pre-DEX administration muscle biopsy has been obtained and used as reference. Seven days after the baseline biopsy, subjects have been administered with a single 8mg dose of DEX i.v. and a post-DEX biopsy has been obtained after 1h and/or 4h. Western blot and real time PCR were used to assess the adaptations of markers related to the ubiquitin-proteasome degradation pathway (UPS), muscle synthesis, autophagy, muscle metabolism, redox status and mitochondrial remodelling. On the basis of the obtained results we suggest that DEX could induce an impairment of the oxidative metabolism. This mechanism could alter cellular functions by producing oxidative stress and leading to mitochondrial fragmentation. This vicious loop results in an increased activation of the autophagy pathway necessary for the removal of damaged cellular constituents, which eventually results in muscle atrophy.

The ability by two-week intake of a mixture of branched chain amino to counteract the effects of DEX on intracellular pathways will be tested.

P.32 Ghrelin peptides and stem cells to counteract sarcopenia

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Sarcopenia is a complex syndrome defined as the irreversible loss of skeletal muscle mass and functionality in aged individuals that results in frailty, mobility disorders, and loss of independence [1]. The pathology is characterized by muscle atrophy and impaired muscle regeneration. The mechanisms involved in its development are not fully understood, although hormonal changes, inflammation, insulin resistance and nutritional deficiencies are surely involved in. In addition, we and other authors showed that aging affect progenitor myogenic cells, including mesoangioblasts (adult vessel-associated stem cells) [2] that are unable to counteract sarcopenic phenotype.

Acylated and unacylated ghrelin (AG and UnAG, respectively) are circulating peptides codified by the ghrelin gene. By acting through its receptor GHSR1a, AG stimulates appetite, adiposity, a strong release of growth

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hormone (GH) and has a broad anti-inflammatory activity. UnAG does not bind to GHSR1a however, similar to AG has a direct anti-atrophic effect on skeletal muscle [3].

Our preliminary results show surprisingly that murine mesoangioblasts treated with recombinant UnAG or AG were able to differentiate spontaneously forming contractile myotubes. In addition, in murine embryonic stem cells and human mesodermal induced pluripotent stem cells subjected to myogenic differentiation, the presence of recombinant proteins resulted in improved myogenic commitment.

Taken together our results show that both AG and UnAG are potent myogenic inducers on murine interstitial cells, human mesodermal derived iPSc, mouse embryonic stem cells and mesoangioblasts, affecting positively muscle differentiation process.

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P.33 Novel data support the use of microencapsulated Sertoli cells as a potential treatment of DMD patients

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Testicular Sertoli cells (SeC) physiologically secrete several trophic and immunomodulatory factors thanks to which they have been used in numerous experimental settings and pre-clinical studies, including treatment of diabetes and neurodegenerative disorders [1,2]. A single intraperitoneal (i.p.) injection of microencapsulated porcine SeC (SeC-MC) resulted in recovery of muscle morphology and performance in *mdx* mice, without any pharmacological immunosuppression [3]. This effect was dependent on the release by SeC-MC of antiinflammatory factors and heregulin beta 1, which from the peritoneal cavity through the circulation reach muscles where they reduce inflammation and induce the expression of the dystrophin paralogue, utrophin at the sarcolemma, respectively [3].

We found that lower doses of SeC-MC than those previously used [3] induced significant reduction of inflammation (MAC3 expression) and percentages of necrotic myofibers, and increased expression of utrophin in muscles of *mdx* mice. Also, transmission electron microscopy analysis showed that SeC inside freshly-prepared microcapsules retained a good morphology of nucleus, cell wall and cytoplasmic organelles. Moreover, SeC protected myotubes against atrophy [i.e., loss of myosin heavy chain (MyHC), reduction of myotube diameters, and increased expression of the atrogenes, atrogen-1 (*Fbxo32*) and Murf-1 (*Trim63*)] induced by treatment with the corticosteroid, dexamethasone (Dex), in a dose-dependent manner *in vitro*. Accordingly, SeC-MC protected muscles against atrophy in an experimental model of cancer-induced cachexia (i.e., LLC tumor-bearing mice), as evaluated by the expression levels of *Fbxo32* and *Trim63*. Interestingly, treatment with SeC-MC did not affect tumor growth *in vivo*, supporting the concept of an immunomodulatory rather than immunosuppressive effect of SeC.

Altogether, our data suggest a role of SeC in counteracting loss of muscle mass in different experimental conditions, and further support the use of i.p. injection of SeC-MC as a potential treatment of DMD patients.

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2. Chiappalupi et al. (2017) Employment of microencapsulated Sertoli cells as a new tool to treat Duchenne muscular dystrophy. *J. Funct. Morphol. Kinesiol.* 2(4):47
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POSTER SESSION:
ALWAYS ON DISPLAY

P.34 New evidences about the involvement of muscle stem cells in the pathogenesis of Amyotrophic Lateral Sclerosis

