Molecular Mechanisms Modulating Skeletal Muscle Development and Homeostasis in Health and Disease

Society for Muscle Biology

Frontiers in Myogenesis

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ABSTRACTS

Program Abstract #1
Muscular Dystrophy: Therapy development and genetic modifiers of severity
Louis Kunkel
Boston Children's Hospital and Department of Genetics and Pediatrics, Harvard Medical School, USA
The muscular dystrophies are a heterogeneous group of genetic disorders of children and adults with the most common form being the X-linked dystrophinopathies. The identification by positional cloning of the dystrophin gene and it's encoded protein lead to improved diagnosis of Duchenne and Becker muscular dystrophy and rational approaches to therapy. The protein dystrophin was shown to co-purify with a unique group of muscle proteins many of which were themselves altered in other forms of muscular dystrophy. The understanding of dystrophin function in normal muscle and the consequences of its absence in diseased muscle lead to ideas of therapy either dystrophin dependent as well as dystrophin independent. These approaches will be highlighted and their rational discussed. Following the identification of dystrophin and the analysis of patients and animal models with muscular dystrophy it was clear that there are likely genetic modifiers of the disease phenotype and progression and the identification of these modifiers could lead to additional targets for therapy. One such modifier was recently described in a dog model of dystrophin deficiency and is currently being targeted for therapy development. Our Wellstone Center for FSHD research also focuses on the clinical variability in FSHD and the likely genetic factors, which might influence disease progression.

Program Abstract #2
Regulation of early myogenesis by microRNAs
Andrea Münsterberg
University of East Anglia, United Kingdom
Myogenesis involves stable commitment of progenitor cells followed by execution of myogenic differentiation, processes coordinated by the myogenic regulatory factors (MRFs). Work in avian embryos has shown that small non-coding RNAs, microRNAs, coordinate the regulation of myogenic development through different activities. In particular, microRNAs that are specifically expressed in the myotome of developing somites, so-called myomiRs (miR-1, miR-206 and miR-133), play important roles and *in vivo* knock-down of myomiRs using specific antagomiRs leads to impaired myogenesis. Previous work showed that myomiRs provide robustness during the progenitor to myoblast transition by targeting Pax3. In addition, they stabilize myoblast differentiation by targeting components of BAF chromatin-remodeling complexes. Recent functional experiments, combined with transcriptome analysis of avian somites, revealed novel functions for miR-133 including interactions with the hedgehog signaling pathway and cell cycle genes. Our studies indicate the broad significance of myomiRs for successful myogenesis.

Program Abstract #3
Symmetric satellite stem cell expansion requires p38γMAPK
Natasha C. Chang, Fabien P. Chevalier, Melanie Lacaria, Michael A. Rudnicki
Sprott Centre for Stem Cell Research, Regenerative Medicine Program, Ottawa Hospital Research Institute, Canada
During skeletal muscle regeneration, resident satellite cells activate and give rise to myogenic progenitors that participate in muscle repair. Concurrently, satellite stem cells maintain the satellite cell pool through their ability to undergo self-renewal via both symmetric and asymmetric cell divisions. A critical balance between symmetric and asymmetric stem cell division is required for homeostatic maintenance of healthy muscle and defects in this balance underlie pathologies associated with muscle degeneration. The intrinsic molecular mechanisms that control these cellular fate decisions, however, have remained elusive. Myogenic commitment in satellite stem cells is regulated during asymmetric cell division by the methyltransferase Carm1. Employing a candidate kinase approach, we identified p38γ as a Carm1 regulatory kinase. siRNA-mediated knock-down of p38γ protein in satellite cells cultured on isolated EDL myofibers revealed a preference for asymmetric cell division and inhibited symmetric satellite stem cell divisions. Depletion of p38γ in prospectively isolated satellite cells abrogated satellite cell engraftment potential when transplanted into regenerating muscle. Along these lines, inactivation of p38γ specifically in satellite cells *in vivo* resulted in a significant reduction in satellite cell number and impaired muscle regeneration following muscle injury. Thus, in contrast to Carm1, which drives asymmetric satellite cell division, p38γ is required for symmetric satellite stem cell expansion. Ultimately, insight into the molecular pathways that regulate satellite stem cell fate decisions and understanding how these pathways are altered in pathological contexts will be essential for the advancement of therapeutic strategies to treat muscle degeneration.

Program Abstract #4
Mechanisms and applications of muscle stem cell fusion
Doug Millay
Cincinnati Children's Hospital Medical Center, USA

Myoblast fusion is a fundamental process for proper skeletal muscle formation during development and regeneration. Our knowledge regarding the molecules and mechanisms by which muscle cells accomplish fusion remains limited. Myomaker, a muscle-specific membrane protein is a central component of the myoblast fusion machinery. Indeed, genetic deletion of myomaker during development and adult regeneration renders myoblasts fusion incompetent, which results in a dramatic inability to form skeletal muscle. Moreover, expression of myomaker in cells that normally do not express this protein causes their fusion with muscle cells. One aspect of our laboratory aims to understand the regulation and biochemical function of myomaker during the fusion process. Our previous work demonstrated that the intracellular C-terminal region of myomaker is required for fusion. More recently, we discovered that a post-translational modification within this C-terminal region is required for proper trafficking and localization. We have also generated genetic mouse models to manipulate fusion *in vivo*, which will allow investigation into the role of satellite cell-derived myomaker during physiological muscle growth and homeostasis. We will discuss our recent findings relating to the cellular trafficking of myomaker and the impact of myomaker-mediated fusion during adult muscle growth.

Program Abstract #5

The mechanobiology of muscle nuclei: mechanisms and functional significance

Talila Volk, Shuoshuo Wang, Karen Fridman, Dana Lorber
Weizmann Institute of Science, Israel

Nuclear shape and morphology is essential to maintain the epigenetic state of the genome and is robust in differentiated cells. In contrast to nuclei in non motile tissues, nuclei in differentiated skeletal and cardiac muscles are facing iterated and altered cytoplasmic mechanical forces, produced by muscle contraction/relaxation waves. Recent findings from several labs (including ours), uncovered a muscle-specific network of nuclear associated cytoskeletal proteins, which is essential to protect muscle nuclei from the variable cytoplasmic strain induced by muscle contraction/relaxation, and consequently is essential for the maintenance of myonuclear shape. We are studying Nesprin-related mechanisms essential for maintenance of robust muscle nuclear structure. Our recent studies identified intra-nuclear alterations in the distribution of chromatin elements and DNA within the muscle nuclei of Nesprin/MSP-300/Klar mutants. These proteins were shown to be essential for linking the nuclear membrane with the microtubule network, as well as with muscle sarcomeres, in order to maintain robust myonuclear shape. Live imaging of muscles within intact *Drosophila* larvae with fluorescently labeled nuclei and Z-lines enabled imaging of myonuclei during muscle contraction/relaxation waves. A general recovery of myonuclear shape is detected in the course of contraction/relaxation waves of wild type larvae. In contrast, mutant nuclei became fluidic and exhibited significant deformation. We suggest that this deformation is the basis for defects in the intra-nuclear organization of chromatin, which further leads to aberrant transcriptional alterations in the mutant muscles. Such alterations might represent the cause for the numerous muscle diseases associated with mutants of the LINC complex in humans.

Program Abstract #6

Age-related Myopathy with Megaconial Mitochondria in Mice Lacking Group I PAKs

Giselle A. Joseph, Robert S. Krauss
Department of Developmental and Regenerative Biology, Graduate School of Biological Sciences, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA

Group I Paks are serine/threonine kinases that function as major effectors of the small GTPases Rac1 and Cdc42. They regulate many cellular functions, including cell polarity, cytoskeletal dynamics, and transcription. Pak1 and Pak2 are redundantly essential for embryonic myoblast fusion in *Drosophila*, with Pak2 playing the more important role (Duan et al. *J. Cell Biol.* 199:169-185, 2012).Both are expressed in mammalian skeletal muscle, but little is known as to their function in myogenesis. We find that Pak1 and Pak2 are expressed in mammalian myoblasts and activated specifically during cell differentiation. Individual genetic deletions of *Pak1* and *Pak2* in mice show no overt defects in muscle development or regeneration. However, young adult mice with muscle-specific deletion of *Pak1* and *Pak2* together (dKO mice) present with reduced muscle mass and a higher proportion of myofibers with smaller cross-sectional area compared to controls. This phenotype is exacerbated after repair to acute injury. Primary myoblasts from dKO animals show delayed differentiation, with lower expression of myogenic markers and inefficient myotube formation. Furthermore, with age, dKO mice develop a chronic myopathy. Histological analyses of resting muscle show the presence of central nuclei in the majority of fibers, as well as significant fibrosis, inflammation, necrosis, and hypertrophy with fiber splitting. Ultrastructural analysis revealed grossly elongated and branched intermyofibrillar mitochondria along with occasional accumulation of subsarcolemmal mitochondria. These characteristics are absent in control animals. We conclude that while Pak1 and Pak2 have redundant roles in regulating myoblast differentiation, their major role appears to be in muscle homeostasis. Few protein kinases have been implicated in muscle disease. Group I Paks have wide roles in cell regulation, so further analysis of these mice should provide new mechanistic insights into muscle maintenance.

Program Abstract #7

lncRNA Chronos is an ageing related repressor of muscle hypertrophy

Ronald L Neppl[1,2], Chia-Ling Wu[2], Kenneth Walsh[2]
[1]*Department of Orthopedic Surgery, Brigham and Women's Hospital, United States;* [2]*Whitaker Cardiovascular Institute, Boston University School of Medicine, United States*
A gradual loss of muscle mass occurs with advancing age, and in a subset of individuals its rapid progression leads to a condition referred to as sarcopenia. Further, muscle mass, independent of body mass, is a prognostic indicator for survival in patients with cancer or chronic disease. Recent advances in RNAseq analysis have shown that the majority of the human genome is actively transcribed into multiple classes of non-coding RNAs, including long non-coding RNAs (lncRNAs). LncRNAs are a relatively new class of RNA transcripts (> 200nt) known to be involved in multiple cellular processes including the maintenance of stem cell pluripotency, cellular differentiation and fate determination, cell cycle progression, and senescence. Though this class of transcripts has limited protein coding potential, lncRNAs are known to regulate gene expression by multiple mechanisms including post transcriptional gene regulation, recruitment of chromatin modifiers, formation of lncRNA-RNPs, and functioning as miRNA sponges. However, the biological function of lncRNAs with respect to normal age-associated muscle loss and sarcopenia has not been reported to date. RNAseq analysis indicates that expression of a highly muscle-enriched 3.6kb lncRNA, referred to as Chronos, is repressed >8-fold in the hypertrophic muscle of transgenic mice expressing a constitutively active form of Akt1. In addition, Chronos expression is reduced nearly 5-fold in regenerating muscle, and increased nearly 5-fold in aged/sarcopenic muscle. Inhibition of Chronos expression results in myocyte hypertrophy both *in vitro* and *in vivo*. At the cellular level, Chronos represses hypertrophy, in part, through inhibition of Bmp7; a known positive regulator of muscle hypertrophy. Currently, studies are focused on uncovering the molecular mechanism whereby Chronos regulates the expression of Bmp7 and other pro-hypertrophic genes.

Program Abstract #8
Developmental progression of skeletal muscle stem cells is mediated by autonomous extracellular matrix remodeling
Alessandra Sacco
Sanford Burnham Prebys Medical Discovery Institute, USA
Skeletal muscle stem cells (MuSC) exhibit distinct behavior during successive phases of developmental myogenesis. However, how their transition to adulthood is regulated is poorly understood. We show that fetal MuSC resist progenitor specification and exhibit altered division dynamics, intrinsic features that are progressively lost postnatally. Following transplantation, fetal MuSC more efficiently expand and contribute to muscle repair. Conversely, niche colonization efficiency increases in adulthood, indicating a balance between muscle growth and stem cell pool repopulation. Gene expression profiling identified several extracellular matrix (ECM) molecules preferentially expressed in fetal MuSC, including tenascin-C, fibronectin and collagen VI. Loss-of-function experiments confirmed their essential and stage-specific role in regulating MuSC function. Finally, fetal-derived paracrine factors were able to enhance adult MuSC regenerative potential. Together, our studies revealed that fetal MuSC possess a remarkable regenerative potential through more efficient expansion, intrinsically regulating their behavior through the selective expression of ECM proteins to remodel their local microenvironment. The identified components of the fetal MuSC niche can be exploited as novel tools to direct adult muscle stem cell activity towards the immediate or long-term aspects of tissue repair, thus facilitating the development of novel regenerative medicine approaches for muscle wasting diseases.

Program Abstract #9
Muscle stem cell regenerative decline with aging
Laura García-Prat[1], Eusebio Perdiguero[1], Antonio L. Serrano[1], Marco Sandri[2], Pura Muñoz-Cánoves[1]
[1]*1Pompeu Fabra University (UPF) and ICREA, Barcelona; Spanish Cardiovascular Research Center (CNIC), Madrid, Spain;* [2]*2University of Padova, Padova, Italy*
Muscle stem cells (satellite cells) are normally quiescent in adult life. Upon injury, satellite cells activate and proliferate, to subsequently differentiate and form new myofibers or self-renew to restore the quiescent satellite cell pool. Through a combination of global gene expression/bioinformatics and molecular/cellular assays, we found that resting satellite cells have basal autophagy activity, which is required to maintain the quiescent state. Impaired autophagy in old satellite cells or Atg7 deletion in young cells provoked loss of proteostasis and regenerative decline. Pharmacological and genetic reactivation of autophagy could restore regenerative functions in aged satellite cells. We will discuss our recent findings on how autophagy may regulate muscle stem cell homeostasis.

Program Abstract #10
Rejuvenating muscle stem cell fate and function
Helen Blau
Stanford University, USA
Muscle stem cell (MuSC) dysfunction is central to muscle pathophysiology, including age-associated loss of muscle regenerative capacity and congenital disorders such as Duchenne Muscular Dystrophy. Despite its anatomically well-

defined niche, relatively little is known about the factors that maintain MuSCs in a quiescent state, poised to respond to damage. Although regulators of asymmetric and symmetric self-renewal must control quiescence and expansion of the muscle stem cell pool during regeneration the signals controlling the balance between muscle stem cell quiescence, proliferation, and differentiation remain incompletely understood. Through library and in silico screens we have discovered metabolic, inflammatory, and cytokine regulators that control the stem cell states. These factors are capable of tipping the balance between quiescent, activated and committed sates, yielding populations with distinct proteomic and epigenetic signatures. Given that aberrant activation and exhaustion of stem cells is seen in a variety of disorders, these regulators constitute attractive therapeutic targets to restore muscle stem cell functions in muscle disease states.

Program Abstract #11

Loss of fibronectin from the aged stem cell niche affects the regenerative capacity of skeletal muscle

Laura Lukjanenko[1,2], Juliane Jung[3], Nagabhooshan Hegde, Claire Perruisseau-Carrier[1], Eugenia Migliavacca[1], Michelle Rozo[4], Sonia Karaz, Guillaume Jacot Karaz[1], Manuel Schmidt[3], Liangji Li[4], Sylviane Metairon, Frédéric Raymond, Umji Lee, Federico Sizzano[1], David H. Wilson[5], Nicolas A. Dumont[5], Alessio Palini[1], Reinhard Fässler[6], Pascal Steiner[1], Patrick Descombes[1], Michael A. Rudnicki[5], Chen-Ming Fan[4], Julia von Maltzahn[3], Jérôme N. Feige[1], C. Florian Bentzinger[1]

[1]Nestle Institute of Health Sciences, Switzerland; [2]Ecole Polytechnique Fédérale de Lausanne (EPFL), Switzerland; [3]Leibniz Institute for Age Research, Germany; [4]Department of Embryology Carnegie Institution of Washington, USA; [5]Regenerative Medicine Program Ottawa Hospital Research Institute, Canada; [6]Department of Molecular Medicine Max Planck Institute of Biochemistry, Germany

Age-related changes in the niche have long been postulated to impair the function of somatic stem cells. Here we demonstrate that the aged stem cell niche in skeletal muscle contains substantially reduced levels of Fibronectin (FN) leading to detrimental consequences for the function and the maintenance of muscle stem cells (MSCs). Genetic deletion of FN from young regenerating muscles reiterates the aging phenotype and leads to a loss of MSC numbers. Using an extracellular matrix (ECM) library screen and pathway profiling we characterize FN as a preferred adhesion substrate for MSCs and demonstrate that integrin mediated signaling through focal adhesion kinase (FAK) and the p38 MAP kinase pathway is strongly deregulated in aged muscle stem cells due to insufficient attachment to the niche. Restoration of FN levels in the aged niche remobilizes stem cells and restores youthful muscle regeneration. Taken together, we identify loss of stem cell adhesion to FN in the niche ECM as a previously unknown aging mechanism.

Program Abstract #12

AMPKa1-LDH pathway regulates muscle stem cell self-renewal by controlling metabolic homeostasis

Marine Theret[1,2,3], Rémi Mounier[1,2,3]

[1]Institut Neuromyogène, Université Claude Bernard Lyon 1, France; [2]INSERM U1217, France; [3]CNRS UMR 5310, France

The decision for a stem cell to enter into terminal differentiation *versus* to return to quiescence (self-renewal) is crucial for tissue repair. Here, we evidenced that AMP-activated protein Kinase (AMPK), the master metabolic regulator of the cell, controls muscle stem cell (MuSC) self-renewal. AMPKa1$^{-/-}$ MuSCs exhibited a high self-renewal rate, which impairs muscle regeneration. AMPKa1$^{-/-}$ MuSCs showed a Warburg-like switch of their metabolism to higher glycolysis. We identified Lactate Dehydrogenase (LDH) as a new functional target of AMPKa1. LDH, which is a non-limiting enzyme of glycolysis in differentiated cells, saw its activity tightly regulated in stem cells. In functional experiments, LDH overexpression phenocopied AMPKa1$^{-/-}$ phenotype, *i.e.* shifting MuSC metabolism towards glycolysis triggering their return to quiescence,while inhibition of LDH activity rescued AMPKa1$^{-/-}$ MuSC self-renewal. Finally, providing specific nutrients (galactose/glucose) to MuSCs directly controlled their behavior through the AMPKa1/LDH pathway, emphasizing the importance of metabolism in stem cell fate.

Program Abstract #13

Rbfox regulated splicing of Calpain 3 is important for maintaining muscle mass

Ravi Singh[1], Arseniy Kolonin[1], William Lagor[2], Marta Fiorotto[2,3], Thomas Cooper[1,2]

[1]Department of Pathology and Immunology, Baylor College of Medicine, Houston, TX, USA; [2]Department of Molecular Physiology Biophysics, Baylor College of Medicine, Houston, TX, USA; [3]Department of Pediatrics, Baylor College of Medicine, Houston, TX, USA

Multiple human conditions are characterized by muscle loss including muscular dystrophies, cachexia, and sarcopenia. Understanding how skeletal muscle is maintained is crucial to discover novel therapeutic intervention points.

The Rbfox family of RNA binding proteins is highly conserved and regulates tissue-specific alternative splicing. Two of the three Rbfox proteins, Rbfox1 and Rbfox2, are expressed in skeletal muscle. Using a loxp-cre system, we have conditionally deleted Rbfox1 and/or Rbfox2 in adult mouse skeletal muscle. We found that Rbfox1/2 double knockout of Rbfox causes severe and rapid loss of skeletal muscle.

RNA-sequencing identified altered splicing of hundreds of transcripts in muscles lacking Rbfox proteins. Alternative

splicing is enriched in transcripts encoding factors involved in muscle contraction, metabolism, and cell signaling. In particular, we identified altered splicing in Capn3 transcripts in Rbfox1/2 double knockout muscle. Capn3 is a highly unstable serine protease because of autolytic degradation. The lack of Rbfox proteins induces expression of a stable Capn3 isoform due to skipping of two constitutive exons in knockout muscle. This altered splicing of Capn3 leads to activation of Calpain 3 protease, likely contributing to muscle loss in Rbfox1/2 double knockout animals.
Our findings underscore the regulation of Calpain 3 activity by developmentally regulated alternative splicing and its role in maintaining muscle mass.

Program Abstract #14

Of Mice and Men: Unexpected insights into muscle development from the Ulnar-Mammary Syndrome gene, Tbx3.

Mary Colasanto[1], Shai Eyal[2], Payam Mohassel[3], Michael Bamshad[4], Carsten Bonnemann[3], Eli Zelzer[2], Anne Moon[5], Gabrielle Kardon[1]

[1]University of Utah, USA; [2]Weizmann Institute of Science, Israel; [3]National Institutes of Health, USA; [4]University of Washington, USA; [5]Weis Center for Research, USA

In the vertebrate limb over 40 muscles are arranged in a precise pattern of attachment via muscle connective tissue and tendon to bone and provide an extraordinary range of motion. How the development of somite-derived muscle is coordinated with the development of lateral plate-derived muscle connective tissue, tendon, and bone to assemble a functional musculoskeletal system is a long-standing question. Unexpectedly, our analysis of the T-box transcription factor, *Tbx3*, has provided new insights into musculoskeletal development. Mutations in *Tbx3* have long been identified as the genetic cause of Ulnar Mammary Syndrome (UMS), characterized by distinctive defects in posterior forelimb bones. Using conditional mutagenesis in mice, we show that *Tbx3* has a broader role in limb development; *Tbx3* is not only required for development of forelimb posterior bones (ulna and digits 4 and 5), but also for a subset of posterior muscles (lateral triceps and brachialis) and their bone eminence attachment sites. *Tbx3* specification of origin and insertion sites determines whether these particular muscles develop and represents a novel mechanism for specification of anatomical muscles. Re-examination of a UMS patient reveals similar previously unrecognized muscle and bone eminence defects and indicates a conserved role for *Tbx3* in regulating musculoskeletal development.

Program Abstract #15

Facioscapulohumeral dystrophy: Molecular mechanisms and therapeutic opportunities

Jennifer Whiddon[1], Amy Campbell[1], Sean Shadle[1], Sujatha Jagannathan[1], Jun Wen Zhong[1], Jong Won Lim[1], Chao-Jen Wong[1], Lauren Snider[1], Rabi Tawil[2], Silvere van der Maarel[3], Robert Bradley[1], Stephen Tapscott[1]

[1]Fred Hutchinson Cancer Research Center, USA; [2]University of Rochester, USA; [3]Leiden University Medical Center, USA

Facioscapulohumeral dystrophy is caused by the mis-expression of the DUX4 retrogene in skeletal muscle. DUX4 is normally expressed in the luminal cells of the testis, most likely the male germline cells, and is epigenetically repressed in skeletal muscle and most somatic tissues. The retrogene is embedded in the D4Z4 macrosattelite repeat, which exists as large direct repeat arrays in the subtelomeric regions of chromosome 4q and 10q. Contractions of the array to ten or fewer D4Z4 units, or mutations in components of the repeat-mediated epigenetic silencing pathway result in inefficient epigenetic repression and de-repression of DUX4 in skeletal muscle. The expression of DUX4 in skeletal muscle has many consequences that might contribute to the muscle disease. When expressed in cultured human muscle cells or expressed in mouse muscle, DUX4 induces apoptosis. DUX4 also activates the expression of many germline genes that have the potential to be immunogenic. DUX4 inhibits nonsense mediated decay and alters splicing of many RNA transcripts, leading to the accumulation of aberrant RNAs and possibly their translation to abnormal or misfolded proteins. The identification of DUX4 as the causative factor in FSHD provides multiple opportunities for therapeutic development and will inform the development of preclinical models. In this regard, it will be important to compare the conservation, and/or divergence, of the transcriptional programs of the human DUX4 and the mouse Dux retrogene, the closest mouse ortholog.

Program Abstract #16

Structural basis of dystroglycan function

Kevin Campbell[1,2]

[1]Howard Hughes Medical Institute, United States; [2]University of Iowa, United States

Dystroglycan is a highly glycosylated extracellular matrix (ECM) receptor that is critical for the structure and function of skeletal muscle. The post-translational modification of α-dystroglycan (α-DG) is essential for its ability to function as a receptor for laminin G (LG) domain-containing ligands, such as laminin, agrin, neurexins, perlecan, pikachurin, and Slit. Reduced ligand binding by α-DG due to perturbed o-glycosylation is a pathological feature common to several distinct forms of congenital/limb-girdle muscular dystrophy known as the dystroglycanopathies. Recent genetic data has shown that mutations in at least 18 genes encoding known and putative glycosyltransferases disrupt the O-glycosylation of α-DG and cause muscular dystrophy. Our previous efforts to understand the molecular mechanism underlying the ability of α-

DG to bind the ECM revealed that LARGE is a bifunctional enzyme with both xylosyltransferase (Xyl-T) and glucuronyltransferase (GlcA-T) activities, and that it generates a novel heteropolysaccharide [-GlcA-β1,3-Xyl-α1,3-]$_n$. However, how the LG domains of laminin and other proteins recognize specifically the LARGE product remained a mystery. Using a novel enzymatic method of digestion, we demonstrated that native α-DG from skeletal muscle contains the unmodified heteropolysaccharide [-GlcA-β1, 3-Xyl-α1,3-]$_n$ and that its presence confers the ability to bind laminin. We have also used a multidisciplinary approach involving NMR binding studies and crystallographic analysis of the laminin LG4-5 region bound to a LARGE-synthesized oligosaccharide to determine the structural basis of high-affinity binding of laminin to α-DG. Our results reveal a novel mechanism of carbohydrate recognition. Moreover, they provide a structural framework for elucidating the mechanisms that underlie the dystroglycanopathies as well as physiological and pathophysiological systems that are dependent on the receptor function of dystroglycan.

Program Abstract #17

Antibody-mediated dysfunction of the neuromuscular synapse

Jan J.G.M. Verschuuren

Leiden University Medical Center, The Netherlands

Myasthenia gravis comprises a group of acquired autoimmune disorders of the neuromuscular junction. Postsynaptic AChR, MuSK, LRP4, Agrin, or ColQ and the presynaptic VGCC are all antigens at this synapse that are accessible to antibodies. Remarkably, each of these antibody-mediated disorders is associated with a distinct clinical phenotype. In myasthenic disorders of the postsynaptic muscle membrane ocular or bulbar weakness predominates, while the presynaptic disorder, Lambert-Eaton myasthenic syndrome, presents with proximal leg weakness. The clinical muscular weakness is the result of a complex interaction between the immunological attack and the capacity of the muscle to compensate for the resulting damage. Immunological parameters are the genetic background, the association with a tumor or thymic abnormalities, the IgG subclass and age. HLA-B8DR3 is associated with early-onset AChR MG, but not with late-onset AChR. Thymoma is mainly found in elderly people. AChR MG is caused by complement activating IgG1 antibodies, while the IgG4 MuSK antibodies mechanically block the MuSK-LRP4 complex. These autoimmune disorders are treated by immunomodulating or immunosuppresive therapies, but about 15% of MG patients has been found refractory to immunotherapy or suffers from severe side effects. An increasing number of new drugs, directed at immunological targets, are being tested in MG, but a more symptomatic approach that strengthens the muscle to withstand the antibody attack is largely lacking. Given the extensive knowledge of the physiology of the neuromuscular junction this approach might result in possibly fruitful new therapeutic options.

Program Abstract #18

Restoration of basement membrane assembly by small linker proteins prevents laminin-alpha2-deficient muscular dystrophy

Markus A. Ruegg

University of Basel, Switzerland

Merosin-deficient congenital muscular dystrophy (MDC1A) is the most frequent form of early-onset and often fatal congenital muscular dystrophies. It is caused by mutations in *LAMA2*, the gene encoding laminin-α2, the long arm of the heterotrimeric laminin-211 (containing laminin-α2, laminin-β1 and laminin-γ1). We here show that the muscle basement membrane of MDC1A patients is structurally labile and that the loss of laminin-α2 causes upregulation and deposition of laminin-α4 around skeletal muscle fibers. Previous *in vitro* studies on basement membrane assembly have shown that laminin-411 (α4, β1, γ1), in contrast to laminin-211, has poor polymerization and cell surface-binding capacity. We now show that basement membrane assembly of laminin-411 can be fully achieved by the addition of two specifically designed linker proteins. One, called αLNNd, consists of the amino-terminal part of laminin-α1 and the laminin-binding site of nidogen-1. The second construct, called mini-agrin, represents a small fusion protein of the amino-terminal and the carboxy-terminal regions of agrin. We furthermore show that transgenic expression of both linker proteins restores basement membrane stability and provides large functional benefits in dy^W/dy^W mice, a mouse model for MDC1A. The functional benefits include regain of muscle force and size, improvements in body weight and the extension of lifespan from 8 weeks to more than 90 weeks. This work therefore provides a conceptual framework to the understanding of MDC1A and is the proof-of-principle that gene therapy introducing αLNNd and mini-agrin might be a new therapeutic avenue for the treatment of MDC1A.

Program Abstract #19

Maturation of human pluripotent stem cells towards skeletal muscle progenitor cells using directed differentiation to deliver CRISPR/Cas9 gene editing therapies for DMD

Michael Hicks[1,2], Julia Hiserodt[1], Katrina Paras[1], Wakana Fujiwara[1], Haibin Xi[1,2], Majib Jan[1,2], Ascia Eskin[2,3], Stan Nelson[2,3], Melissa Spencer[2], Ben Van Handel[4], April Pyle[1,2]

[1]Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research, University of California, Los Angeles, United States; [2]Center for Duchenne Muscular Dystrophy, University of California, Los Angeles, United States;

[3]*Department of Human Genetics University of California, Los Angeles, United States;* [4]*Novogenix Laboratories, United States*

Satellite cells (SCs) are the endogenous muscle stem cells capable of repairing damaged skeletal muscle. Strategies to harness the regenerative potential of SCs could improve disease course for many muscle wasting conditions including the most prevalent pediatric muscle disorder Duchenne Muscular Dystrophy (DMD). However, SCs are difficult to obtain and *ex vivo* expansion results in loss of self-renewal. Recent reports have successfully directed the differentiation of human pluripotent stem cells (hPSCs) to skeletal muscle progenitor cells (SMPCs). However, we demonstrate existing methods to differentiate hPSCs to SMPCs result in SMPCs that are morphologically and functionally less mature than fetal or adult SCs. To define the developmental status of hPSC-SMPCs relative to fetal (week 9-17) and adult SCs, we evaluated *in vitro* fusion, *in vivo* engraftment, and gene expression by RNA-SEQ. In fusion assays, hPSC-SMPCs formed significantly fewer myofibers *in vitro* and *in vivo* compared to fetal and adult SCs. RNA-SEQ analyses of differential gene expression between hPSC-SMPCs, fetal SCs, and myotubes enabled identification of novel candidates to improve myogenic activity of hPSC-SMPCs. Compared to unsorted SMPCs, hPSC-SMPCs enriched for fetal receptors Nerve Growth Factor Receptor (NGFR) or Epidermal Growth Factor Receptor 3 (ERBB3) improved fusion *in vitro* and engraftment *in vivo*. Further, aberrant TGFβ signaling was identified as a key barrier to hPSC-SMPC fusion, and inhibition of TGFβR1 improved myogenesis of all hPSC lines tested to levels of fetal and adult SCs. Engraftment of SMPC subpopulations derived from CRISPR/Cas9 gene-edited DMD hiPSCs co-delivered with TGFβ inhibitor restored dystrophin to levels of cultured fetal SCs in mdx mouse models of DMD. In summary, this is the first identification of candidates important for maturing SMPCs to enable robust myogenic activity *in vitro* and *in vivo* and provides insight for using these cells to treat muscle diseases.

Program Abstract #20

Imaging fusion *in vivo* reveals roles of two distinct Pax7 stem cell populations in larval zebrafish muscle repair

Tapan G. Pipalia[1], Jana Koth[1,2], Shukolpa D. Roy[1], Christina L. Hammond[1], Koichi Kawakami[3], Simon M. Hughes[1]

[1]*King's College London, UK;* [2]*Oxford University, UK;* [3]*National Institute of Genetics, Japan*

How are fibres formed from muscle precursor cells (mpcs) during muscle wound regeneration? Time-lapse 3D confocal imaging was employed to visualize the process of new fibre formation and fusion during muscle repair *in vivo*. Zebrafish have two Pax7 genes, *pax7a* and *pax7b*. Using fluorescent reporters for each gene, distinct mpc populations were shown to contribute to muscle wound repair in different ways. Mpcs marked by *pax7a*-only enter wounds early, proliferate and are observed to initiate, or found, new fibres. *Pax7b*-marked cells, in contrast, accumulate in wounds, align and then frequently to fuse to both nascent fibres and pre-existing fibres adjacent to the wound, thereby rapidly regenerating muscle. Ablation of a substantial portion of nitroreductase-expressing *pax7b* cells with metronidazole prior to wounding triggered rapid *pax7a*-only cell accumulation, but this neither inhibited nor augmented *pax7a*-only cell-derived myogenesis and thus altered the cellular repair dynamics during wound healing. Moreover, *pax7a*-only cells did not regenerate *pax7b* cells, suggesting a lineage distinction. We propose a modified founder cell/fusion competent cell model in which *pax7a*-only cells initiate fibre formation and *pax7b* cells contribute to fibre growth. This novel cellular complexity in muscle wound repair raises the possibility that distinct populations of myogenic cells contribute differentially to repair in other vertebrates.

Program Abstract #21

Pw1/Peg3 regulates skeletal muscle growth and satellite cells self-renewal

Rosamaria Correra[1], David Ollitrault[1], Alessia Mazzola[1], Bjorn Adalsteinsson[2], Anne Ferguson-Smith[2], Giovanna Marazzi[1], David Sassoon[1]

[1]*Stem Cells and Regenerative Medicine, Institute of Cardiometabolism and Nutrition (ICAN), UMRS 1166 INSERM, University of Pierre and Marie Curie Paris VI, France;* [2]*Department of Genetics, University of Cambridge, UK*

Pw1/Peg3 is a parentally imprinted gene expressed from the paternal allele. In the adult, it is expressed in all adult progenitor/stem cell populations examined to date including muscle satellite cells. Several parentally imprinted genes, including Pw1/Peg3, have been shown to play a role in the regulation of overall body growth and have been found to be expressed in adult stem cells. We examined the impact of loss-of-function of Pw1/Peg3 in skeletal muscle that makes up the majority of body mass. We found that constitutive loss of Pw1/Peg3 results in reduced muscle mass (~20% of WT) resulting from a decrease in muscle fiber number, but not a reduction in fiber CSA. The reduced fiber number is present at birth. Mice lacking both the paternal and maternal alleles display a lower fiber number as compared to mice carrying the paternal deletion suggesting that the maternal allele is functional during prenatal development and hybrid analyses (C57BL6J and Cast/Ei) of muscle tissue reveals ~ 10% bi-allelic expression of Pw1/Peg3. Pw1/Peg3 is strongly up-regulated in response to muscle injury. Using the constitutive Pw1/Peg3 knock out mouse, we observed that satellite cells display a reduced self-renewal capacity following muscle injury coupled with an increase in fat deposition. Pw1/ Peg3 is expressed in satellite cells as well as a subset of muscle interstitial cells (PICs) that constitute a mixture of progenitor populations in adult skeletal muscle. In order to determine the specific role of Pw1/Peg3 in satellite cells, we crossed our conditional Pw1/Peg3 allele with the Pax7-CreER line. Interestingly, these mice displayed a more pronounced phenotype

of impaired regeneration revealing a clear and direct role for Pw1/Peg3 in satellite cells. Taken together, our data reveal that Pw1/Peg3 plays a role during fetal development in the determination of muscle fiber number that is gene-dosage dependent and plays a specific role in muscle satellite cell self-renewal.

Program Abstract #22
Regulation of muscle stem cells during organ growth
Peter Currie[1,2]
[1]*Australian Regenerative Medicine Institute, Australia;* [2]*EMBL Australia, Australia*
Organ growth requires a careful balance between cell commitment and stem cell self renewal to maintain tissue growth trajectories. While the processes that regulate resident stem cells during regeneration and disease have received much attention, the basis of stem cell deployment during organ growth remains poorly defined. This knowledge is critical for advancing efforts to generate functioning organ systems *in vitro*. Here we utilize lineage analysis and timelapse imaging to characterize the mechanisms that control stem cell behaviours during skeletal muscle growth in zebrafish. We identify a lifelong stem cell pool that exhibits extensive clonal drift, shifting from the random deployment of a large population of stem cells during larval growth, to the reliance on a small number of dominant stem cell clones to fuel adult muscle growth. We further reveal that self renewal and clonal drift of growth specific muscle stem cells requires the activity of specific genes and cell cycle control. We define a distinct mechanism for the regulation of the stem cells required for organ growth and in the process provides a molecular understanding of the mechanisms underlying clonal drift *in vivo*.

Program Abstract #23
Molecular regulation of muscle stem cell asymmetric division
Michael A. Rudnicki
Ottawa Hospital Research Institute, Canada
We discovered that a subset of satellite cells in skeletal muscle are self-renewing stem cells that give rise to myogenic progenitors through asymmetric apical-basal cell divisions. Our identification of *satellite stem cells* has facilitated important insights into satellite cell biology. For example, we discovered Wnt7a/Fzd7 signaling as important intrinsic control mechanism that plays a central role in regulating the pool size of the satellite stem cell compartment by stimulating symmetric stem cell expansion. Direct injection of recombinant Wnt7a protein into muscle significantly augments regeneration. Wnt7a treated muscles were larger, contained higher numbers of satellite cells, larger caliber myofibers, and were able to generate more force upon stimulation. Thus, the regulation of asymmetric stem cell division is a key control point that impacts the efficacy of the entire regenerative program. Stem cell polarity is established by the PAR complex, comprised of PAR3/PAR6/aPKC, to regulate self-renewal and expansion. We have discovered that full-length dystrophin is expressed in satellite stem cells in skeletal muscle. We have made the seminal discovery that dystrophin regulates the establishment of PAR-mediated polarity in satellite cells. In the absence of dystrophin, the polarity effector Par1b is dysregulated, leading to the failure of Par3 to become localized to the cortex associated with the basal lamina. Importantly, this results in an abnormal increase in centrosome number, a 10-fold reduction in the numbers of satellite stem cells undergoing asymmetric divisions, and a marked decrease in the generation of myogenin-expressing progenitors. Accordingly, our data suggests that the failure of regenerative myogenesis to keep pace with disease progression in DMD is not due to muscle stem cell exhaustion, but rather is due to a cell-autonomous deficiency in asymmetric division.

Program Abstract #24
A novel role for the Agrin receptor Lrp4 in peripheral nerve regeneration
Gribble Katherine[1], Juliane Bremer[1], John Kuwada[2], Michael Granato[1]
[1]*University of Pennsylvania, USA;* [2]*University of Michigan, USA*
The low-density lipoprotein like receptor (Lrp4) is best known as the Agrin receptor and co-receptor for the muscle specific receptor tyrosine kinase (MuSK) critical for vertebrate neuromuscular synapse development. Following injury, peripheral nerves have retained the capacity to reestablish synaptic connections with their original targets. We have established zebrafish as a vertebrate model in which to study *in vivo* the cellular and molecular mechanisms critical for peripheral nerve regeneration (Rosenberg et al 2012, 2014; Issacman-Beck et al 2015). From an ongoing shelf screen we identified mutations in the Lrp4 gene to cause a strong reduction in motor axonal regeneration. We find that the obligate Lrp4 co-receptor MuSK, while essential for zebrafish neuromuscular synapse development, is dispensable for motor axon regeneration, demonstrating that Lrp4 promotes axon regeneration through a MuSK-independent pathway. Moreover, using a second *lrp4* mutant allele we find that the Lrp4 transmembrane domain, while critical for neuromuscular synapse development, is dispensable for axon regeneration, consistent with the idea that Lrp4 promotes regeneration through a novel, tether-independent pathway. Restoring *lrp4* expression only to muscle cells in animals otherwise lacking *lrp4* does not rescue motor axon regeneration, suggesting that Lrp4 acts in another cell type to promote axon regeneration. We will present ongoing efforts to further characterize this novel MuSK-independent pathway through which Lrp4 promotes peripheral nerve regeneration.

Program Abstract #25

Presynaptic neurotrophin co-receptor p75 is required for the maturation of the neuromuscular junction

Viviana Pérez[1,3], Francisca Bronfman[2,3], Margarita Calvo[2,3], Felipe Court[2,3], Claudio Cabello-Verrugio[4], Marco Fuenzalida[5], Ariel Ionescu[6], Eran Perlson[6], Juan Pablo Henríquez[1,3]

[1]Universidad de Concepcion, CHILE; [2]P. Universidad Católica de Chile, CHILE; [3]Millenium Nucleus in Regenerative Biology, CHILE; [4]Universidad Andrés Bello, CHILE; [5]Universidad de Valparaíso, CHILE; [6]Tel Aviv University, ISRAEL

Disruption of neurotrophin-mediated signaling alters the maintenance of the neuromuscular junction (NMJ), but the underlying molecular mechanisms have not yet been fully elucidated. Here, we have analyzed the neuromuscular system of mice null for the neurotrophin co-receptor p75 at the morphological and functional levels. Compared to controls, p75 mutants display a marked walking phenotype and impaired motor coordination in four tests, in spite of exhibiting similar sensory responses (four tests). p75 disruption also resulted in muscle weakness as well as in decreased muscle fiber diameter and increased number of slow-twitch fibers. In addition, contractile activity recording showed impaired muscle resistance in mutant muscles upon high-frequency presynaptic stimulation. Confocal z-stack and 3D analyses revealed that p75 null animals display a delayed maturation of the NMJ, evidenced by morphological changes and reduced area and perimeter of postsynaptic structures. Muscle-derived p75 is likely not involved in these phenotypes, as primary cultures from p75 mutant and control satellite cells showed no differences in their ability to differentiate into myotubes or to develop complex postsynaptic structures. In turn, ultrastructural analyses by EM showed a significant reduction in the number of presynaptic vesicles at the motor terminal of p75 null NMJs. Remarkably, chronic administration of acetylcholinesterase inhibitors significantly rescued the motor coordination performance of p75 null mice. Our findings reveal that motor neuron-derived p75 regulates the number of synaptic vesicles, and thereby the maturation of the NMJ and the motor performance. (Funded by FONDECYT 1110321 and MINREB RC120003, Chile).