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Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the selective degeneration of upper and lower motor neurons, and progressive wasting and paralysis of voluntary muscles. This disease is currently incurable despite intense research and numerous but unsuccessful clinical trials. Until very recently, ALS was considered as a pure motor neuron disease. However, data obtained by different groups, including ours, suggest that motor neuronal protection is not sufficient to prevent the disease. Early symptoms in ALS involve muscle atrophy, wasting and fasciculation with mitochondrial dysfunction and bioenergetic alterations as recognized hallmarks of muscle pathology. In parallel to early signs of muscle pathology, there is ample evidence of defective energy homeostasis in patients with ALS. Hypermetabolism has been described in familial and sporadic forms of ALS as well as before disease onset in animal models both excessive energy expenditure and loss of fat mass are significant predictors of poor prognosis and reduced survival. In addition, increased fuel metabolism is associated with disease progression in both ALS patients and animal models of ALS. These changes are seen at the macroscopic level as a switch from glycolysis (use of glucose as the main energy source) to β -oxidation (use of fat as main energy source) in ALS mouse models. The involvement of the muscle stem cell compartment in the disease onset and progression has not been investigated. Our aim is to look into the impact of the metabolic alterations occurring in ALS on the differentiation and homeostasis of skeletal muscle satellite cells (MuSCs) and fibro/adipogenic progenitors (FAPs), two cell populations playing a prominent role in muscle regeneration. Preliminary results show that MuSCs isolated from G93A-SOD1 mice display an altered differentiation phenotype. This alteration is accompanied by a reduction in the mitochondrial oxidative capacity.

P.35 “Noisy” field electrical stimulations promote muscle cell differentiation

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Innovative “noisy” field electrical stimulations (ES) obtained by electromyogram recordings (EMGstim) from a human skeletal muscle during locomotion, have been used as a template to stimulate fiber cultures *in vitro*. EMGstim is characterized by biphasic voltage pulses of various duration, amplitude and frequency. The voltage output was the minimum to elicit 60% of contracting fibers.

Isolated single myofibers from mouse *Flexor Digitorum Brevis* (FDB) muscle, were cultured in DMEM, in the absence of nerve inputs. In such a preparation, the satellite are present in their native position. After cell plating, they start to divide, detach from the skeletal fiber surface, differentiate and fuse into multinucleated myotubes. 72 hours after plating the cultures, satellite cell differentiation was determined by counting the myogenin-positive cells with respect to the total number of DAPI-positive nuclei. Compared with control conditions, in response to EMGstim delivered for 1 hour, a significantly higher proportion of myogenin-positive satellite cells was observed (58 ± 1.3 % vs 49.6 ± 1.15 %) as well as the proportion of elongated cells, resembling the postnatal muscle differentiation contributing to muscle regeneration.

The patterns of field ES have a crucial role in determining the effects on the muscle cell properties. Single cell twitching could be monitored during EMGstim, revealing a single contraction each second. Nevertheless, such noisy stimulation was found to be more efficient in inducing satellite cell differentiation compared with 1 Hz regular electrical pulses. Conditioned medium collected from EMGstim-stimulated cell cultures was equally able to favor cell differentiation, suggesting that soluble factors released by the cultured muscle cells would be responsible for promoting satellite cell differentiation. ES effect on cell differentiation was also mimicked by the exogenous application of ATP (0.1 μ M). Carbenoxolone (CBX), an antagonist of the pannexin hemichannel, reduced the EMGstim-induced satellite cell differentiation (38.8 ± 1.7 % in CBX vs 50.9 ± 2.4 % in control stimulated cells). Similarly, the ATP diphosphohydrolase, Apyrase (10 U/ml) reduced the EMGstim-induced muscle cell differentiation (38.3 ± 1.2 % vs 42.4 ± 1.1 %).

In conclusion, the experimental model represented by FDB fibers, allowed us to discover properties induced by field ES which are intrinsic to the muscle itself. Thus, we suggest that “noisy” ES, even in absence of neural trophic influence, could be used to promote muscle regeneration in neuropathological diseases or after traumatic injury.

POSTER SESSION:
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P.36 Evo-Devo approach to study Pax3/7 functions

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Pax3 and Pax7 are paired-homeobox transcription factors regulating progenitor and stem cells of several tissues. To analyse Pax3/7 conservation during evolution, we have replaced Pax3 by Amphioxus and Lamprey ancestral Pax3/7 gene. Lampreys and Amphioxus do not form limbs, lack migratory neural crest cells and possess only a single Pax3/7 gene.

Our results show that Amphioxus and Lamprey Pax3/7, similar to mouse Pax7, can compensate for Pax3 deficiency in dorsal neural tube, and somite development. Surprisingly, muscle progenitor cell migration and neural crest cell migration are also restored. Our results have implications for the study of the gene regulatory network of somites and crest cells during evolution.

P.37 Drug Repurposing for Duchenne Muscular Dystrophy: the Monoamine Oxidase B Inhibitor Safinamide Ameliorates the Pathological Phenotype in mdx Mice and in Myogenic Cultures from DMD Patients

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Oxidative stress and mitochondrial dysfunction play a crucial role in the pathophysiology of muscular dystrophies. We previously reported that the mitochondrial enzyme monoamine oxidase (MAO) is a relevant source of reactive oxygen species (ROS) not only in murine models of muscular dystrophy, in which it directly contributes to contractile impairment, but also in muscle cells from Collagen VI-deficient patients. Here we now assessed the efficacy of a novel MAO-B inhibitor, safinamide, using in vivo and in vitro models of Duchenne muscular dystrophy (DMD). Specifically, we found that administration of safinamide in 3-month old mdx mice reduced myofiber damage and oxidative stress, and improved muscle functionality. In vitro studies with myogenic cultures from mdx mice and DMD patients showed that even cultured dystrophic myoblasts were more susceptible to oxidative stress than matching cells from healthy donors. Indeed, upon exposure to the MAO substrate tyramine or to hydrogen peroxide, DMD muscle cells displayed a rise in ROS levels and a consequent mitochondrial depolarization. Remarkably, both phenotypes normalized when cultures were treated with safinamide. Given that safinamide is already in clinical use for neurological disorders, our findings could pave the way towards a promising translation into clinical trials for DMD patients as a classic case of drug repurposing.