Program Abstract #26

Localized LoxL3-dependent fibronectin oxidation regulates myofiber stretch and integrin-mediated adhesion

Ortal Kraft-Sheleg[1], Shelly Zaffryar-Eilot[1], Olga Genin[2], Wesal Yaseen[1], Sharon Soueid-Baumgarten[1], Ofra Kessler[3], Tatyana Smolkin[3], Gal Akiri[3], Gera Neufeld[3], Yuval Cinnamon[2], Peleg Hasson[1]

[1]The Rappaport Faculty of Medicine and Research Institute, Technion – Israel Institute of Technology, Israel; [2]Institute of Animal Sciences, Department of Poultry and Aquaculture, Agricultural Research Organization, The Volcani Center, Israel; [3]Cancer Research and Vascular Biology Center, The Rappaport Faculty of Medicine and Research Institute, Technion – Israel Institute of Technology, Israel

For muscles to function, myofibers have to stretch and anchor at the myotendinous junction (MTJ), a region rich in extracellular matrix (ECM). Integrin signaling is required for MTJ formation, and mutations affecting the cascade lead to muscular dystrophies in mice and humans. Underlying mechanisms for integrin activation at the MTJ and ECM modifications regulating its signaling are unclear. We show that lysyl oxidase-like 3 (LoxL3) is a key regulator of integrin signaling that ensures localized control of the cascade. In LoxL3 mutants, myofibers anchor prematurely or overshoot to adjacent somites, and are loose and lack tension. We find that LoxL3 complexes with and directly oxidizes Fibronectin (FN), an ECM scaffold protein and integrin ligand enriched at the MTJ. We identify a mechanism whereby localized LoxL3 secretion from myofiber termini oxidizes FN, promoting FN polymerization thus priming it for integrin activation at the tips of myofibers and ensuring correct positioning and anchoring of myofibers along the MTJ.

Program Abstract #27

Mechanobiology of skeletal muscle during development

Joana Esteves de Lima[1,2,3], Marie-Ange Bonnin[1,2,3], Delphine Duprez[1,2,3]

[1]CNRS UMR 7622, IBPS-Developmental Biology Laboratory, F-75005, Paris, France; [2]Sorbonne Universités, UPMC Univ Paris 06, IBPS-Developmental Biology Laboratory, F-75005, Paris, France; [3]Inserm U1156, F-75005, Paris, France

Skeletal muscle is essential for the mobility of the human body and consequently for the quality of life, health and survival. Skeletal muscle development, homeostasis and regeneration rely on muscle stem cells. Skeletal muscle is very plastic and adapts in size according to upload and download demands with hypertrophy or atrophy, respectively. Both muscle fibres and progenitors respond to loading changes, however, their respective contributions in muscle adaptation are not well established. Moreover, the molecular mechanisms underlying the interplay between muscle fibres and muscle progenitors during foetal muscle growth in the context of immobilization are not known. We set up a model of immobilisation by blocking muscle contraction in chick foetuses. We analysed the molecular and cellular consequences for foetal myogenesis when muscle contraction was blocked. We established that the activity of the NOTCH signalling pathway, known to be a central regulator muscle stem cells, was decreased in foetal muscles following immobilization. Moreover, the inhibition of muscle contraction mimicked a NOTCH loss-of-function phenotype, *i.e.* dramatically decreased the number of foetal muscle progenitors and concomitantly increased differentiation. Forced-NOTCH activation prevented the diminution in the number of foetal muscle progenitors and the increase of differentiation in immobilized embryos. We further showed that muscle fibres rather than muscle progenitors first responded to the block

of mechanical forces. We also provide molecular insights into how the muscle fibres sense the mechanical forces through the transcriptional co-activator YAP and affect the number of muscle progenitors via the NOTCH signalling pathway.

Program Abstract #28

Failure to up-regulate transcription of genes necessary for muscle adaptation underlies limb girdle muscular dystrophy 2A (calpainopathy)

Irina Kramerova[1], Natalia Ermolova[1], Ascia Eskin[1], Andrea Hevener[1], Oswald Quehenberger[2], Aaron Armando[2], Ronald Haller[3], Stanley Nelson[1], Melissa Spencer[1]

[1]University of California, Los Angeles, USA; [2]University of California, San Diego, USA; [3]UT Southwestern Medical Center, USA

Limb girdle muscular dystrophy 2A is due to loss-of-function mutations in the Calpain 3 (*CAPN3*) gene. Our previous data suggest that CAPN3 helps to maintain the integrity of the triad complex in skeletal muscle. In *Capn3* knock-out mice (C3KO), Ca^{2+} release and Ca^{2+}/calmodulin kinase II (CaMKII) signaling are attenuated. We hypothesized that calpainopathy may result from a failure to transmit loading-induced Ca^{2+}-mediated signals, necessary to up-regulate expression of muscle adaptation genes. To test this hypothesis, we compared transcriptomes of muscles from wild-type and C3KO mice subjected to endurance exercise. In wild-type mice, exercise induces a gene signature that includes myofibrillar, mitochondrial and oxidative lipid metabolism genes, necessary for muscle adaptation. C3KO muscles fail to activate the same gene signature. Furthermore, in agreement with the aberrant transcriptional profile, we observe a commensurate functional defect in lipid metabolism whereby C3KO muscles fail to release fatty acids from stored triacylglycerol. In conjunction with the defects in oxidative metabolism, C3KO mice demonstrate reduced exercise endurance. Failure to up-regulate genes in C3KO muscles is due, in part, to decreased levels of PGC1a, a transcriptional co-regulator that orchestrates the muscle adaptation response. Destabilization of PGC1a is attributable to decreased p38 MAPK activation via diminished CaMKII signaling. Thus, we elucidate a pathway downstream of Ca^{2+}-mediated CaMKII activation that is dysfunctional in C3KO mice, leading to reduced transcription of genes involved in muscle adaptation. These studies identify a novel mechanism of muscular dystrophy: a blunted transcriptional response to muscle loading resulting in chronic failure to adapt and remodel.

Program Abstract #29

a-Actinin-3 is a novel genetic modifier of Duchenne muscular dystrophy (DMD)

Peter Houweling[1,2], Marshall Hogarth[2,3], Kristen Thomas[2], Heather Gordish-Dressman[3], Luca Bello[3,4], Elena Pegoraro[4], Eric Hoffman[3], Stewart Head[5], Kathryn North[1,2]

[1]Murdoch Childrens Research Institute, Australia; [2]Institute for Neuroscience and Muscle Research, Australia; [3]Children's National Medical Centre, United States of America; [4]University of Padova, Italy; [5]University of New South Wales, Australia

Duchenne muscular dystrophy (DMD) is characterized by muscle degeneration and progressive weakness. There is considerable inter-patient variability in disease onset and progression that often confounds clinical trial results. It has been hypothesized that genetic variation at loci independent of dystrophin (i.e. genetic modifiers) are responsible for the unexplained variability in onset, progression and response to therapies. To date, two modifying genes, osteopontin (*SPP1*) and *LTBP4*, have been identified that influence strength and age at loss of ambulation (LoA) in DMD. Here we show that a common polymorphism in the *ACTN3* gene (R577X) also alters muscle strength and disease progression in DMD. Homozygosity for a common null polymorphism (R577X) in *ACTN3* results in absence of the fast muscle fiber protein α-actinin-3 in ~18% of the general population. *ACTN3* genotype significantly influences muscle performance in elite athletes and the general population. On this basis we hypothesized that *ACTN3* may act as a genetic modifier in patients with inherited muscle disorders. To test this we developed a double knockout (*Actn3* KO/mdx; dKO) mouse model that shows reduced muscle force, but protection from stretch-induced eccentric damage with age. This suggests that α-actinin-3 deficiency reduces muscle strength, but ameliorates disease progression. Furthermore, α-actinin-3 deficiency significantly reduces muscle strength and results in a longer 10m walk test time in young, ambulant DMD patients. Longitudinal analyses of two natural history cohorts (CINRG and Padova) also suggest that *ACTN3* genotype alters age at LoA. Mechanistically, α-actinin-3 deficiency triggers an increase in oxidative muscle metabolism through activation of calcineurin and AMPK, which likely confers the protective effect in dKO mice. Identifying genetic modifiers such as *ACTN3* have immediate importance in the stratification of clinical trials and provide insight into the molecular pathogenesis of DMD.

Program Abstract #30

54 Years of myogenesis and counting: Unresolved myogenic questions from stem cells to gene regulation

Steve Hauschka

University of Washington, USA

This Keynote address will begin with a brief review of myogenic clonal analysis studies during early developmental periods to point out unresolved questions pertaining to transitional states within vertebrate skeletal muscle lineages.

The talk will then describe *in vitro* myogenic commitment assays in somites and myoblasts that identify yet-to-be-understood temporal changes and inductive effects of growth factors and other external signals during myogenesis. The presentation will then transition to studies of muscle-specific gene regulation, and will highlight studies that identified transcription factors not previously known to play significant roles in muscle gene expression. The presentation will conclude by describing recent progress in designing miniaturized muscle-specific regulatory gene cassettes for the *in vivo* expression of therapeutic and experimental products in adult skeletal muscle fiber types and cardiac muscle over several orders of magnitude, and that can far exceed transcription rates of the most active viral promoters and enhancers in striated muscles.

Program Abstract #32

Exercise-like effects by Estrogen-related receptor gamma in muscle do not prevent insulin resistance in db/db mice

Pierre-Marie Badin[1], Isabelle Vila[2], Danesh Sopariwala[1], Vikas Yadav[1], Sabina Lorca[1], Katie Louche[3], Eun-Ran Kim[1], Qingchun Tong[1], Min Sup Song[2], Cedric Moro[2], Vihang Narkar[1]

[1]University of Texas Medical School at Houston, US; [2]University of Texas MD Anderson Cancer Center, Houston, US; [3]Institut National de la Santé et de la Recherche Médicale, Inserm UMR 1048, France

Dissecting exercise-mimicking pathways that can replicate the benefits of exercise in obesity and diabetes may lead to promising treatments for metabolic disorders. Muscle estrogen-related receptor gamma (ERRg) is induced by exercise, and when over-expressed in the skeletal muscle mimics exercise by stimulating glycolytic-to-oxidative myofiber switch, mitochondrial biogenesis and angiogenesis in lean mice. The objective of this study was to test whether muscle ERRg in obese mice mitigates weight gain and insulin resistance. To do so, ERRg was selectively over-expressed in the skeletal muscle of obese and diabetic db/db mice. Muscle ERRg over-expression successfully triggered glycolytic-to-oxidative myofiber switch, increased functional mitochondrial content and boosted vascular supply in the db/db mice. Despite aerobic remodeling, ERRg surprisingly failed to improve whole-body energy expenditure, block muscle accumulation of triglycerides, toxic diacylglycerols (DAG) and ceramides or suppress muscle PKCε sarcolemmal translocation in db/db mice. Consequently, muscle ERRg did not mitigate impaired muscle insulin signaling or insulin resistance in these mice. In conclusion, obesity and diabetes in db/db mice are not amenable to selective ERRg-directed programming of classic exercise-like effects in the skeletal muscle. Other biochemical pathways or integrated whole-body effects of exercise may be critical for resisting diabetes and obesity.

Program Abstract #33

Brown and beige fat promote rotator cuff muscle regeneration through paracrine signaling

Anna Bryniarski, Gretchen Meyer

Washington University in St. Louis, United States

Adipose tissues in the body influence distant tissues through endocrine signaling in a phenotype-specific manner. Specifically, “browning” of beige fat results in improved muscle insulin sensitivity and increased bone growth. The “brownness” of beige fat may also influence neighboring tissues through paracrine signaling, especially in depots that hug the periphery of tissues. The purpose of this study is to investigate the potential for regulation of muscle regeneration by a recently identified beige fat depot, epimuscular fat, in the rotator cuff through paracrine signaling. To this end, we developed a novel fat transplant model in the mouse rotator cuff where small pieces of known adipose phenotypes (white, beige and brown) are transplanted between muscles in the rotator cuff in a similar location to where the beige depot is found in the human cuff. We then induced muscle regeneration adjacent to the graft with an intramuscular injection of cardiotoxin and evaluated the influence of the fat graft on morphological and functional measures of muscle recovery. Transplanted fat engrafted well as evidenced by maintenance of tissue size, morphology and vascularity. Muscles adjacent to brown and (to a lesser extent) beige grafts have significantly higher wet weights and fiber sizes compared with muscles adjacent to white grafts indicating a larger degree of hypertrophy during regeneration. This effect was local as distal muscles showed no size differences between groups. Increased functional hypertrophy in brown- and beige-adjacent muscles was also supported by increased hypertrophy-associated gene expression and increased active tension generation in these groups. Grafting brown and beige fat into the rotator cuff improved muscle regeneration associated hypertrophy. This finding suggests “browning” of human epimuscular fat in the rotator cuff could be a novel therapeutic intervention to promote muscle hypertrophy and recovery of function following surgery.

Program Abstract #34

Osteopontin ablation ameliorates muscular dystrophy by shifting macrophages to a pro-regenerative phenotype

Joana Capote[1], Irina Kramerova[1], Leonel Martinez[1], Sylvia Vetrone[3], Elisabeth Barton[2], Lee Sweeney[2], Carrie Miceli[1], Melissa Spencer[1]

[1]University of California, Los Angeles, United States; [2]University of Florida, Gainesville, United States; [3]Whittier College, United States

In the degenerative disease Duchenne muscular dystrophy, inflammatory cells enter muscles in response to repetitive muscle damage. Immune factors are required for muscle regeneration, but chronic inflammation creates a pro-fibrotic

milieu that exacerbates disease progression. Osteopontin (OPN) is an immunomodulator highly expressed in dystrophic muscles. Ablation of OPN correlates with reduced fibrosis and improved muscle strength as well as reduced NKT cell counts. Here, we demonstrate that the improved dystrophic phenotype observed with OPN ablation does not result from reductions in NKT cells. OPN ablation skews macrophage polarization towards a pro-regenerative phenotype by reducing M1 and M2a and increasing M2c subsets. These changes are associated with increased expression of pro-regenerative factors IGF-1, LIF and uPA. Furthermore, altered macrophage polarization correlated with increases in muscle weight and muscle fiber diameter, resulting in long-term improvements in muscle strength and function in mdx mice. These findings suggest that OPN ablation promotes muscle repair via macrophage secretion of pro-myogenic growth factors.

Program Abstract #35

A reduction in Selenoprotein S (SEPS1) amplifies the inflammatory profile of fast twitch skeletal muscle and reduces fibre size distribution in the mdx dystrophic mouse

Craig Wright[1], Giselle Keefe[1], Alex Addinsall[2], Natasha McCrae[2], Sof Andrikopoulos[3], Nicole Stupka[2]
[1]Institute for Physical Activity and Nutrition (IPAN), School of Exercise and Nutrition Sciences, Deakin University, Waurn Ponds, VIC, Australia; [2]School of Medicine, Deakin University, Waurn Ponds, VIC, Australia; [3]Department of Medicine - Austin Health, The University of Melbourne, Heidelberg, VIC, Australia

Dysregulated inflammation is a hallmark of Duchenne Muscular Dystrophy (DMD). Novel regulators of inflammation are of interest, due to their potential to alleviate the dystrophic muscle pathology. Selenoprotein S (SEPS1) is highly expressed in skeletal muscle and can protect against inflammatory stress. Here, we examined the role of SEPS1 on the inflammatory profile of the mdx dystrophic mouse. Male C57BL6 mice with a global heterozygous deletion of SEPS1 were mated with female mdx mice to produce F1 male mdx mice with or without a heterozygous deletion of SEPS1 (mdx:SEPS1-/+). At 12 weeks, EDL and soleus muscles underwent ex vivo contractile function testing, and were then frozen for gene expression analysis. Contralateral muscles were excised for histology. All procedures were approved by the Deakin University Animal Ethics Committee (AEC#: G29/2014). mdx:SEPS1-/+ mice had a 51% reduction in SEPS1 protein in fast twitch tibialis anterior muscles ($p=0.034$). EDL muscles of the mdx:SEPS1-/+ mice had a 2.8 fold increase in MCP-1 mRNA ($p=0.034$), a two-fold increase in macrophage marker F4/80 mRNA ($p=0.029$) and a trend for elevated TGF-β1, IL-1β, iNOS, arginase, CD163 and CD68. mRNA markers of inflammation were not altered in slow twitch soleus muscles of mdx:SEPS1-/+ mice. Histological analysis of the EDL using min ferets diameter, suggested a reduction in fibre size in mdx:SEPS1-/+ mice ($p=0.06$). Further analysis revealed a significant interaction (genotype*size $p=0.0017$) in fibre size distribution, with mdx:SEPS1+/- mice having greater proportions of small fibres and fewer large fibres. The genetic reduction of SEPS1 had no effect on force production in EDL and soleus muscles. Thus, reduced SEPS1 expression in mdx mice appears to exacerbate the inflammatory profile of fast twitch EDL muscles and reduce fibre size, but no change in strength. Further analysis is required to determine whether these results are beneficial or detrimental to disease progression.

Program Abstract #36

Lymphoid regulators of muscle inflammation and regeneration during muscular dystrophy

Armando Villalta[1], Wendy Rosenthal[2], Leonel Martinez[3], Amanjot Kaur[2], Tim Sparwasser[4], James G. Tidball[3], Marta Margeta[2], Melissa Spencer[3], Jefffrey A Bluestone[2]
[1]University of California Irvine, USA; [2]University of California San Francisco, USA; [3]University of California Los Angeles, USA; [4]Twincore, Germany

We previously demonstrated that regulatory T cells (Tregs) suppress muscle inflammation and injury in the mdx mouse model of Duchenne muscular dystrophy (DMD). Tregs were largely absent in wild type mouse and normal human muscle, but were elevated in mdx and dystrophic patient muscle. Tregs were present in necrotic lesions and displayed an activated phenotype. Moreover, the elevated number of Tregs correlated with increased expression of IL-10, which was further upregulated when mdx mice were treated with IL-2/anti-IL-2 antibody complex (IL-2c). IL-2c reduced myofiber injury and muscle inflammation, reflected by reduced expression of cyclooxygenase-2. More recently, we discovered that IL-2c also increased the number of innate lymphoid cells (ILCs), a specialized subset of immune cells not previously implicated in the pathogenesis of muscular dystrophy but known to promote tissue repair. ILCs were increased in muscle of mdx mice compared to controls, and displayed phenotypic characteristics of group 2 ILC (ILC2s) that express the type 2 cytokines IL-5 and IL-13. These findings indicate that IL-2c ameliorates the severity of dystrophinopathy by increasing the recruitment of Tregs that suppress muscle inflammation, likely through the production of IL-10, and also increase ILC2s in mdx muscle that may participate in muscle repair.

Program Abstract #37

Single-cell based analysis of functional populations in aged and dystrophic muscle

Lorenzo Giordani[1], Fabien Le Grand[1,2]
[1]Centre de Recherche en Myologie, 105, bld de l'hôpital - G.H. Pitié-Salpétrière, Paris, France; [2]Institut National de la Santé et de la Recherche Médicale (INSERM), France

Skeletal muscle is a complex structure endowed with extreme regenerative capability; this ability relies on the orchestrated interplay between different muscle populations that reside within the tissue. Functional changes occurring at the microenvironmental level during aging or pathological conditions however, interfere with this ability leading to fibrosis and fat infiltration. Despite a large body of work still we are far from completely understanding these changes; even when genetic cause is known (e.g. Duchenne muscular Dystrophy) we are still unable to pinpoint the steps that lead from the molecular cause to the outcome of the disease.The main reason for this bottleneck is that our knowledge has been limited so far by the lack of technical tools to dissect the heterogeneity of these populations. The use of bulk-scale methods able only to provide averaged information has frustrated our effort to characterize those pathological changes leaving those dysfunctional, disease-specific subpopulation to remain hidden within the bulk.
Here we present a novel approach based on single cell mass spectrometry to study the populations that reside in the muscle. Using CyTOF technology we have profiled at single cell resolution resident populations in healthy, dystrophic and aged muscle. Our data allowed us to identify and map functional subfractions thus enabling us to track how these subpopulations dynamically respond to aging and dystrophic condition. Ultimately our goal is to identify dysfunctional subsets involved in the regeneration defect. This study will not only shed light on the mechanisms underpinning muscle regeneration but would provide a solid ground for the future identification of diagnostic biomarkers through the study of disease-specific subpopulations.

Program Abstract #38

Development of a High-throughput Screen for Drugs to Treat Limb Girdle Muscular Dystrophy Type 2A

Jian Liu[1], Irina Kramerova[1], Ascia Eskin[2], Robert Damoiseaux[3], Carrie Miceli[4], Stanley Nelson[2], Melissa Spencer[1]
[1]Department of Neurology, University of California, Los Angeles, United States; [2]Department of Human Genetics, University of California, Los Angeles, United States; [3]California Nanosciences Institute, University of California, Los Angeles, United States; [4]Department of Microbiology, Immunology and Molecular Genetics, University of California, Los Angeles, United States

Limb girdle muscular dystrophy type 2A (LGMD2A) is caused by mutations in the non-lysosomal, cysteine protease calpain 3 (*CAPN3*). Muscles from patients and mice lacking CAPN3 have greatly reduced muscle bulk; however, unlike the dystrophinopathies, their sarcolemmal membrane is stable, suggesting that pathogenic mechanisms of LGMD2A differ from the dystrophinopathies. CAPN3 localizes to several subcellular compartments, including triads, where it is activated by calmodulin and plays a yet undefined role in calcium release. Previous studies in our lab have shown that muscles from CAPN3 knockout (C3KO) mice do not grow following a bout of atrophy. Concomitantly, Ca-calmodulin protein kinase II (CaMKII) signaling is compromised. While muscles of WT mice subjected to exercise training increase expression of several sarcomeric genes such as Myl2, Mybph and Ckmt2, muscles of C3KO mice do not exhibit these adaptive changes to exercise. Thus, enhancing the expression of CaMKII induced genes represents a novel target for LGMD2A. We hypothesize that chemical compounds that activate these slow genes may be of therapeutic potential for LGMD2A. To identify such compounds, we designed a high throughput screen using C2C12 cells with a stable Myl2 promoter reporter. We confirmed that reporter expression reflects the endogenous Myl2 expression pattern during C2C12 differentiation. Using these cells, we performed high throughput screens to identify drugs to over-ride the CAMKII signaling block and treat LGMD2A. Positive hits were validated in secondary screens and will be tested in mouse models. These studies represent the first high throughput screening approach to identify drugs for LGMD2A. Future studies will be directed towards understanding the relationship between CAPN3 and CaMKII.

Program Abstract #39

Loss of Anoctamin 5 leads to membrane-associated defects in a mouse model of Limb-Girdle Muscular Dystrophy

Ryan Johnson[1], Danielle Griffin[1], Eric Pozsgai[1], Kristin Heller[1], Jared Whitlock[2], Zarife Sahenk[1], Criss Hartzell[2], Louise Rodino-Klapac[1]
[1]The Research Institute at Nationwide Childrens Hospital, USA; [2]Emory University School of Medicine, USA

Limb Girdle Muscular dystrophies (LGMDs) are a group of degenerative muscle disorders that that initially present with shoulder and hip muscle weakness but eventually affect all muscles. Of the 14 defined recessive LGMDs, LGMD type 2L is caused by mutations in the anoctamin 5 (*ANO5*) gene, which encodes an 8-pass transmembrane protein of unknown function. Some members of the anoctamin family of proteins act as calcium-activated chloride channels, while others function in phospholipid scrambling at the plasma membrane. Evidence indicates that ANO5 exhibits neither activity, and we hypothesize that it functions to promote the normal behavior of membranes in skeletal muscle.
In the present study, we used our *Ano5*$^{-/-}$ mouse model to examine multiple aspects of membrane health. We analyzed membrane structures via histopathology and electron microscopy. We assessed the ability of ANO5-deficient muscle to undergo membrane repair following damage, and examined the behavior of known membrane repair proteins. Lastly, we assessed membrane fusion during myoblast differentiation *in vitro* and *in vivo*.
Histological staining and ultrastructural analysis of *Ano5*$^{-/-}$ tibialis anterior (TA) muscle revealed inclusion bodies of membranous tubular aggregates and additional abnormalities. Functionally, we demonstrated that, relative to wild type, *Ano5*$^{-/-}$ muscle fibers exhibited impaired membrane resealing after laser-induced injury, as well as altered abundance and

mislocalization of the repair-associated protein AHNAK. *Ano5*$^{-/-}$ muscle also displayed regeneration deficits following cardiotoxin injection. Consistent with these findings, cultured *Ano5*$^{-/-}$ myotubes leaked greater amounts of CK following bulk injury by glass beads, and exhibited reduced fusion capacity.
This work highlights the dysregulation of membrane organization and behavior in skeletal muscle due to a deficit in ANO5, and will serve as a framework for understanding how ANO5 deficiency leads to disease.

Program Abstract #40
A tunable phenotypic FSHD-like mouse model
Takako Jones, Peter Jones
University of Massachusetts Medical School, USA
Muscle weakness in facioscapulohumeral muscular dystrophy (FSHD) patients typically becomes noticeable in the second or third decade of life, followed by a progressive decline with ~20% of patients ultimately becoming wheelchair bound. However, there is great variability in disease onset, severity, and progression, including a severe infantile form, suggesting no single animal model may sufficiently recapitulate the disease. Fortunately, the aberrant expression of the primate transcription factor DUX4 is the key mediator of all forms of FSHD and its expression correlates with severity. Thus, DUX4 expression is a prime target for therapeutic intervention and FSHD models should be based on DUX4. *In vitro* studies have identified numerous DUX4-mediated events that have adverse effects on cell viability and function. However, it is unknown which, if any, of these pathways has pathogenic relevance, due to the lack of a phenotypic FSHD-like model to test efficacy of directed therapeutics. Here, we report the first successful generation of a viable, fertile, and highly tunable phenotypic FSHD-like mouse model based on the controlled expression of DUX4. For the first time, this allows the interrogation of downstream effects of DUX4 expression as to potential roles in pathophysiology and validation as therapeutic targets. With flexible design of this mouse model, we can initiate DUX4 expression in either young or adult animals as well as control the degree of pathology and rate of disease progression, thus allowing for the assessment of prevention, inhibition, or reversal of pathology, as desired. We can essentially tailor the model to the type of therapeutic being tested or pathway being studied. In addition, we are able to distinguish early, initiating events from those cumulative, chronic effects, which may help to inform which biomarkers correlate more directly with DUX4 expression and are the best to follow as the field moves towards clinical trials.

Program Abstract #41
PAX3 and PAX7 target genes are globally repressed in skeletal muscle in Facioscapulohumeral muscular dystrophy
Peter Zammit[1], Maryna Panamarova[1], Robert White[1], Frédéric Relaix[2], Simone Severini[3], Christopher Banerji[1,3]
[1]King's College London, UK; [2]Paris Est-Creteil University, France; [3]University College London, UK
Facioscapulohumeral muscular dystrophy (FSHD) is a prevalent, incurable, degenerative myopathy. FSHD is linked to hypomethylation of *D4Z4* repeats on chromosome 4q, resulting in expression of the DUX4 transcription factor encoded by the ORF in the last D4Z4 repeat, stabilised when on a permissive 4q haplotype generating a poly(A) signal. However, it is debatable if global changes in expression of *DUX4* target genes is a biomarker of skeletal muscle in FSHD. An alternative hypothesis is that DUX4 competitively inhibits the related transcription factors *PAX3/7* or down-regulates their target genes, since *PAX3/7* can rescue *DUX4*-mediated apoptosis in mouse myoblasts *in vitro* (Bosnakovski *et al.* 2008. *The EMBO journal* **27**, 2766-2779). Using meta-analysis over five published FSHD muscle biopsy gene expression studies, we found that suppression of *PAX3/7* target genes is a hallmark of FSHD, but found no evidence for global changes in expression of *DUX4* target genes. These findings were confirmed by RNA-sequencing on myoblasts and myotubes derived from a mosaic FSHD patient. We also report that *DUX4* inhibits *Pax7* from activating its transcriptional targets. Furthermore, *PAX3/7* target gene repression can explain oxidative stress sensitivity in FSHD muscle via HIF1a, as we previously proposed (Banerji et al. 2015. Journal of the Royal Society, Interface / the Royal Society 12, 20140797). In summary, suppression of *PAX3/7* transcriptional targets is a hallmark of FSHD skeletal muscle, while we find no evidence for global over-expression of *DUX4* transcriptional targets. This does not mean that DUX4 does not directly activate/repress target genes in FSHD and that they do not contribute to pathology, rather that there is not a global transcriptional DUX4 signal in FSHD.
Support is gratefully acknowledged from: FSH Society Shack Family and Friends research grant (FSHS-82013-06), Association Française contre les Myopathies (AFM 17865), Muscular Dystrophy UK (RA3/3052/1).

Program Abstract #42
Genetic reduction of the extracellular matrix protein versican modulates the function and pathology of fast and slow hindlimb muscles from dystrophic mdx mice
Natasha R. McRae[1], Leonard G. Forgan[1], Chris van der Poel[2], Daniel R McCulloch[1], Nicole Stupka[1]
[1]School of Medicine, Deakin University, Australia; [2]School of Life Science, La Trobe University, Australia
Versican is a transitional ECM protein. Cleavage of pericellular versican by the ADAMTS-5 facilitates myoblast fusion. Versican also regulates inflammation and cytokine bioavailability. Excess versican is seen in fibrosis, and expression is

increased in dystrophic muscles from DMD patients and *mdx* mice. To test the hypothesis that versican reduction improves dystrophic muscle function, *mdx* mice were bred with *hdf* mice which are haploinsufficient for the versican allele. F1 male pups, *mdx-hdf* and *mdx*, were confirmed by genotyping and immunohistochemistry for versican. Muscle strength normalised to muscle size (specific force; sP_o) and fatigability were assessed in isolated fast EDL and slow soleus muscles at 6 and 25 wk (N=8-11 mice). Mice were also placed in metabolic cages at 25 wk. A 20% increase in physical activity was observed in *mdx-hdf* mice (p=0.024), suggesting a protective effect of versican reduction. However, targeting versican in dystrophic muscles is complex and effects depend on age and fibre type. In *mdx-hdf* soleus muscles, sP_o was increased at 6 wk (p=0.002), but decreased at 25 wk (p<0.001). This decrease in sP_o at 26 wk may be due to greater fibrosis, as mRNA transcripts of *collagen A1* (p=0.045) and *TGF-β1* (p=0.045) were higher in *mdx-hdf* soleus muscles. In EDL muscles, the genetic reduction of versican did not affect sP_o at 6 and 25 wk. Nonetheless, *mdx-hdf* EDL muscles had more undamaged fibres/mm^2 (p=0.01), which was associated with less degeneration and mononuclear infiltration (p=0.07) and reduced mRNA transcripts of the macrophage marker *F4/80* (p=0.003). EDL muscles of *mdx-hdf* mice were more resistant to fatigue at 6 wk (p<0.001), but not at 25 wk. Versican reduction did not affect the fatigability of soleus muscles at 6 or 25 wk. To gain a mechanistic understanding of these muscle function findings, current work is characterising the effects of versican reduction on inflammation, myogenesis and fibrosis in dystrophic EDL and soleus muscles.

Program Abstract #43

Genetic reduction of versican improves fatigability and strength of diaphragm muscles from dystrophic mdx mice

Natasha McRae[1], Alex Addinsall[1], Chris van der Poel[2], Nicole Stupka[1]
[1]*Deakin University, Australia;* [2]*La Trobe University, Australia*

Versican is a transitional matrix protein, and its synthesis and remodelling modulate cellular processes relevant to inflammation, fibrosis and myogenesis (e.g. myoblast proliferation and fusion). Excess versican is associated with pathology and is upregulated in dystrophic muscles from patients with DMD. Glucocorticoid treatment, the only therapeutic intervention with clinical efficacy in DMD, increased fusion and myotube formation in C2C12 myoblasts and was attributed to a 50% reduction in versican gene and protein expression. Thus versican is a highly relevant target in DMD. The diaphragm of dystrophic *mdx* mice best models DMD pathology, with versican being highly upregulated in *mdx* diaphragm muscles and nearly non-existent expression in wild type diaphragm muscles (p=0.0002). To investigate whether the genetic reduction of versican improves *mdx* diaphragm function, *mdx* mice were bred with *hdf* mice, which are haploinsufficient for the versican allele. F1 male pups, *mdx-hdf* and *mdx*, were confirmed by genotyping. As diaphragm degeneration is progressive in *mdx* mice, muscle strength normalised to muscle size (specific force as determined from the force frequency curve; sP_o) and fatigability were assessed in isolated diaphragm muscle strips from 6 and 21 wk old *mdx-hdf* and *mdx* mice (N=11 mice). In *mdx-hdf* diaphragm muscles, sP_o tended to increase at 6 wk (p=0.10), and by 21 wk this increase was highly significant (p<0.001). At 6 and 21 wk, *mdx-hdf* diaphragm muscles fatigued less following 4 min of intermittent, submaximal stimulation compared to *mdx* diaphragm muscles (60 Hz every 5 s; p<0.001), and force recovery post fatigue was also improved (p<0.001). It is interesting that versican reduction improves strength and endurance in diaphragm muscles, as respiratory failure is a significant morbidity in DMD. Analysis is ongoing to determine how versican reduction modulates myogenesis, inflammation and fibrosis within the dystrophic diaphragm muscles.

Program Abstract #44

The contribution of nuclear and cytoplasmic CELF1 protein to muscle wasting in myotonic dystrophy type 1

Diana C. Cox, Thomas A. Cooper
Baylor College of Medicine Departments of Pathology and Immunology and Biochemistry and Molecular Biology, Houston, TX, United States

Myotonic dystrophy type 1 (DM1) is the most common form of muscular dystrophy in adults. Skeletal muscle wasting is a primary cause of mortality in DM1, yet the cause of muscle wasting remains unknown. DM1 is caused by expansion of a CTG repeat in the 3' untranslated region of the Dystrophia Myotonica-Protein Kinase (DMPK) gene. The primary cause of pathogenesis is the expanded repeat (CUGexp) RNA expressed from the expanded allele. The CUGexp RNA accumulates in nuclear foci and affects the functions of at least two families of RNA binding proteins: muscleblind-like (MBNL) and CUGBP Elav-like family (CELF). MBNL is sequestered on CUGexp RNA foci producing a loss of function. The contribution of MBNL to DM1 has been well established through the generation of MBNL knockout mice. In addition, CUGexp RNA induces post-transcriptional up regulation of the CELF family member, CELF1, producing a gain of function. Our lab used inducible transgenic mice that overexpress CELF1 in adult skeletal muscle to demonstrate that CELF1 up regulation is pathogenic and reproduces DM1-like muscle phenotypes, including muscle wasting and dystrophic muscle histology. CELF1 functions in the nucleus as a splicing regulator and in the cytoplasm as a regulator of mRNA stability and translation. Previous results from the Mahadevan lab illustrated that a Celf1-/- background partially rescued muscle pathology in a DM1 mouse model and affected CELF1 cytoplasmic targets but not nuclear. However, it is unknown to what degree CELF1 up regulation contributes to DM1 pathogenesis. These results

establish the basis for creating transgenic mice that inducibly express CELF1 specifically in adult skeletal muscle that localize either to the nucleus or cytoplasm to determine whether a gain of function of CELF1 nuclear and/or cytoplasmic function(s) contributes to DM1 pathogenesis. Transgenic lines have been generated and characterization of molecular and phenotypic effects will be presented.

Program Abstract #45

An inducible mouse model expressing expanded CUG RNA repeats recapitulates skeletal muscle phenotypes of myotonic dystrophy type 1

Ginny Morriss, Thomas Cooper
Baylor College of Medicine, United States

Skeletal muscle wasting is a prominent cause of morbidity and mortality in myotonic dystrophy type 1 (DM1). Clinical manifestations of DM1 are caused by expression of expanded CUG-DMPK RNA from the DMPK 3'-UTR but the mechanisms that directly underlie muscle wasting in the disease remain unknown. We have developed a Tet-inducible, skeletal muscle-specific mouse model (CUG_{960}) containing 960 interrupted-CUG repeats in the context human DMPK exons 11-15. We show that after 12 weeks of induction, the CUG_{960} model shows clear signs of skeletal muscle wasting based on decreased muscle weight and increased severity of skeletal muscle histological defects. CUG_{960} mice had higher percentage of fibers containing centralized nuclei in gastrocnemius, quadriceps, soleus, and tibialis anterior muscles and decreased median cross-sectional area in gastrocnemius and tibialis anterior muscles, compared with littermate controls. CUG_{960} mice also showed alternative splicing changes consistent with those typically observed in DM1 that were reversed upon removal of doxycycline; however, the extent of splicing changes was mild relative to DM1 muscle. RNA-seq analysis is being performed during a time course following induction to identify changes in gene expression and alternative splicing in repeat-expressing CUG_{960} mice. Motif analysis will be performed to identify potential RNA binding proteins that facilitate splicing changes during wasting. Gene ontology analysis of identified molecular changes will be performed to determine the most significant pathways affected by expression of CUG_{960} RNA over time. The CUG_{960} model will allow for detailed mechanistic studies of skeletal muscle wasting and potentially can be used to test therapeutic approaches for prevention and reversal of molecular and physiological features of DM1.

Program Abstract #46

The transcriptional landscape of inherited skeletal muscle disease in zebrafish

Jane Patrick, Samantha N. Carruthers, Neha Wali, Ian M. Sealy, John E. Collins, Elisabeth M. Busch-Nentwich
Wellcome Trust Sanger Institute, UK

Inherited skeletal muscle diseases are a genetically and phenotypically heterogeneous spectrum of disorders characterised by muscle weakness and/ or degeneration, frequently with joint and neurological problems. Many patients have a rare or unique mutation and are undiagnosed, making identification of therapeutic targets challenging; there are currently only symptomatic treatments. Accurate diagnosis is also difficult since patients are generally diagnosed based on the analysis of morphological characteristics on a muscle biopsy into a specific skeletal muscle disease category, whereas the boundaries between different disease subtypes are ill defined. To investigate the genotype-phenotype relationship in detail we generate and characterise zebrafish knockout models of skeletal muscle disorders, to gain insight into underlying mechanisms of the phenotype at both the morphological and the transcriptional level. The external fertilisation, translucency and genetic tractability of zebrafish embryos make them an ideal model for studying early muscle development, whilst the rapid generation of mutants enables high-power transcriptional analysis. We have performed quantitative genome-wide messenger RNA sequencing on nine zebrafish skeletal muscle mutants, comprising both dystrophic and non-dystrophic phenotypes, including myofibrillar and nemaline myopathies, to generate a catalogue of transcriptional profiles. Comparative analysis of these profiles will allow us to build a transcriptional landscape of the spectrum of skeletal muscle phenotypes and identify specific signatures or gene regulatory networks underlying the molecular manifestation of particular phenotype subsets, which may be independent of the specific underlying genetic mutation. This approach has promise in clinical application to identify common features for therapeutic targets amongst a subset of patients with particular molecular phenotype characteristics, independently of the underlying causal mutation.

Program Abstract #47

Regulation of skeletal muscle development and disease by an actin-dependent transcriptional circuit

Bercin K. Cenik, Ning Liu, Beibei Chen, Svetlana Bezprozvannaya, Rhonda Bassel-Duby, Eric N. Olson
UT Southwestern Medical Center, USA

Myocardin-related transcription factors (MRTFs) play a central role in the regulation of actin expression and dynamics. We have recently shown that loss of Leiomodin-3 (Lmod3), an actin-binding protein and a component of the sarcomere thin filament, is required for the development and maintenance of the sarcomere. Loss of Lmod-3 led to compromised sarcomere integrity and nemaline myopathy (NM), a severe congenital myopathy. Transcription of the Lmod3 gene in skeletal muscle is controlled by the SRF and MEF2 transcription factors. MRTFs, which function as SRF coactivators, serve as sensors of actin polymerization and are sequestered in the cytoplasm by actin monomers. Conversely, conditions

favoring actin polymerization de-repress MRTFs, leading to activation of SRF-dependent genes. Consistent with this actin-dependent regulatory circuit, the actin nucleator Lmod3 enhances MRTF-SRF activity. In turn, SRF cooperates with MEF2 to sustain the expression of Lmod3 and other components of contractile apparatus, thereby establishing a regulatory circuit to maintain skeletal muscle function. We next explored the role of MRTFs in muscle development *in vivo* by generating mutant mice harboring a skeletal muscle-specific deletion of MRTF-B and a global deletion of MRTF-A. These double knockout (*dKO*) mice were able to form sarcomeres during myofibrillogenesis. However, the sarcomeres were abnormally small and disorganized, causing skeletal muscle hypoplasia and perinatal lethality. Transcriptome analysis demonstrated dramatic dysregulation of actin genes in *MRTF dKO* mice, highlighting the importance of MRTFs in actin cycling and myofibrillogenesis. MRTFs were also necessary for the survival of skeletal muscle myoblasts and for the formation of intact myotubes. Our findings provide insights into how actin cycling networks interact with skeletal muscle specific transcripts and/or proteins to contribute to myogenesis and myopathies.

Program Abstract #48

Is the epidermal growth factor signalling pathway a regulator of skeletal muscle fibre type plasticity?

Margherita Ciano

Imperial College London, UK

Skeletal muscle consists of oxidative, insulin-sensitive, **slow-twitch (ST)** and glycolytic, less insulin-sensitive, **fast-twitch (FT)** fibres. Adult skeletal muscle fibres retain the ability to switch between a ST and FT type. A high proportion of ST fibres in skeletal muscle protects against **insulin resistance (I_R)**:the problem underpinning **type 2 diabetes mellitus (T2DM)**. Our *in vitro* study on C2C12 muscle cells suggests that inhibition of **epidermal growth factor receptor (EGFR)** promotes the ST, and suppresses the FT, fibre phenotype. ***Hypothesis.***Inhibiting EGFR in skeletal muscle will reduce the risk of developing I_R and T2DM and will treat established T2DM, by promoting ST fibre specification in adult skeletal muscle. ***Objectives.***To investigate if EGFR inhibition increases ST gene expression *in vitro* and increases ST fibre proportion *in-vivo* ***Methods.***C2C12 mouse myoblasts were treated with an EGFR inhibitor, RNA was extracted and qPCR used to quantify gene expression. Six 8-week old mice were treated with an EGFR inhibitor (n=6). mRNA and protein expression of ST and FT genes in lower limb skeletal muscles were quantified by western blot and qPCR. Two global *EGFR*-knockout zebrafish were generated by using the CRISPR/Cas9 system, and identified EGFR expression in skeletal muscle of zebrafish embryos by *in-situ* hybridisation; egfr+/- and wt fish were immunostained for ST and FT fibre. ***Results.***EGFR inhibitor decreases FT, and increases ST and GLUT4, gene expression *in-vitro*. EGFR inhibitor induces a significant increase in GLUT4 expression *in-vivo*. EGFRis expressed at early stages in zebrafish larvae. Progeny of mutated fish develop abnormalities in ST fibre organisation. ***Conclusions.***Inhibition of EGFR signalling increases ST and GLUT4, and decreases FT, gene expression in mouse myotubes and increases GLUT4 expression in treated mice. EGFR mutation in zebrafish appears to disrupt normal ST fibre formation; more detailed description of which is being undertaken currently.

Program Abstract #49

Exosome-driven EMT causes skeletal muscle degeneration and fibrosis in the mouse model of the lysosomal disease sialidosis

Alessandra d'Azzo, Diantha van de Vlekkert

St. Jude Children's Research Hospital, USA

Abnormal processing of terminal sialic acids (SIAs) on membrane glycoproteins changes their biochemical and functional properties, which in turn may influence cell–cell and cell–ECM communication, cell migration, adhesion, and intracellular signaling, thereby negatively affecting tissue and organ homeostasis. The pathological consequences of glycoprotein oversialylation are evident in the severe systemic phenotypes seen in sialidosis, a lysosomal storage disease due to deficiency of the SIA-cleaving enzyme NEU1. As in patients with sialidosis, the *Neu1*$^{-/-}$ mice develop severe atrophy, which is caused by progressive fibrosis of the connective tissue that eventually invades the myofibers, leading to their fragmentation and degeneration. We linked this fibrotic phenotype to exacerbated lysosomal exocytosis, a calcium-mediated process negatively regulated by NEU1. Increased exocytosis of lysosomal hydrolases and exosomes by *Neu1*$^{-/-}$ fibroblasts initiates a cascade of events ultimately affecting ECM integrity and tissue microenvironment. We demonstrate that *Neu1*$^{-/-}$ fibroblasts behave as myofibroblasts or mesenchymal cells, are proliferative, migratory and invasive, and have increased exocytosis of exosomes. Microarray and proteomic analyses of WT and *Neu1*$^{-/-}$ muscle and exosomes showed upregulation of the TGFβ and β-catenin/WNT signaling pathways, as well as of canonical markers of the epithelial-mesenchymal transition (EMT). Interestingly, human and murine WT fibroblasts can be educated to become proliferative and invasive, by culturing them in medium conditioned with exosomes derived from *Neu1*$^{-/-}$ myofibroblasts. Thus, these *Neu1*$^{-/-}$ microvesicles are loaded with signaling molecules which can induce and maintain an EMT phenotype. An ongoing EMT in the *Neu1*$^{-/-}$ connective tissue explains the increased proliferation of the *Neu1*$^{-/-}$ myofibroblasts and the abnormal expression and deposition of ECM components, leading to a full-blown fibrosis and ultimately to muscle degeneration.

Program Abstract #50

Locus colocalization is essential for the formation of the PAX3-FOXO1 fusion gene in alveolar rhabdomyosarcoma

Irina Lagutina[1], Virginia Valentine[1], Fabrizio Picchione[1], Frank Harwood[1], Marcus Valentine[1], Barbara Villarejo-Balcells[2], Jaime Carvajal[2,3], Gerard Grosveld[1]

[1]St Jude Children's Research Hospital, USA; [2]The Institute of Cancer Research, UK; [3]Centro Andaluz de Biologia del Desarrollo, Spain

Many recurrent chromosome translocations in cancer result in the generation of fusion genes that are directly implicated in the tumorigenic process. Precise modeling of the effects of cancer fusion genes in mice has been inaccurate as constructs of fusion genes often completely or partially lack the correct regulatory sequences. The reciprocal t(2;13)(q36.1;q14.1) in human alveolar rhabdomyosarcoma (A-RMS) creates a pathognomonic *PAX3-FOXO1* fusion gene. We first determined that in mouse fore limb myoblasts the *Pax3* and *Foxo1* loci physically co-localize in 10-20% of cells by using FISH. To reenact the translocation in mouse myoblasts we inverted a 4.5 Mb *Foxo1* gene including mouse/human syntenic region on mouse chromosome 3 in ES cells using *Cre* recombination. This allows for the formation of a productive *Pax3-Foxo1* fusion gene form a fusion chromosome carrying a single centromere. We used these ES cells to generate mice homozygous for the inverted 4.5Mb syntenic region. We used myoblasts of these mice to mimic the *PAX3-FOXO1* translocation in the human ARMS cell line RH30. We used targeted CRISPR-Cas9 nuclease-induced DNA double strand breaks in intron 7 of *Pax3* and intron 1 of *Foxo1* in sequences homologous to the breakpoints in RH30. This showed that spatial proximity of the *Pax3* and *Foxo1* loci in these myoblasts is a prerequisite for *Pax3-Foxo1* fusion gene formation as no chromosome translocations were generated in MEFs of these mice, which show no co-localization of the *Pax3* and *Foxo1* loci.

Program Abstract #51

Insights into the origins and pathogenesis of embryonal rhabdomyosarcoma

Catherine J. Drummond, Matthew R. Garcia, Daniel J. Devine, Jennifer Peters, Victoria Frohlich, David Finkelstein, Mark E. Hatley

St. Jude Children's Research Hospital, USA

Rhabdomyosarcoma (RMS) is the most common pediatric soft tissue sarcoma. Despite aggressive therapy, survival for RMS has not improved for three decades, emphasizing the need to uncover the molecular underpinnings of the disease. RMS includes two histolopathologic subtypes: alveolar RMS, driven by the fusion protein PAX3/7-FOXO1, and embryonal RMS (ERMS), which is genetically heterogeneous. RMS has been presumed to originate from derailed muscle progenitors based on the histologic appearance and gene expression pattern of the tumors. However, an origin restricted to skeletal muscle does not explain RMS occurring in tissues devoid of skeletal muscle such as the prostate, bladder, biliary tree and the omentum. Previously, we showed that activation of Sonic Hedgehog signaling through expression of a conditional, constitutively active Smoothened allele, *SmoM2*, under control of an adipocyte-restricted *adipose protein 2 (aP2)-Cre* recombinase transgene in mice gives rise to aggressive skeletal muscle tumors that display the histologic and molecular characteristics of human ERMS. We illustrated the transcriptome of the *aP2-Cre;SmoM2* tumors recapitulates both other mouse ERMS models as well as human ERMS. With the short latency and anatomic restricted tumor location, we sought to leverage this model to explore the cell of origin. Lineage tracing the *aP2-Cre* with reporter mice illustrated *aP2-Cre* expression in both brown and white adipose tissue as well as a discrete population of cells lying between skeletal muscle fibers. These aP2-lineage cells are distinct from Pax7-positive skeletal muscle stem cells or satellite cells. The aP2-lineage cells do not contribute to myotube formation. When compared to *aP2-Cre;R26-Tom* mice, the addition of oncogenic SmoM2 (*aP2-Cre;R26-Tom;SmoM2*) results in embryonic expansion of the aP2-lineage interstitial muscle cells. Our findings suggest that non-skeletal muscle progenitors are a potential cell of origin for Sonic Hedgehog-driven ERMS.

Program Abstract #52

Novel roles for EPH-RTKs in rhabdomyosarcoma

Veronica LaCombe, Morgan Seibert, DDW Cornelison

University of Missouri, United States

Rhabdomyosarcoma (RMS), the most common soft tissue cancer in children, develops to resemble immature skeletal muscle. It is an aggressive cancer with a 5-year survival rate of 33% if it has metastasized. Current treatment options involve surgery, chemotherapy and radiation, so there is a significant unmet need for targeted therapies. Eph receptors, the largest family of RTKs, have been implicated in development and progression of many other tumor types. Recent results from our group identified several Eph receptors expressed by primary muscle stem cells (satellite cells), which led us to screen for protein expression of Ephs in canine RMS primary tumors as well as mouse and human RMS cell lines. Intriguingly, we noted strong expression but abnormal localization of one Eph receptor: in multiple tumors from all three species, we detected EphA1 in the nucleus of interphase cells. Mislocalization of RTKs to the nucleus in other tumor types has been shown to promote cancer progression, with the classic case being ErbBs. No such activity had been described for Ephs, however, until last year when two other nuclear-localized Ephs were identified in tumor cells from

lung (EphA5, which affects the DNA damage response via protein-protein interactions) and prostate (EphB4, acting as a transcriptional regulator). Our current research is focused on determining whether nuclear EphA1 is a full-length protein or a truncated or cleavage product; how it is trafficked to the nucleus in RMS cells and when in tumorigenesis this first occurs; and whether it promotes tumorigenesis by interacting with nuclear proteins, nucleic acid, or both. These studies have the potential to identify novel avenues of therapy for this disease.

Program Abstract #53

Dietary flavonoids prevent ubiquitin ligases-mediated muscle wasting in tumor-bearing mice

Katsuya Hirasaka[1], Takeshi Nikawa[2], Yao Wang[1], Takayuki Uchida[2], Reiko Nakao[3], Shigeto Taniyama[1], Katsuyasu Tachibana[1]

[1]Graduate School of Fisheries and Environmental Sciences, Nagasaki University, Japan; [2]Department of Nutritional Physiology, Institute of Medical Nutrition, Tokushima University Medical School, Japan; [3]National Institute of Advanced Industrial Science and Technology, Japan

Proinflammatory cytokines contribute to the progression of muscle wasting caused by ubiquitin-proteasome-dependent proteolysis. We have previously demonstrated that isoflavones, such as genistein and daidzein, prevent tumor necrosis factor-α-induced muscle atrophy in C2C12 myotubes. In this study, we examined the effect of dietary flavonoids on the wasting of muscle. C57BL/6 mice were subcutaneously inoculated with 1×10^6 Lewis lung carcinoma cells. After tumor cell inoculation, mice were fed either a normal or an isoflavone diet containing 0.4% soya flavone for 3 weeks. There were no significant differences between normal and isoflavone diet groups in the intake of food or body weight. The wet weight and myofiber size of skeletal muscle in tumor-bearing mice significantly decreased, compared with that of vehicle-injected control mice. Interestingly, the wet weight and myofiber size of skeletal muscle in tumor-bearing mice fed the isoflavone diet were nearly the same as those in vehicle-injected control mice, although isoflavones supplementation did not affect the increased tumor mass and concentrations of proinflammatory cytokines, such as TNF-α and IL-6, in blood. Moreover, increased expression of muscle-specific ubiquitin ligase genes encoding MAFbx/Atrogin-1 and MuRF1 in skeletal muscle of tumor-bearing mice was inhibited by the intake of isoflavone. In parallel with the expression of muscle specific ubiquitin ligases, dietary isoflavone significantly suppressed phosphorylation of ERK in tumor-bearing mice. These results suggest that dietary isoflavone suppresses muscle wasting in tumor-bearing mice. Thus, isoflavone may be efficacious in a therapeutic approach against the wasting of muscle.

Program Abstract #54

Culture media for expansion and differentiation of human skeletal muscle myogenic progenitors

Robert Judson[1], Kimberly Feng[1], Crystal Chau[1], Arthur Sampaio[1], Terry Thomas[1], Allen Eaves[1,2], Stephen Szilvassy[1], Sharon Louis[1]

[1]STEMCELL Technologies Inc., Vancouver, BC, Canada; [2]Terry Fox Laboratory, BC Cancer Agency, Vancouver, BC, Canada

In vitro cultures of human myogenic progenitors (myoblasts) are useful tools for modelling human skeletal muscle biology and disease, and for drug screening. Following expansion, myoblasts are differentiated into multinucleated myotubes to recapitulate skeletal muscle formation *in vitro*. A common challenge in the field, however, is to ensure consistent culture conditions for optimal expansion of these undifferentiated cells. To address this issue, we developed two defined culture media that support consistent expansion and differentiation of myoblasts that can then be used for downstream applications. Commercially available cryopreserved myoblasts were seeded into T75 flasks (6.5x104 cells/cm2) and maintained for >6 passages. Net cell expansion was superior to two commercially available myogenic media. Quantitative PCR analysis showed that myoblasts grown in the new medium expressed low levels of differentiation markers *MYOGENIN* (9 ± 1 fold), *MYH1* (86 ± 5 fold) and *MCK* (331 ± 23 fold), and maintained expression of naïve progenitor markers *MYF5* and *PAX3* when compared to other commercial media. Flow cytometric analysis showed that these myoblasts were CD45/31-, CD29/56+, indicating that the new medium supports effective expansion of myoblasts that maintain a progenitor phenotype. For differentiation, expanded human myogenic progenitors were seeded onto plates coated with an extra-cellular matrix and upon reaching >90% confluency switched to differentiation medium for 5-8 days. Immunocytochemistry revealed that our differentiation media induced robust formation of MHC+ multinucleated myotubes with a fusion index of 65 ± 11%, comparable to competitor medium. In summary, we have developed two media that support human myogenic progenitor expansion and differentiation that complement *in vitro* modelling of muscle biology.

Program Abstract #55

Derivation of myogenic cells from human induced pluripotent stem (iPS) cells using a stirred bioreactor

Yuko Miyagoe-Suzuki

National Institute of Neuroscience, Japan

Transplantation of skeletal muscle stem cells/progenitors is one of the strategies to treat severe genetic muscle diseases. Human induced pluripotent stem cells (hiPSCs) are expected to be a cell source for muscle stem cells/progenitors,

because hiPSCs have nearly unlimited self-renewal capability *in vitro* and have the ability to differentiate into skeletal lineage *in vitro* and *in vivo.* To obtain skeletal muscle stem cells/progenitors from hiPSCs on a large scale, we modified a recently reported "EZ-sphere" method by introducing continuous, low frequent agitation of the culture medium with a magnetic stirrer. The modified EZ-sphere method supported robust growth of spheres and deviation of myogenic cells. qPCR and FACS analysis revealed that gene expression patterns and cell surface markers of myogenic cells derived from hiPSCs are similar to primary adult human myoblasts. Furthermore, M-Cadherin antibody together with CD56, CD271, CD57, CD108, EESA4 was useful to enrich iPSC-derived myogenic cells. Our results suggest that stirred suspension culture in combination with FACS-sorting is applicable to preparation of clinical-grade myogenic cells for cell therapy of muscular dystrophies.

Program Abstract #56

Precise genome editing in human induced pluripotent stem cell models of FKRP-deficient muscular dystrophy

Beatrice Lana[1], Jihee Kim[1], David Ryan[2], Evangelos Konstantinidis[1], Derek Stemple[2], Francesco Muntoni[3], Yung-Yao Lin[1]

[1]Blizard Institute, Queen Mary University of London, 4 Newark Street, London E1 2AT, UK; [2]Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SA, UK; [3]UCL Institute of Child Health, 30 Guilford Street, London WC1N 1EH, UK

Allelic mutations in the *FKRP* gene cause a wide range of muscular dystrophies associated with hypoglycosylation of alpha-dystroglycan, commonly referred to as dystroglycanopathies. The most severe forms are characterized by an early onset of muscle degeneration associated with central nervous system (CNS) involvement. Molecular and cellular mechanisms underlying FKRP-deficient muscular dystrophies are not well understood. Currently there is no cure or effective treatment. Patient-specific induced pluripotent stem cells (iPSCs) can be differentiated to neural and myogenic lineage cells to investigate pathological mechanisms. Nevertheless, one major challenge is the lack of appropriate isogenic control cells, which minimize the variability between different genetic backgrounds during analysis. We have successfully developed a protocol to carry out targeted gene correction in FKRP-deficient iPSCs and targeted gene mutation in wildtype iPSCs using CRISPR/Cas9-mediated genome editing technology. We show that CRISPR/Cas9 induced homologous recombination in combination with the *piggyBac* positive/negative selection cassette is a powerful and versatile technology, which allows precise modification of the mammalian genome at single base-pair levels without leaving footprints. Our isogenic pairs of human iPSC-derived cellular models will further elucidate mechanisms underlying the CNS involvement and muscle pathology due to FKRP deficiency. Moreover, we will exploit our unique cellular models for genetic and chemical screens aimed at identifying therapeutic targets in FKRP-deficient muscular dystrophies.

Program Abstract #57

Cytosolic Ca2+ dynamics through the SR is associated with pathology of muscular dystrophy

Jun Tanihata

Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Japan

[Background] Duchenne muscular dystrophy (DMD), the most common form of muscular dystrophy, is caused by lack of dystrophin. Exon skipping is a promising treatment for DMD and "exon 45–55 skipping" strategy is though to be one of the goals of this therapy, partly because Becker muscular dystrophy (BMD) patients with exon 45-55 in-frame deletion show very mild skeletal muscle symptoms. Moreover, this strategy theoretically rescue up to 63% of DMD patients with a deletion mutation. However the function of exon 45-55 deleted dystrophin is not well investigated. **[Methods]** We generated novel transgenic (Tg) mice expressing the exon 45-55 deleted human dystrophin. We, then, mated the Tg male mice with *mdx* female mice to obtain Tg/*mdx* male mice and extensively analyzed their phenotypes. **[Results and Discussion]** This Tg/*mdx* was found to express only the human truncated dystrophin, accompanied with recovery of dystrophin-glycoprotein complex at the muscle sarcolemma. The functional improvement of serum creatine kinase level, muscle pathology and specific tetanic force of Tg/*mdx*, to a level of the wild type were also confirmed. These results suggest that the truncated dystrophin with exon 45-55 deletion has an equivalent function to the full length dystrophin. On the other hand, neuronal NOS (nNOS) of Tg/*mdx* exists as an activated form and its localization was changed from sarcolemma to cytosol. We found that activated nNOS in Tg/*mdx* led to hyper-nitrosylation of the ryanodine receptor type-1 (RyR1) and subsequent constant Ca^{2+} release from sarcoplasmic reticulum (SR) to cytosol. These phenomena were also confirmed in *mdx*. However, the function of RyR1 stimulated by caffeine and SERCA were maintained in Tg/*mdx* and wild type but not in *mdx*. These results suggest that cytosolic Ca^{2+} dynamics through the SR is associated with dystrophic pathology and our Tg/*mdx* is useful model for analyzing pathogenesis of BMD.

Program Abstract #58

Integrative therapeutics: a novel approach to discover new treatments for muscle disease

Sahar Tavakoli[1], Leonard Zon[1,3,4], Amy Wagers[1,2,4]

[1]*Harvard Department of Stem Cell and Regenerative Biology, USA;* [2]*Joslin Diabetes Center, USA;* [3]*Boston Children's Hospital, USA;* [4]*Harvard Medical School, USA*
Duchenne muscular dystrophy (DMD) is a recessive X-linked muscular dystrophy caused by either a frame shift (60%) or point mutation (40%) in the dystrophin gene. This gene is the largest gene in nature with 2.4 Mb of DNA and 79 exons. Dystrophin is a link between the internal cytoskeletal system of myofibers and extracellular matrix structural proteins. Consequently, an unstable cell membrane causes an abnormal calcium homeostasis and degeneration of the muscle cell. A potential treatment for DMD is replacing the mutated gene. But, the low engraftment capacity of cultured myoblasts is a major stumbling block. Through this work, we aimed to develop an optimal pre-clinical procedure for transplanting engraftable skeletal muscle progenitors. We developed a method to find chemicals that stimulate muscle formation (myogenesis) using zebrafish embryos. This system has significant advantages for chemical screening, including the greater ease and lower cost of cell isolation, and the significantly more rapid *in vitro* development of zebrafish blastomere cells as compared to mammalian induced pluripotent stem cells (iPSCs) or adult muscle progenitors. Using this screening method we have identified bFGF is capable of expanding myogenic progenitors from fish, mouse and human in culture. We also identified a cocktail of three chemicals (forskolin, GSK3β inhibitor and FGF2) that specifies skeletal muscle production from human induced pluripotent stem cells (iPSCs). Treating the zebrafish embryonic stem cells with bFGF increased the engraftment capacity of newly differentiated muscle cells significantly in the adult recipient zebrafish. Similarly, the muscle-specified iPSC-derived cells could be transplanted into DMD model mice, and contributed to functional muscle fibers and muscle progenitors (satellite cells). Our cross-species approach suggests a new potential opportunity for therapy for DMD, using iPS-derived muscle progenitors for autologous or allogeneic cell transplant.

Program Abstract #59
six-family genes and mylpfa are required to maintain trunk fast muscle integrity
Jared Talbot[1], Emily Teets[1], Casie Jingle[1], David Langenau[2,3], Sharon Amacher[1]
[1]*The Ohio State University, Columbus, OH, USA;* [2]*Massachusetts General Hospital, Boston, MA, USA;* [3]*Harvard University, Boston, MA, USA*
Fast muscle fibers are particularly vulnerable in human Duchenne Muscular Dystrophy (DMD) patients. To better understand the dynamics of dystrophic degeneration, we used time-lapse imaging to follow fast and slow muscle fibers in zebrafish *dystrophin* (*dmd*) mutants with sub-cellular resolution. We find that degeneration is widespread among trunk fast fibers, first appearing as small sarcolemmal blisters which can sometimes be repaired, but sometimes proceed to fiber death. In contrast, trunk slow fibers are almost normal in *dmd* mutants; slow muscles appear distorted only when they are adjacent to large lesions in fast muscle. To find additional genes essential for fast muscle integrity, we created mutations in fast muscle-expressed candidates including the homeodomain transcription factors *six1a*, *six1b*, *six4a*, and *six4b*. Although *six1a;six1b;six4a;six4b* quadruple mutants initially have surprisingly normal trunk musculature, their trunk muscles deteriorate over time; *six*-mutant degeneration begins with fast muscle sarcolemmal defects, and within a week degeneration spreads to include slow fibers. A proposed target of Six proteins is the fast-muscle-specific myosin light chain gene, *mylpfa* (also called *mylz2*). Similar to the *six*-family quadruple mutant, we find that *mylpfa* mutant trunk muscle deteriorates within one week, suggesting that *six*-family genes may promote fast muscle integrity in part by activating structural genes like *mylpfa*. Gene requirements can differ in separate muscle groups. For instance migratory muscles are primarily fast-type, yet they are spared in *dmd* mutants for the first week, whereas in *mylpfa* mutants migratory muscles degenerate. In *six1a;six1b;six4a;six4b* mutants, migratory muscles fail to develop in the first place, precluding degeneration analysis. Altogether we find that like *dmd*, *mylpfa* and four *six*-family genes promote fast muscle integrity in zebrafish trunks, yet unlike *dmd*, *mylpfa* is essential for integrity of migratory muscles.

Program Abstract #60
Impaired satellite cells differentiation in children with cerebral palsy
Andrea Domenighetti[1,2], Margie Mathewson[3], Henry Chambers[3,4], Richard Lieber[1,2]
[1]*Rehabilitation Institute of Chicago, USA;* [2]*Northwestern University, Chicago, USA;* [3]*UC San Diego, USA;* [4]*Children's Hospital and Health Center, San Diego, USA*
Cerebral Palsy (CP) is a group of permanent disorders that are caused by a non-progressive malformation/insult to the developing brain. Development of muscle contractures and atrophy are among the most debilitating comorbidities that affect quality of life in patients with CP. Prevalence is estimated to be 2-3 cases/1000 births in US and Europe. We previously showed that muscle impairment was associated with reduced satellite cells number in children with CP. In this study, we tested the capacity of these cells to differentiate and to form multinucleated muscle fibers *in vitro*. NCAM-positive satellite cells (SC) were enzymatically isolated from semitendinosus muscle biopsies from CP and typically developing (TD) children [n= 8 patients/group]. We observed decreased fusion indexes and inhibited myotube formation in CP after *in vitro* exposure to low-serum conditions. These defects were associated with downregulation of multiple protein markers important for myoblast fusion and differentiation. In particular, we demonstrated a ~10-fold decrease in the Itgb1 gene expression and a ~75% downregulation of ITGB1D protein expression levels, associated with ~25% loss of integrin-mediated FAK kinase activity (phosphorylation) in early (4h) and late-stage (48h) differentiation. Analysis of

the Itgb1 promoter showed ~7-fold increase in DNA methylation in CP. Together, these data suggest that muscle defects may be associated with an intrinsic dysregulation of the SC pool in children with CP. Inhibited cell differentiation could be dependent on transmissible changes in gene expression associated with epigenetic modifications. Such ability loss to differentiate could lead to atrophy and contractures formation in CP.A better understanding of CP satellite cell dysfunction at the epigenetic and protein expression levels could lead to treatments that rescue and normalize their physiology, leading to improvement of muscle function in patients with CP.

Program Abstract #61

Effects of dietary soy protein on skeletal muscle volume and strength in humans with various physical activities

Takeshi Nikawa[1], Rie Hashimoto[1], Katsuya Hirasaka[2], Chiaki Yano[1], Kana Aibara[1], Ayako Ohno (Maita)[1], Kazuhito Akama[3]

[1]Department of Nutritional Physiology, Tokushima University Graduate School, Japan; [2]Graduate school of Fisheries Science and Environmental Studies, Nagasaki University, Japan; [3]Faculty of Life and Environmental Science, Shimane University, Japan

In recent years, the number of bedridden people is rapidly increasing due to aging or lack of exercise in Japan. This problem is becoming more serious. Many ubiquitin ligases, such as Muscle RING-Finger Protein-1 (MuRF-1) and Muscle atrophy F-box protein-1 (MAFbx-1)/atrogin-1, are associated with skeletal muscle atrophy. We also showed that Casitus B cell lymphoma-b (Cbl-b) ubiquitinated and degraded insulin receptor substrate-1 (IRS-1) in skeletal muscles during unloading conditions, suggesting that Cbl-b is one of enzymes associated with muscle atrophy. However, there is no effective drug or diet on such muscle atrophy in human. To conquer this problem, we found that the phosphorylated pentapeptide, DGpYMP, which named Cblin (Cbl-b inhibitor), inhibited Cbl-b-mediated degradation of IRS-1, resulting in suppression of denervation-mediated muscle atrophy in mice. We also showed that dietary soy glycinin, which contained the Cblin-like sequence, DIYNP, effectively Cbl-b mediated IRS-1 ubiquitination *in vitro*. Dietary soy glycinin protein also prevented denervation-induced muscle atrophy in mice by inhibiting the ubiquitination and degradation of IRS-1. Based on these findings, we demonstrate that soy is an effective protein source against muscle atrophy at least in rodent animals. In the present study, we designed to investigate whether dietary proteins, especially soy, had beneficial effects on skeletal muscle in volunteers with various physical activities. Soy protein supplementation tended to increase the volume and strength of quadriceps femoris muscle in participants with low physical activity and bedridden patients, compared with no supplementation or casein protein supplementation. Our results suggest that soy protein is a beneficial dietary source for human health under muscle proteolytic conditions, such as low physical activity and bedrest.

Program Abstract #62

GAA deficiency in Pompe disease is alleviated by exon inclusion in iPS cell-derived skeletal muscle cells

Erik van der Wal[1], Atze J. Bergsma[1], Joon Pijnenburg[1], Tom J.M. van Gestel[1], Holm Zaehres[2], Marcos J. Araúzo-Bravo[2], Hans R. Schöler[2], Ans T. van der Ploeg[1], WWM Pim Pijnappel[1]

[1]Erasmus Medical Center, The Netherlands; [2]Max Planck Institute for Molecular Biomedicine, Germany

Pompe disease is a monogenic disorder caused by mutations in the acid-alpha glucosidase (GAA) gene and leads to progressive skeletal muscle wasting. Current enzyme replacement therapy has disadvantages including incomplete efficacy, heterogeneous response, and very high costs. The common Caucasian c.-13-32T>G (IVS1) GAA mutation occurs in 90% of adults and 50% of children with Pompe disease. It causes exon 2 skipping during pre-mRNA splicing resulting in mRNA degradation, but also allows some residual wild type splicing. We hypothesized that the promotion of exon inclusion in IVS1 patients may restore wild type GAA expression and provide a basis for an alternative treatment option. To this end, a screen was performed using lentiviral-mediated U7 snRNA expression of antisense oligonucleotides. Hits were tested using PMO-based antisense oligonucleotides in patient-derived primary fibroblasts. In addition, patient-derived iPS cells were differentiated using a transgene free procedure to generate purified myotubes. The U7 snRNA screen resulted in a number of target sequences in the GAA pre-mRNA that showed enhancement of exon inclusion and GAA enzymatic activity when repressed by an antisense oligonucleotide. Two of these sequences enabled the design of PMO-based antisense oligonucleotides. These restored canonical GAA splicing: splice product-specific RT-qPCR showed increased expression of wild type GAA mRNA and a concomitant decreased expression of aberrantly spliced products. Importantly, the GAA enzymatic activity in Pompe patient-derived myotubes was enhanced to levels above the disease threshold. We anticipate that antisense oligonucleotide-mediated exon inclusion may be developed into an alternative treatment option for childhood/adult onset Pompe disease.

Program Abstract #63

The satellite cell paradox in Pompe disease: an increased regenerative potential combined with a failure to respond to disease-mediated damage

Gerben Schaaf, Tom van Gestel, Stijn in t Groen, Ans van der Ploeg, WWM Pim PIjnappel

Erasmus MC Dept of Pediatrics, The Netherlands

Background: Pompe disease is a metabolic myopathy caused by acid alpha glucosidase (GAA) deficiency. Patients

experience general muscle weakness affecting mobility and respiration. The progressive muscle wasting suggests that the regenerative response is insufficient. We recently reported that satellite cells were present at healthy levels in biopsies from Pompe patients, but failed to become activated in response to the damage. Actively regenerating eMyHC-positive myofibers were rarely detected. The **aim** of our current work is to identify the mechanism behind the failing regenerative response using the mouse model of Pompe disease. **Methods and results:** Muscles from GAA-knockout (GAAko) animals showed progressive lysosomal glycogen accumulation and associated myofiber damage. An increase of ~25 new central nucleated myofibers per week indicated a modest response to the damage. To investigate the functional capacity of GAA-deficient satellite cells TA muscles were injured with $BaCl_2$. The levels of Pax7-positive cells increased rapidly in GAA-deficient muscle and peaked earlier as compared to WT muscle (5 and 9 days after injury, respectively). A similar rapid increase in activated, Pax7+/Ki67+ satellite cells and eMyHC-expressing myofibers was observed in regenerating GAAko muscle. Isolated GAAko myofibers generated increased numbers and myogenic commitment of fiber-derived cells compared to isolated WT fibers. **Interpretation:** Our data demonstrate that GAA-deficient satellite cells are not functionally compromised and that the regenerative machinery is preserved in Pompe disease. The failure of the GAA-deficient satellite cells in human and mouse Pompe muscle to become activated during the natural course of disease suggest a failure in the generation of proper regeneration cues from the niche. The preservation of the satellite cell pool and function offers a venue for the development of novel treatment strategies aimed at activating endogenous satellite cells.

Program Abstract #64

Development of a CRISPR/Cas9-mediated gene editing platform to restore the reading frame for 60% of Duchenne muscular dystrophy patients

Courtney Young, Michael Hicks, Natalia Ermolova, Haruko Nakano, Majib Jan, Shahab Younesi, Atsushi Nakano, Stanley Nelson, Carrie Miceli, Melissa Spencer, April Pyle
University of California, Los Angeles, USA

Duchenne muscular dystrophy (DMD) is typically due to frame-shifting mutations in the DMD gene encoding dystrophin. Loss of dystrophin protein results in progressive muscle degeneration and premature death. Approximately 60% of DMD patients have out-of-frame mutations in a hotspot region within exons 45-55 in the rod domain of dystrophin. Genotype/phenotype assessments have revealed that in-frame deletion of exons 45-55 leads to the milder, allelic disease, Becker muscular dystrophy. This finding suggests that restoration of the reading frame by targeting exons 45-55 could treat ~60% of DMD patients to greatly reduce disease severity. We have developed a platform using clustered regularly interspaced short palindromic repeats (CRISPR) and- associated protein (Cas9) gene editing to achieve this purpose. We have utilized CRISPR/Cas9-mediated deletion and rejoining of up to 725kb to restore the reading frame in DMD human induced pluripotent stem cells (hiPSCs). This is the largest deletion shown to date in DMD. Clonal hiPSC lines containing the exon 45-55 deletion were differentiated to disease relevant types, such as cardiomyocytes and skeletal muscle myotubes, which had restored dystrophin protein. We demonstrated, for the first time, that the internally deleted dystrophin generated by CRISPR/Cas9 was functional and improved membrane integrity, reduced miR31 expression, and restored the dystrophin glycoprotein complex *in vitro* and after engraftment of skeletal muscle cells *in vivo*. This gene editing platform restores the reading frame for the majority of DMD patients and offers potential as an ex vivo correction for stem cell therapy or for use in muscle *in vivo*.

Program Abstract #65

Analyses of muscle diversification processes by cell specific approaches in *Drosophila*

Benjamin Bertin, Yoan Renaud, Krzysztof Jagla, Guillaume Junion
GreD, INSERM U1103, CNRS UMR6293, University of Clermont Ferrand, France

Cell diversification is an essential process for proper organism development. Understanding genetic and molecular mechanisms conferring the individual characteristics of each muscle during development remains a major challenge. Our current knowledge about the control of cell diversification is usually based on functional analyses of individual genes in whole embryo. However, this strategy is not adapted to define with high precision gene networks that control the acquisition of specific properties of muscle cells. For studying muscle diversification process we apply cell specific approaches and use them in *Drosophila* embryo model. Indeed, muscle network in *Drosophila* embryos represents an attractive system for studying cell diversification. It is composed of 30 muscle fibers and 6 stem cells (AMP cells) per hemisegment, which are easy to detect and to follow during development. Currently we took advantage from tested drivers to perform translational ribosome affinity purification (TRAP) on Slou, Lms and Duf positive muscle cells at 3 different time windows to isolate mRNA engaged in translation. The preliminary results from microarray analyses show enrichment of muscle specific genes. Furthermore biological process gene ontology showed that the majority of enriched genes are linked to targeted stages of muscle development. According to those results, TRAP method seems to be efficient to identify with high sensitivity transcriptional signatures in subsets of muscle cells. With the collected data we identified temporal and spatial signatures of genes controlling the acquisition of individual properties of muscles cells and perform functional analyses to establish their role in muscle diversification processes. This study should provide insights

into genes acting as realisators of muscle identity code and required for setting fusion programs, attachment or innervation of individual muscles

Program Abstract #66

The cellular pathways that direct myotube pathfinding

Brenna Clay, Aaron Johnson
University of Colorado, Denver, USA

Skeletal movement requires a tight physical interaction between myofibers and tendon cells. Compared to our understanding of myoblast cell fate specification and myoblast fusion, relatively little is known about the pathways that direct nascent myotubes to the correct tendon. We have taken a comprehensive approach to identify the molecules and mechanisms that regulate myotube pathfinding. By using forward genetics, expression screening, and live imaging in *Drosophila*, we have identified extracellular regulators and novel intracellular effectors that direct myotube pathfinding. Embryonic tendon cells express FGF8 orthologues and myotubes in *FGF8* mutant embryos failed to generate filopodia and migrate to tendon cells. FGFs are thus essential chemotactic signals during myogenesis. We have also identified a novel serine/threonine kinase that we named Out-of-step (Ofs). Similar to *FGF* mutants, *ofs* myotubes showed defects in filopodial dynamics and myotube migration. Our preliminary results suggest that Ofs regulates the intracellular response to multiple guidance signals. Lastly, we have made inroads toward understanding the actin regulatory pathways that direct filopodial dynamics during myotube pathfinding. Surprisingly, Tropomyosin works with actin polymerizing proteins, including WASP and ARP3, to regulate myotube pathfinding. Tropomyosin alleles are associated with several myopathies, and our results argue that myotube pathfinding defects contribute to the clinical phenotypes associated with congenital myopathies. Our approach has thus established a scaffold for understanding myotube pathfinding in which intracellular kinases transduce specific external signals to regulate actin-mediated filopodial dynamics.

Program Abstract #67

Understanding the role of the novel protein CG1674 in muscle structure and function in *Drosophila melanogaster*

Marilyn Cisneros, Richard Cripps
University of New Mexico, USA

Drosophila is used as a genetic tool that allows us to study muscle formation and structure to gain insight of the factors that induce muscle diseases in humans. Our current goal is to determine the role of the novel protein CG1674 in muscle formation, as well as the localization of this protein within the muscle cell. CG1674 was discovered through proteome sequencing of dissected flight muscles, suggesting that this protein is a functional component of the flight muscles of *Drosophila*. To determine the requirement of CG1674 in normal muscle formation, we expressed in the flight muscles an RNAi targeting the *CG1674* transcript. When crossing *UAS-CG1674 RNAi* with the drivers *1151Gal4, Mef2Gal4*, and *Act88FGal4* the flies are flightless. Immunohistochemical staining of the muscles revealed defects in myofibril formation and structure indicating that the presence of the CG1674 protein is required for sarcomere assembly. In parallel, we generated a CG1674-FLAG fusion protein to determine the localization of our protein within the muscle cell. Immunohistochemical staining of the flight muscles revealed that CG1674 is localized to the Z-discs of the sarcomeres. To further determine the importance of CG1674, we are creating a knockout using the CRISPR-Cas9 method. In addition, we are trying to determine if there are other proteins that interact with the CG1674 protein and affect its localization within the muscle cell. These studies identify a potentially novel muscle protein that is required for normal muscle assembly and function.

Program Abstract #68

Defining the regulatory network of fiber specific alternative splicing in *Drosophila* muscle development

Sandy Oas
University of New Mexico, United States

We have used adult fruit flies to understand the genetic mechanisms determining differentiation of somatic muscles into specific fiber types. *Drosophila* flight and jump muscles are distinct functionally and biochemically. This distinction is due to differentially expressed genes and differentially spliced mRNA transcripts unique to each muscle type. From our previous work, we showed that Aret is an important and novel alternative splicing regulator in the adult fly musculature. Endogenous Aret protein is localized to the nuclei of the flight muscles within the thorax, and promotes flight muscle patterns of alternative splicing. Without this regulator, the flies are flightless due to ultra-structural changes within the flight muscles resulting from changes in splicing patterns. Remarkably, ectopic expression of Aret in the jump muscle and in cultured cells promotes flight muscle specific splicing. This indicates Aret works autonomously as a regulator of alternative splicing in flight muscles. In search for interactive partners of Aret, we describe a potential role for the DEAD box RNA helicase, Vasa, in *Drosophila* muscle development. Aret and Vasa co-localize in the adult flight muscles, and Vasa knockdown flight muscles show defects in alternative splicing and in muscle structure. Additionally, knockdown of Vasa leads to cytoplasmic localization of the Aret protein. Vasa was previously shown to bind to Importin α2 (Kpna2)

which functions as a shuttle for proteins between the nucleus and cytoplasm (Yajima and Wessel 2015). We hypothesize that Vasa may function in a similar manner with Aret, by shuttling Aret from the cytoplasm back to the nucleus. Aret's mammalian ortholog, the Celf proteins, are implicated as regulators of alternative splicing, and improper dosages result in cardiomyopathies and muscular dystrophies. In contrast, the mammalian Vasa have not been implicated in muscle development, and this study suggests a novel role for Vasa in muscle development.

Program Abstract #69

Anchoring of the muscle nuclei to the plasma membrane is mediated by α2δ Amphiphysin-MSP300/Nesprin complex

Adriana Reuveny, Talila Volk
Weizmann Institute of Science, Israel

The nuclei in the multinucleated muscle fiber display a stereotypic morphology. Their appearance is uniform; they are flat and exhibit an oval shape. This morphology is tailored to the nucleus architecture for the accommodation of chromosomes, chromatin factors and the nuclear lamina. In order to maintain this stereotypic morphology the LINC complex (The linker of nucleoskeleton and cytoskeleton) connects the nucleus to cytoskeletal elements and performs diverse functions including nuclear positioning as well as mechanotransduction. In this work, we identified the muscle specific α2δ (Mα2δ), the L-type Ca2+ channel auxiliary subunit in *Drosophila* and reveal a novel developmental role for this protein in muscles. Our study demonstrates that α2δ forms a complex with MSP30/nesprin, a LINC complex member, and the BAR protein Amphiphysin that connects the nucleus to the plasma membrane. Our analysis demonstrates that Mα2δ is enriched in the plasma membrane above the nucleus, and it is required for proper positioning of the nuclei in the larval muscle fibers. Further analysis indicates that in *Mα2δ* mutant muscles the distribution of MSP300, a *Drosophila* homologue of mammalian Nesprin protein 1 and 2, is impaired at the outer nuclear level. Moreover, *Mα2δ* mutant muscles exhibit accumulation of MSP300-Kash containing isoforms in the ER. Mechanistically, we find that Mα2δ forms a protein complex with the BAR domain protein amphiphysin, shown recently to interact with N-WASP in promoting proper nuclear positioning in mammalian muscle fibers. Taken together we suggest that Mα2δ, positioned at the plasma membrane, forms a complex with amphiphysin and MSP-300/Nesprin thereby connecting the nucleus from above to the plasma membrane. The connection of the nucleus to the plasma membrane appears to be crucial for proper function of MSP300 in promoting position and spacing of the muscle nuclei.

Program Abstract #70

Differential usage of nuclear import pathways among myonuclei

Alicia Cutler, Anita Corbett, Grace Pavlath
Emory University, United States of America

Regional differences in transcription and protein expression within multinucleated myofibers are critical for proper muscle function. Proteins that regulate gene expression are synthesized in the cytoplasm and cross the nuclear envelope through the nuclear pore to access their targets in the nucleus. To enter the nucleus, proteins larger than 40 kDa must be bound to a nuclear transport receptor, which recognizes a nuclear localization signal (NLS) within the cargo protein. Transport then proceeds through distinct import pathways determined by the transport receptor. Regulation of which import pathways are active in individual nuclei could be one way of regulating differences in transcription of specific genes along the length of myofibers. To investigate whether heterogeneity exists in the usage of nuclear import pathways among nuclei within single muscle cells, we examined the activity of four well-defined pathways in primary mouse myotubes *in vitro*. By pairwise comparison using fluorescent reporters for each pathway in a microscopy-based import assay, we identified three subsets of nuclei: nuclei with more than one active import pathway, nuclei with one detectable active import pathway, and nuclei with no detectable import. The pathway which imports proteins containing a classical NLS (cNLS) was the most active pathway in these cells. To investigate when differences in protein import among myonuclei are established, we examined import of a cNLS reporter protein. We found that cNLS import was high in myoblasts, fell drastically in myocytes, and gradually increased again in myotubes. We are currently analyzing the correlation between active import pathways and transcriptional activity. We are also pursuing post-translational modifications of the nuclear pore as a mechanism to broadly regulate import pathway activity. Together, our results suggest that spatial and temporal regulation of distinct nuclear import pathways may be important in myofiber regionalization.

Program Abstract #71

Epigenetic mechanisms of histone deacetylase 1 and 2 regulate skeletal muscle differentiation

Hosouk Joung[1,2], Hyun-Ki Min[1,2], Gwang Hyeon Eom[1], Hyun Kook[1,2]
[1]Department of Pharmacology, Chonnam National University Mediacal School, Gwangju 501-746, South Korea; [2]Brain Korea 21 Project, Center for Biomedical Human Resources, Chonnam National University, Gwangju 501-746, South Korea

Epigenetics is defined as heritable information other than the DNA sequence itself. The concept implies that the

regulation of gene expression is a highly complex process in which epigenetics plays a major role that ranges from fine-tuning to permanent gene activation/deactivation. HDAC1 and 2 are highly homologous proteins that show redundant or specific roles in different cell types or in response to different stimuli and signaling pathways. As previous studies have demonstrated that HDAC1 and 2 are associated with the repressors of myogenesis. However, the molecular details of this dual regulation including post-translational modifications (PTMs) during myogenesis are largely unknown. C2C12 myoblasts were exposed to trichostatin A (TSA), one of the most potent HDACis, enhanced the expression of skeletal specific gene. TSA increased skeletal specific promoter activity. In TSA treated C2C12 myoblasts, HDAC1 was expressed 55- and 70-kDa protein and HDAC2 was degradated by ubiquitin-proteasome system, in contrast to other HDAC inhibitors. Treatment with 2-D08(SUMO inhibitor) inhibited a modification of HDAC1, while other inhibitors had no effect. *In vivo* SUMOylation assay, HDAC1 is modified by SUMO-1. In differentiation conditions, HDAC1 protein, but not HDAC2, was modified and HDAC1,2 activity was decreased. In differentiated C2C12 myoblasts, MyoD-HDAC1 interactions were significantly attenuated, whereas MyoD binds to HDAC2 in differentiation conditions. The protein amount of HDAC1 and 2 was significantly increased in denervated muscles. Taken together, our results show that HDAC1 and 2 played a major role in skeletal myoblast cells and seem to have important roles in epigenetic regulation.

Program Abstract #72

PKN2 and Cdo interact to activate AKT and promote myoblast differentiation

Sang-Jin Lee[1], Jong-Sun Kang[2], Gyu-Un Bae[1]

[1]Research Center for Cell Fate Control, College of Pharmacy, Sookmyung Women's University, Republic of Korea; [2]Department of Molecular Cell Biology, Sungkyunkwan University School of Medicine, Samsung Biomedical Research Institute, Republic of Korea

Skeletal myogenesis is coordinated by multiple signaling pathways that control cell adhesion/migration, survival and differentiation accompanied by muscle specific gene expression. A cell surface protein Cdo is involved in cell contact-mediated promyogenic signals through activation of p38MAPK and AKT. Protein kinase C-related kinase 2 (PKN2/PRK2) is implicated in regulation of various biological processes, including cell migration, adhesion and death. It has been shown to interact with and inhibit AKT thereby inducing cell death. This led us to investigate the role of PKN2 in skeletal myogenesis and the crosstalk between PKN2 and Cdo. Like Cdo, the expression of PKN2 was elevated in C2C12 myoblasts during differentiation and decreased in cells with Cdo depletion caused by shRNA or cultured on integrin-independent substratum. This decline of PKN2 levels resulted in diminished AKT activation during myoblast differentiation. Consistently, PKN2 overexpression enhanced C2C12 myoblast differentiation, whereas PKN2-depletion impaired it, without affecting cell survival. PKN2 formed complexes with Cdo, APPL1 and AKT via its C-terminal region and this interaction appeared to be important for induction of AKT activity as well as myoblast differentiation. Furthermore, PKN2 enhanced MyoD-responsive reporter activities by mediating the recruitment of BAF60c and MyoD to the Myogenin promoter. Taken together, PKN2 plays a critical role in cell adhesion-mediated AKT activation during myoblast differentiation.

Program Abstract #73

Loss of HuR in striated muscle mediates changes in muscle fibers type composition and oxidative capacity

Brenda Janice Sanchez[1], Patricia L. Hallauer[3], Erzsebet Kovacs[2], Derek Hall[1], Jennifer F. Ma[1], Sergio Di Marco[1], Imed-Eddine Gallouzi[1]

[1]Dept. of Biochemistry McGill University, Canada; [2]Rosalind & Morris Goodman Cancer Research Centre, Canada; [3]Montreal Neurological Institute, Canada

The Human antigen R (HuR) protein, a member of the ELAV family of RNA-binding proteins (RBPs), has been highly implicated in the process of muscle fiber formation (Myogenesis). Indeed, our *in vitro* studies have demonstrated that the HuR mediated post-transcriptional regulation of key myogenic factors such as MyoD, Myogenin, HMGB1 and p21, is essential to ensure the proper formation of muscle fibers. The physiological relevance of these findings, however, has yet to be determined. In this study, to address the *in vivo* role of HuR in myogenesis, we used the Cre-LoxP system to generated HuR skeletal muscle-specific knockout mice (MyoD Cre^{+};$Elav1^{fl/fl}$). To our surprise, the ablation of HuR in skeletal muscle showed no clearly visible phenotype. Thoroughly characterization of their muscle tissue, however, showed a reduced muscle mass with decreased cross-sectional area. Furthermore, our histological assessment, indicate that the knockout of HuR in skeletal muscle promotes a shift in the composition of muscle fiber types towards an oxidative phenotype. RNA-seq and Western blot analyzes exhibited an increase in Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) levels in our HuR muscle-specific knockout mice when compared to control littermates. Given the importance of PGC-1α in muscle biology, and its potential role in the metabolic basis of disease, we are currently delineating the relationship between HuR deficiency, PGC-1α and muscle fiber type alterations as well as its repercussions in muscle physiology.

Program Abstract #74

Spatiotemporal control of BAI3 signaling during myoblast fusion

Noumeira Hamoud[1,3], Ariane Pelletier[1], Artur Kania[1], Michel Bouvier[2,3], Jean-Francois Cote[1,3]
[1]Institut de Recherches Cliniques de Montréal (IRCM), Canada; [2]Institut de Recherches en Immunologie et Cancérologie (IRIC), Canada; [3]Université de Montréal, Canada
Fusion of myoblasts during embryonic myogenesis, or of satellite cell-derived myoblasts during regeneration, is crucial to the formation and maintenance of muscle fibers. The signaling involved in myoblast fusion is poorly defined. We began to uncover the important players when we identified Dock1 as the first molecule required for fusion in mice. Studies on Myomaker and the GPCR BAI3 recently revealed the first cell surface proteins essential for myoblast fusion. Here, we aim to identify the ligands that act on BAI3 and to define the mechanistic details of how heterotrimeric G-Proteins and ELMO/DOCK1 operate downstream of this GPCR to promote fusion. We initially focused on C1q-Like proteins, the only known ligands of BAI3. Profiling experiments revealed that C1qL4 is expressed in muscle progenitors and in the neural tube in developing chick embryos. Surprisingly, depletion of C1qL4 in C2C12 enhanced, while exogenous C1qL4 blocked, myoblast fusion. Expression of C1qL4 in chick muscle progenitors *in vivo*, but not of a C1qL4 mutant defective in BAI3-binding, impaired myoblast fusion. Hence, we propose that C1qL4 acts in a spatiotemporal manner to repress BAI3 activity prior to the exact timing when fusion is to be deployed. We also provide evidence for the existence of a ligand of BAI3 promoting fusion and we have identified a number of novel candidate BAI3 ligands that are now being characterized. To define downstream signaling, we performed a BRET screen to identify if BAI3 activates canonical G-proteins. This work uncovered that BAI3 mediates a specific activation of $G\alpha_i$. We also determined that the BAI3-mediated activation of heterotrimeric G-proteins contributes to recruit the ELMO/DOCK complex to the membrane, suggesting for a two-step recruitment mechanism. Collectively, these studies are revealing the layers of BAI3 regulation, which could ultimately be exploited to hijack the activity of this GPCR for regenerative purposes.

Program Abstract #75
Investigating microRNA-target interactions in skeletal muscle during chick development
Camille Viaut, Andrea Münsterberg
School of Biological Sciences, University of East Anglia, Norwich, NR4 7TJ, United Kingdom
MicroRNAs (miRNAs), short non-coding RNAs acting post-transcriptionally, are involved in the regulation of gene expression. Of widespread significance, they have been implicated in many biological processes during development and disease, including muscle disease. The myomirs are miRNAs highly enriched in striated muscles, including skeletal muscles in developing somites. With the recent advances in sequencing technology and bioinformatics, new miRNAs expressed in somites have been identified. For many of these miRNAs specific roles, in particular during myogenesis, have not yet been determined. We aim to better understand interactions between miR-128 and one of its predicted targets, Eya4, specifically during skeletal muscle development in chick. To determine miRNA expression patterns, chick embryos were collected at different stages of development, fixed and LNA in situ hybridisations (ISH) were performed. Putative miRNA target genes were identified using miRBase and TargetScan databases, and GO term analysis allowed short-listing of candidates. For selected genes expression patterns were determined using RNA in situ hybridisation. To generate sensor constructs, 3'UTR regions were cloned downstream of the luciferase reporter gene, mutants were generated in putative miRNA binding sites, and both constructs were used in luciferase assays. Validated *in vitro* miRNA-target interactions were tested *in vivo* by performing loss-of-function experiments followed by in situ hybridisation for genes of interest and muscle markers. LNA ISH revealed that miR-128 is expressed in the myotome. RNA ISH indicated a strong expression of Eya4 in the myotome. Luciferase assays showed that miR-128 can interact with Eya4 3'UTR leading to a decrease in luciferase activity, which is rescued by mutating the miR-128 target site. Inhibition of miR-128 in developing somites by antagomir injection results in altered Eya4 expression and affects myogenesis.

Program Abstract #76
IGF regulation of limb muscle development
Rabeea Mohammed, Helen Anderton, Dylan Sweetman
University of Nottingham, UK
Insulin like growth factors are well characterised regulators of limb muscle growth and can regulate both myoblast proliferation and differentiation. However the question of how these signals can promote both these activities in the same cells remains open. To address this we have used the developing chicken embryo limb as a model. During limb development myogenic precursors delaminate from the ventrolateral somite and migrate into the limb. In the limb they form two tissues blocks, the dorsal and ventral muscle masses and only then begin to express muscle specific markers such as the MRFs. The expression of these muscle markers follows a stereotypical and well defined pattern making limb muscles an excellent system to determine how myogenic regulators operate *in vivo*. We have grafted beads soaked in IGF-I or IGF-II into developing chicken limbs and determined their effects on myogenesis. Both IGFs induce Pax3 expression and cell proliferation but also induce MyoD expression. To understand this paradoxical result we have combined growth factor soaked beads with various small molecule inhibitors. IGF-IR and MEK inhibitors block both Pax3 and MyoD induction; however FGFR and PI3K inhibitors specifically block induction of MyoD by IGFs. We

propose a model where IGF signaling through MEK directly increases myoblast proliferation and Pax3 expression while affecting MyoD expression indirectly through induction of FGF

Program Abstract #77
Vezatin, an AChR-associated protein, is essential for myogenic differentiation.
Natasha Permaul, Matthew Friese, Thomas Neubert, Steven Burden
Skirball Institute of Biomolecular Medicine, New York University Medical School, United States
Vezatin is an integral membrane protein that is associated with cell adhesions junctions and the actin cytoskeleton. By the use of SILAC, Stable Isotope Labeling with Amino Acids in Culture, vezatin has been identified as an AChR-associated protein. Here we show that vezatin associates with AChR complexes and is enriched at neuromuscular synapses. We used a combination of myogenic regulatory factors, Myf5 and MyoD, to drive cre recombinase mediated ablation of vezatin in skeletal muscle. This strategy demonstrates an increased efficacy over the use of Myf5-Cre/+ alone. Loss of vezatin in skeletal muscle causes postnatal lethality. While formation of AChR clusters are defective, this lethality is due to a large reduction of myofiber number and possible degeneration of remaining myofibers suggesting that vezatin may have two roles in skeletal muscle. One that is important in myogenic differentiation and another important for proper post-synaptic differentiation. Because vezatin has been shown to interact with beta-catenin at adhesions junction and beta-catenin associates with AChRs, we assayed the consequences, using the same genetic approach, of muscle specific deletion of beta-catenin. In the absence of beta-catenin there is again a striking loss of muscle fibers resulting in regions of complete myofiber loss in dorsal and medial portions of diaphragm muscle. Taken together, vezatin is important during embryonic myogenesis and may act through an interaction with beta-catenin.

Program Abstract #78
Abl2 kinase regulates myoblast fusion
Jennifer Lee
Skirball Institute of Biomolecular Medicine, NYU Medical Center, USA
Formation of skeletal muscle requires differentiation of muscle precursor cells that ultimately fuse to form multinucleated muscle fibers. Myoblast fusion is essential for muscle development but the signaling pathways remain largely unknown. Abl2 is a non-receptor tyrosine kinase that is ubiquitously expressed during development. We find that *abl2* null mice form strikingly elongated muscle fibers in several hypaxial muscle groups. Mutant muscle fibers that are dissociated from end to end possess an increased number of nuclei, suggesting that lengthened muscle results from an increase in myoblast fusion during embryonic development. Importantly, *abl2* is required cell autonomously in myoblasts to regulate myoblast fusion since muscle-specific deletion of a*bl2* display elongated muscles that are identical to those of *abl2* null mice. Unique from other non-receptor tyrosine kinases, Abl2 contains two F-actin binding domains and a microtubule binding domain. We hypothesize that Abl2 may be a kinase that regulates cytoskeletal rearrangements that are necessary for myoblasts to fuse and form multinucleated muscle fibers.

Program Abstract #79
The muscle-less jerboa foot as a novel system to understand muscle degeneration
Mai Tran
UC, San Diego, USA
Since the first terrestrial vertebrates crawled out of water 350 million years ago, natural selection has shaped limbs in extraordinary ways for diverse functions. Within the rodents, the ancestral quadrupedal “mouse-like” animals have forelimbs and hindlimbs with muscles in hands and feet that enable grasping and climbing. In contrast, the three-toed jerboa (*Jaculus jaculus*), a close relative of laboratory mouse, can no longer climb and instead has dramatically elongated lever-like hindlimbs as an adaptation to leap bipedally through open desert habitats. The jerboa has all of the muscles in the thigh and calf but entirely lacks muscles distal to the ankle, possibly because the presence of unnecessary muscles in a non-grasping limb would contribute to weight that may hinder redirection of limbs in a forward stride. Immunohistochemical staining of jerboa foot sections shows that newborn jerboas have fully differentiated muscles, suggesting that muscle migration and patterning are normal in early development. By postnatal day 5, the muscles are less prominent, but TUNEL staining shows that apoptosis is not involved in this muscle cell loss. Instead, non-aligned sarcomeres and areas of disorganized muscle filaments in electron micrographs suggest that these muscles degenerate. How are muscles specifically lost in the jerboa foot while more proximal muscles are maintained? We are currently investigating if activation of non-apoptotic cell death pathways like autophagy or necroptosis in the foot could provide a potential mechanism for this process. We are also assessing the organization of core sarcomeric proteins over time to track the trajectory of maturation and disassembly during the foot muscle degeneration. The muscle-less jerboa foot serves as a model to understand the cellular processes involved in muscle maturation and degeneration and shed light on the dynamic developmental processes that sculpt the musculoskeletal system.

Program Abstract #80

The emergence of vertebrate head muscle stem cells

Julia Meireles Nogueira[1,2], Katarzyna Hawrot[1], Colin Sharpe[1], Anna Noble[1], William M. Wood[3], Erika C. Jorge[2], David J. Goldhamer[3], Gabrielle Kardon[4], Susanne Dietrich[1]

[1]University of Portsmouth, UK; [2]Universidade Federal de Minas Gerais, Brazil; [3]University of Connecticut, USA; [4]University of Utah, USA

Past research established that trunk and head myogenesis follow different programs, and even in the adult, the associated muscle stem cells are quite distinct. To shed light on the development of head muscle stem cells and to understand their special properties, we traced the emergence of these cells in the key vertebrate models for myogenesis, chicken, mouse, frog and zebrafish. We show that the mechanism of head muscle stem cell deployment is the same in all models and quite different from the trunk. Head muscle stem cells do not have a history of Pax3/Pax7 expression. Instead, they have a history of MyoD expression, and Pax7 expression emerges de-novo. The cells are being deployed late, and well after the head mesoderm committed to myogenesis and initiated differentiation. We propose that this unique mechanism of muscle stem cell development is a legacy of the evolutionary history of the chordate head mesoderm.

Program Abstract #81

Evolution and development of muscles in the head/trunk interface of vertebrates

Rie Kusakabe, Shigeru Kuratani

Evolutionary Morphology Laboratory, RIKEN, Japan

The head/trunk boundary of vertebrates is associated with the distinction of muscle morphology that differs between head and trunk. In crown vertebrates, an additional category of skeletal muscles arises in the interface, generally recognized as cervical muscles. Cyclostomes (i.e., lampreys and hagfishes) do not have a clear counterpart of the cervical muscles nor the paired fin muscles. In jawed vertebrates, many of skeletal muscles in the neck or shoulder developmentally originate in the anterior somites, similarly to the muscles in the fore- and hindlimbs. During embryogenesis, the crescent region surrounding posterior portion of the pharynx (also called the circumpharyngeal region) serves as the paths through which the muscle precursor cells extend and/or migrate distally into the sites of differentiation. In the circumpharyngeal region also occurs the interactions of mesenchymal cells with different origins (neural crest cells, cranial mesoderm and lateral plate mesoderm) that give rise to the pharyngeal muscles and skeletons, the shoulder/limb structure, and the heart. We have reported that, in the lamprey, the hypobranchial muscle derives from ventral portions of rostral somites and develops in the circumpharyngeal region, a developmental process shared with the tongue muscles of jawed vertebrates. The comparable process is also observed in the hypopharyngeal muscles of the elasmobranchs, of which the detailed myogenetic pathway has yet to be clarified. Comparison of expression of developmental markers has delineated the temporal order of differentiation of various muscular tissues, such as the hypobranchial, posterior pharyngeal and cucullaris (trapezius) muscles as well as the myocardium. These analyses will shed new light on the evolutionary history of skeletal/cardiac musculature and illustrate how it contributed to the complex vertebrate morphology.

Program Abstract #82

Understanding skeletal myogenesis from early human embryos to enhance skeletal muscle progenitor cell specification from human pluripotent stem cells

Haibin Xi[1], Wakana Fujiwara[1], Simone Liebscher[2], Ben Van Handel[3], Katja Schenke-Layland[1,2,4], April Pyle[1]

[1]University of California Los Angeles, USA; [2]Eberhard Karls University Tübingen, Germany; [3]CarthroniX Inc., USA; [4]Fraunhofer Institute for Interfacial Engineering and Biotechnology, Germany

Skeletal muscle progenitor cells (SMPCs) derived from human pluripotent stem cells (hPSCs) are promising sources for regenerative medicine in treating muscle wasting disorders including muscular dystrophies and sarcopenia. In the past few years, major breakthroughs in generating hPSC-derived SMPCs have been achieved by mimicking the developmental cues critical for early myogenic mesoderm specification in model organisms. However, there are likely differences between myogenesis in model organism and human development. Using early human embryos at somitogenesis stages (Carnegie stage (CS) 13-14; week 4-5), we have discovered novel pathway(s) which can be modulated to enhance *in vitro* specification of hPSCs toward the fate of somite, the common ancestor of most skeletal muscles. During early myogenesis in model organisms, a population of myogenic cells within the developing somites at limb bud levels migrates to give rise to limb muscles. Thus, to better understand this migratory population, we extended our studies to human embryos at early stages of limb bud development (CS 16; week 6). We found PAX3 but not PAX7 positive cells in the hindlimb bud (HLB), which might represent the migrating myogenic populations. In order to isolate and characterize these potential migratory SMPCs, we performed immunofluorescent staining of PAX3 in combination of surface markers reportedly to label mouse or human SMPCs. Preliminarily, we found that nearly all PAX3+ cells are also CMET+ and *in vitro* myogenesis is observed in the CMET positively but not negatively sorted cells. In the future, we will refine surface markers to allow better purification and characterization of the migratory SMPCs. Moreover, we will evaluate developed limb buds where myogenic cells have completed migration and turn on PAX7 expression. These

studies will shed light on human myogenesis as well as generate useful information on deriving optimal SMPC populations from hPSCs for cell-based therapies.

Program Abstract #83
Role of p53 during muscle stem cells activation
Francesca Boscolo Sesillo, Alessandra Sacco
Sanford Burnham Prebys Medical Discovery Institute, United States
Skeletal muscle stem cells (MuSCs) are responsible for the homeostatic maintenance and tissue repair of skeletal muscle. In healthy adult muscles MuSCs reside in quiescent state and upon injury they are activated, start proliferating and give rise to committed progenitors that differentiate and fuse to repair damaged myofibers. However, in chronic degenerative conditions the ability of MuSCs to sustain repair is progressively decreased. Significant effort has been recently devoted to understand the mechanisms associated with the functional exhaustion of MuSCs in these conditions to identify novel targets for therapeutic purposes. We aim to define how p53, a tumor suppressor protein implicated in different processes in adult stem cells compartments, influences MuSCs behavior. Utilizing knockout animals we showed that p53 is not required for skeletal muscle development. However, upon injury p53 null muscles exhibit a delay in regeneration. This is characterized by a dramatic reduction in MuSCs number early after injury, and a decrease in myofibers cross-sectional area at late time points. This finding suggests that p53 is implicated in the regulation of MuSCs activation. Therefore we further investigated the specific role of p53 in this process. Through FACS, we first isolated MuSCs from injured mice. We observed increased expression of MyoD correlated to reduction of Pax7 mRNA levels in p53 KO MuSCs. This indicates that lack of p53 induces premature commitment. Consistently, analysis of MuSCs on single myofibers cultures shows a significant reduction of Pax7 positive cells associated to an increase in MyoD proliferating cells. These results are consistent with the loss of MuSCs we observed *in vivo* after injury. We hypothesize that p53 is involved in maintaining a timely activation of MuSCs balancing self-renewal and progression through the myogenic lineage. We are now investigating p53 downstream effectors to identify new targets for develop innovative therapies.

Program Abstract #84
Regulating the satellite cell population proportional to the severity of a muscle injury
Adam Cadwallader, Tiffany Antwine, Bradley Olwin
University of Colorado Boulder, USA
Skeletal muscle tissue is repaired and maintained for the lifetime of mammalian organisms by the adult muscle stem cell or satellite cell (SCs). When injured, the quiescent SCs responds by sequentially "activating," entering the cell cycle, proliferating as progenitors and then undergoing terminal differentiation and fusion to repair damaged myofibers. When regeneration is completed, SC numbers are restored to pre-injury levels by an as yet unidentified mechanism. We have identified a cell-cell interaction between Jam2 expressing SCs and Jam3 expressing cells that appear between 3 days and 7 days following muscle injury. The number of Jam3 expressing cells directly correlates with the severity of a muscle injury, where injection of Jam3 expressing cells into injured muscle increases satellite cell numbers and reduces muscle fiber size. Jam 3 expressing cells interact with SCs, polarize Jam2 and in turn the Par complex, which regulates asymmetric division in satellite cells. Thus we propose that a cell non-autonomous response to muscle injury communicates the degree of damage directly to the SC population, scaling the numbers of SCs appropriately to repopulate the stem cell pool.

Program Abstract #85
Deciphering the functional interactions between distinct subpopulations of Fibro-Adipogenic Progenitors and Satellite Cells
Usue Etxaniz[1], Barbora Malecova[1], Sole Gatto[1], Pier Lorenzo Puri[1,2]
[1]*Development, Aging and Regeneration Program, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA, USA;* [2]*IRCCS Fondazione Santa Lucia, Rome, Italy*
While muscle satellite cells (SCs) have been established as the main cell type contributing to muscle regeneration, recent evidence supports that mesenchymal fibro-adipogenic progenitors (FAPs), among other several cell types, provide an important source of signals that regulate SC activity.
FAPs are located in skeletal muscle interstitium and contribute to both compensatory regeneration and fibro-adipogenic degeneration of muscles in Duchenne Muscular Dystrophy (DMD). During acute injury, FAPs promote muscle regeneration by releasing pro-myogenic paracrine factors. In contrast, in conditions favoring degeneration, such as those induced by chronic damage and muscular dystrophies, FAPs are the major drivers of fibrotic scarring and intramuscular fatty infiltration. This disease stage-dependent activity indicates an intrinsic heterogeneity of FAPs. By using transcriptome analysis at the single cell level, we have identified three distinct subpopulations of FAPs that display specific gene co-expression patterns, exhibit distinct phenotypes and are dynamically regulated in response to regeneration cues. We are currently investigating the differential ability of each of these subpopulations to regulate SC

activity by combining several assays designed to study cell-to-cell interactions, such as the culture of myofibers in FAPs conditioned media, co-culture experiments of isolated SC and FAPs, and co-transplantation experiments.

Program Abstract #86
Integration of inflammation and myogenesis during skeletal muscle repair
Cherie Alissa Lynch
Arizona State University, USA
Efficient tissue repair in response to acute muscle injury is dependent on the innate immune system for clearance of necrotic cells as well as promoting the production of new muscle fibers. Our lab has previously identified the transcription factor Mohawk (Mkx) as an important regulator of muscle differentiation. Preliminary studies performed in adult Mkx deficient mice reveal a failure to efficiently activate an inflammatory response following acute skeletal muscle damage, as measured by clearance of necrotic muscle fibers and expression of key chemokines and cytokines. In culture, we found that the inflammasome could be activated in muscle cells, however this response was dampened in the absence of Mkx. Recently, our lab has demonstrated a deficit in the macrophage subpopulations involved in muscle repair and an increased susceptibility to infection in Mkx deficient mice. These studies reveal a role for muscle in sensing damage and identify Mkx as a key regulator of muscle repair and the innate immune response.

Program Abstract #87
Lysyl oxidase is required for muscle regeneration
Ravit Gabay, Shelly Zaffryar-Eilot, Peleg Hasson
The Rappaport Faculty of Medicine and Research Institute, Technion – Israel Institute of Technology, Israel
The muscular system is a mosaic tissue composed of different components such as myofibers, fibroblasts, extracellular matrix (ECM) and satellite cells (SC). Dynamic homeostasis between these components is essential for normal development and function of the muscle. Following muscle injury, remodeling of the injured tissue takes place and feedback mechanisms ensure that the amount and organization of the myofibers, fibroblasts, ECM and SC returns to normal. The critical role of maintaining this homeostatic balance is largely observed in various myopathies, such as Duchenne Muscular Dystrophy (DMD), in which a perturbation to the balance and organization of muscle components occurs, leading to muscle dysfunction. Among the molecules involved in obtaining this homeostatic balance are the extracellular matrix remodeling enzyme Lysyl Oxidase (Lox) and the TGF-β pathway, a key signaling pathway involved in normal but also in pathological muscle regeneration and in the DMD-related fibrosis. Notably, work from our lab has demonstrated that during embryogenesis Lox modulates TGF-β signaling in the muscles. Nevertheless, careful examination of Lox activities during muscle regeneration and in DMD have not been carried out. To identify Lox contribution to muscle regeneration, we examined its expression following muscle injury (achieved by injecting cardiotoxin to the right gastrocnemius of mice). We find that during regeneration Lox is up-regulated not only in the ECM, but also in regenerating myofibers, satellite cells and in muscle connective tissue fibroblast. In addition, *in vitro* experiments demonstrate that SC are unable to differentiate in the absence of Lox activity. These results suggest that Lox plays a role in muscle regeneration. Current work is aimed at identifying the mechanisms and processes underlying Lox activity during muscle regeneration.

Program Abstract #88
Effects of ßcatenin activation in FAPs on skeletal muscle homeostasis
Marcela Low, Andrew Wu, Elena Groppa, Jack Yuan, Fabio Rossi
The Biomedical Research Centre, University of British Columbia, Canada
Fibrosis impairs tissue function, homeostasis, and regeneration and interferes with therapeutic strategies. Yet, little is known about the mechanisms underlying these effects. Our goal is identify pathways involved in the development and maintenance of fibrosis. RNA sorted FAPs from mdx and $CCR2^{KO}$ mice subjected to acute damage, which triggers transient matrix production or chronic damage, which leads to fibrosis, were subjected to next generation sequencing. B-catenin targets such as Sfrp4, a soluble inhibitor of WNTs and Axin2, were highly upregulated in FAPs from chronically damaged tissue, suggesting that the Wnt pathway is active in FAPs from a fibrogenic environment. To mimic these conditions, we bred mice a CRE-ERT2 cDNA specifically in FAPs and an allele of *ctnnb1* in which exon 3 (Gsk3 phosphorylation sites) is floxed, and whose excision results in b-catenin constitutive activation.FAPs sorted from tamoxifen-induced mice showed upregulation of b-catenin target genes as expected. Concomitantly, we observed deposition of matrix in the interstitial space in the absence of tissue damage and a dramatic drop in the cross sectional area of myofibers in affected muscle. ddPCR analysis revealed the upregulation of a number of autophagy related genes such as FoxO, MURF-1 and Atrogin following tam treatment. To provide an initial assessment of possible changes in the muscle vasculature in our model, we harvested animals one and eight weeks after tam treatment and quantified vessel numbers in muscle sections. Interestingly, we found that the number of CD31 positive structures significantly increased over time. Our results strongly suggest that changes related to interstitial fibrosis and taking place in FAPs have a

profound effect on tissue function. This reveals the so far unrecognized importance of FAPs in modulating muscle function during homeostasis.

Program Abstract #89

Cellular dynamics of regeneration reveals role of two distinct Pax7 stem cell populations in larval zebrafish muscle repair

Tapan Pipalia[1], Jana Koth[1,2], Shukolpa Roy[1], Christina Hammond[1,3], Koichi Kawakami[4], Simon Hughes[1]
[1]King's College London, UK; [2]Oxford University, UK; [3]University of Bristol, UK; [4]National Institute of Genetics and SOKENDAI, Japan

Heterogeneity of stem cells or their niches is likely to influence tissue regeneration. Here we reveal stem/precursor cell diversity during wound repair in larval zebrafish somitic body. In skeletal muscle with incision wounds a swift immune response is followed by an increase in cells at the wound site, many of which express the muscle stem cell marker Pax7. $Pax7^+$ cells proliferate and then undergo terminal differentiation involving Myogenin accumulation and subsequent loss of Pax7 followed by elongation and fusion to repair fast muscle fibres. Analysis of *pax7a* and *pax7b* transgenic reporter fish reveals that cells expressing each of the duplicated *pax7* genes are distinctly localized in un-injured larvae. Cells marked by *pax7a* only or by both *pax7a* and *pax7b* enter the wound rapidly and contribute to muscle wound repair, but each behaves differently. Low numbers of *pax7a*-only cells form nascent fibres. Time-lapse microscopy revealed that the more numerous *Pax7b*-marked cells frequently fuse to pre-existing fibres, contributing more strongly than *pax7a*-only cells to repair of damaged fibres. *Pax7b*-marked cells are more often present in rows of aligned cells that are observed to fuse into a single fibre, but more rarely contribute to nascent regenerated fibres. Ablation of a substantial portion of nitroreductase-expressing *pax7b* cells with metronidazole prior to wounding triggered rapid *pax7a*-only cell accumulation, but this neither inhibited nor augmented *pax7a*-only cell derived myogenesis and thus reduced wound healing. Moreover, *pax7a*-only cells did not regenerate *pax7b* cells, suggesting a lineage distinction. We propose a modified founder cell/fusion competent cell model in which *pax7a*-only cells initiate fibre formation and *pax7b* cells contribute to fibre growth. This novel cellular complexity in muscle wound repair raises the possibility that distinct populations of myogenic cells contribute differentially to repair in other vertebrates

Program Abstract #90

Study of the multiple functions of Nfix in skeletal muscle regeneration: a focus on macrophage biology

Marielle Saclier[1], Giuliana Rossi[1], Stefania Antonini[1], Stefania Monteverde[1], Chiara Bonfanti[1], Rémi Mounier[2], Bénédicte Chazaud[2], Graziella Messina[1]
[1]Department of Biosciences, University of Milan, Via Celoria 26, 20133 Milan, Italy; [2]CGPhyMC, CNRS UMR5534, Villeurbanne; and Université Claude Bernard Lyon1, Villeurbanne, France

Muscle development and skeletal muscle regeneration are processes which required specific and synchronized controlled steps. In the laboratory it has been shown that the transcription factor Nfix is necessary for their complete and successful execution. During muscle development Nfix is expressed by embryonic myoblasts and regulates the switch from embryonic to fetal myogenesis by acting on specific myogenic genes repression and activation. During muscle regeneration, Nfix is expressed by satellite cells and Nfix KO mice display delayed regeneration due to a defect of satellite cells differentiation. It was observed that not only satellite cells but also macrophages (MPs) express Nfix. We focus this work on Nfix expression and function in MPs during skeletal muscle regeneration. *In vitro*, we observed more Nfix positive macrophages in BMDM alternatively activated MPs. We then confirmed this data *in vivo*: we observed more Nfix positive MPs at the later stages of skeletal muscle regeneration. More precisely, Ly6C+ pro-inflammatory MPs exhibit the same level of positive cells while the percentage of Ly6C- anti-inflammatory MPs Nfix positive cells always increases over the time of regeneration. As Nfix is differently expressed by MPs depending on their phenotype, we tried to understand if Nfix could be involved in the acquisition of MPs phenotype. Using shRNA infection on BMDMs, we observed that Nfix silencing leads to a defect of anti-inflammatory phenotype acquisition and maintain of pro-inflammatory phenotype upon M2 polarization. Satellite cells also express Nfix, thus we are now working on specific deleted Nfix mice in MPs: LySM-Cre:Nfix flox mice. We will look at the progression of skeletal muscle regeneration and the trophic functions of Nfix null MPs on myogenesis processes as proliferation and differentiation of myogenic cells. With this work we will look at the specific function of Nfix in MPs during skeletal muscle regeneration.

Program Abstract #91

Regulator of G Protein Signaling-12 (RGS12) in satellite cell-mediated skeletal muscle regeneration

Adam Schroer, Junaith Mohamed, Vincent Setola, Stephen Alway, David Siderovski
West Virginia University, United States

Regulators of G Protein Signaling (RGS proteins) are a family of proteins that negatively regulate G protein-coupled receptors, acting to catalyze the hydrolysis of GTP by G-alpha subunits. Some RGS proteins are endowed with other signaling features, and RGS12 is one such protein with five additional, functional domains: a PDZ domain, a phosphotyrosine-binding domain, two tandem Ras-binding domains, and a G-alpha-binding GoLoco motif. RGS12

expression is temporo-spatially regulated in developing mouse embryos, with strong expression in somites and developing skeletal muscle. We therefore hypothesized that RGS12 is involved in coordinating signaling during myogenesis and/or skeletal muscle regeneration. We have found that RGS12 is expressed in adult tibialis anterior and is increased three days following cardiotoxin-induced injury, supporting a role in skeletal muscle regeneration. Consistent with its coordination of myogenic programming signals, RGS12 is expressed in primary myoblasts. Further, as primary myoblasts undergo differentiation and fusion into myotubes, RGS12 protein abundance is reduced. Myoblasts isolated from mice lacking *Rgs12* have an impaired ability to differentiate into myotubes compared with myoblasts isolated from wild-type mice, suggesting that RGS12 may play a role in myogenic signaling as a "go/no-go" modulator/switch for differentiation. We next assessed the regenerative capacity of mice conditionally lacking *Rgs12* from PAX7-expressing cells following cardiotoxin-induced damage. Analyses revealed that eight days post-damage, mice conditionally lacking RGS12 had attenuated repair of muscle fibers as compared to wild-type mice. These data support the hypothesis that RGS12 coordinates signaling of myogenic programming and suggests RGS12, and the signaling network it regulates, may serve as a therapeutic target that can be applied to pharmacologic and/or cell-based strategies for ameliorating syndromes of muscle weakness and muscle wasting.

Program Abstract #92

MMP-13 is required for efficient myoblast migration and muscle regeneration in a mouse skeletal muscle

Lucas Smith[1], Boshi Zhang[2], Du Chung[2], Bryan Wheeler[2], Hanqin Lei[3], Elisabeth Barton[3]
[1]Department of Medicine, University of Pennsylvania, United States; [2]Department of Anatomy and Cell Biology, University of Pennsylvania, United States; [3]Department of Applied Physiology and Kinesiology, University of Florida, United States

Skeletal muscle regeneration requires coordinated remodeling of the extracellular matrix (ECM), with matrix metalloproteinases (MMP)s playing a critical role. MMP-13, a collagenase, was shown to have high expression during regeneration and regulate C2C12 myoblast migration, yet MMP-13's function in skeletal muscle *in vivo* is has not been studied. Under baseline conditions the *MMP13*$^{-/-}$ mouse does not exhibit a muscle phenotype; including muscle mechanics, fiber size, capillary density, or ECM area. However, in cardiotoxin injured muscle regeneration is delayed at 2 weeks. Regenerating *MMP13*$^{-/-}$ mice have 25% smaller peripherally nucleated fibers, 44% reduced capillary density, and 0.81% of IGG+ indicating fiber degeneration, which was absent in wildtype muscle. However, the phenotype *in vivo* may be muted by a 5.8 fold overexpression of *MMP2*. Crossing the *MMP13*$^{-/-}$ mouse onto the mdx line produced a mouse with chronic muscle degeneration and regeneration cycles. Surprisingly, there was no significant difference in either functional parameters or histological features of *MMP13*$^{-/-}$ x *mdx* compared to *mdx*; save for the recurring presence of 1.8% IGG+ fibers. To determine if these effects were mediated by satellite cell deficiencies when lacking MMP-13, live cell imaging of single fiber cultures was conducted to directly measure migration velocity. *MMP13*$^{-/-}$ satellite cells had 33% lower migration velocity than controls. To test migration in 3D, invasion assays through basement membrane extract (BME) or collagen were used. *MMP13*$^{-/-}$ cells had a 53% reduced ability to migrate through BME substrate while neither cell type migrated through collagen. These data extend evidence of MMP-13 being a critical component of myoblast migration in primary cells in 2D and 3D. The results support a role for this migration defect slowing *in vivo* regeneration and increasing degeneration in acutely and chronically regenerating skeletal muscle of mice lacking MMP-13.

Program Abstract #93

Hereditary myasthenic syndromes: new genes and better treatment

David Beeson, Judith Cossins, Kate Belaya, Pedro Rodriguez-Cruz, Jacqueline Palace
University of Oxford, UK

The congenital myasthenic syndromes (CMS) are hereditary disorders of neuromuscular transmission. The number of cases recognised, at around 1:100,000 in the UK, is increasing with improved diagnosis. The advent of next-generation sequencing has facilitated the discovery of many genes that harbour CMS-associated mutations, and to date at least 22 have been identified. We have identified an emerging group of CMS, characterised by a limb-girdle pattern of muscle weakness which are caused by mutations in genes that encode proteins involved in the initial steps of the N-linked glycosylation pathway. Surprisingly although this pathway occurs in all mammalian cells, symptoms in our patients are largely restricted to neuromuscular transmission which suggests that the neuromuscular junction is particularly sensitive to defects in biochemical pathways affecting glycosylation. We also report a syndrome due to mutations in *COL13A1* which encodes an extracellular matrix protein that is concentrated at the neuromuscular junction and highlights the role of these proteins in maintaining synaptic stability that is independent of the AGRN/MUSK clustering pathway. Knowledge of the neuromuscular synapse and the different proteins involved in maintaining its structure as well as function has now enabled us to tailor treatments to the underlying pathogenic mechanisms that can often be life-transforming for the patients. Examples of these transformational treatments will be given if requested.

Program Abstract #94

The muscle-derived Wnt receptor Frizzled-9 regulates the morphology and activity of the post-natal

neuromuscular junction

Jorge Ojeda[1,2], Francisca Bermedo-García[1,2], Rocío Tejero[3], Mario López[3], Lucía Tabares[3], Juan Pablo Henríquez[1,2]
[1]Universidad de Concepción, CHILE; [2]Millenium Nucleus in Regenerative Biology, CHILE; [3]Universidad de Sevilla, ESPAÑA

We have previously shown that the muscle Wnt receptor Frizzled-9 (Fzd9) inhibits agrin-induced acetylcholine receptor (AChR) clustering in cultured myotubes. To analyze the potential *in vivo* role of Fzd9 at the neuromuscular junction (NMJ), we first studied its endogenous distribution and that of the Wnt pathway effector beta-catenin throughout the postnatal remodeling occurring during NMJ maturation in whole-mounted mouse Levator auris longus (LAL) muscles. Immunohistochemical co-localization studies by confocal z-stacks and 3D reconstructions showed that Fzd9 distributes in postsynaptic AChR rich-areas from P0 to P28. In turn, beta-catenin displays pre- and postsynaptic distribution; in regions devoid of AChRs at early stages and apposed to AChR rich-areas as NMJ maturation proceeds. *In vivo* electroporation of P21 LAL muscles showed that exogenous HA-tagged Fzd9 displays a distribution pattern similar to the endogenous receptor. Remarkably, when analyzed at P42, NMJs of Fzd9-overexpressing muscles showed a significant increase in the proportion of multiperforated postsynaptic shapes, which also display increased area and perimeter than controls, suggesting that NMJ maintenance is compromised. Electrophysiological postsynaptic recording of *ex vivo* LAL preparations showed a significantly depressed synaptic transmission in Fzd9-expressing fibers, evidenced by reduced spontaneous and evoked (0.5Hz, 300sec) end-plate potentials, as well as lower input resistance than controls. In turn, high stimulation (100Hz, 1sec) experiments showed that synaptic plasticity was not affected by the postsynaptic expression of Fzd9. Together, our findings suggest that activation of Wnt signaling through Fzd9 alters the normal distribution of AChRs, which is required to maintain functional postsynaptic apparatuses at the NMJ. (Funded by FONDECYT 1130321 and MINREB RC120003, Chile).

Program Abstract #95

Preloading induced stress relaxation and membrane current changes in denervation intact smooth muscle strip

Xia Li
Hai Nan Medical College, Hai Nan Island China

Objective In this study, we use force sensor and BL-420S recording system, observed denervation intact smooth muscle passive tension pattern under the several preload conditions. Glass microelectrodes method was used to measure the membrane electric current changes during high preload in aorta smooth muscle strip samples.Methods Denervation intact smooth muscle preparations were from Kunming mice aorta and urinary bladder wall. Smooth muscle preparations were fixed on a micro positioning device and the first stretch for inducing a passive tension up to 1 gram, this position was determined as the initial length (L0). Intermittently stretch and increasing sample length 10 steps, 50μm for each step. Recording and analyze the preload L0+1 and L0+10 induced passive tension myograph. The spontaneous tension during relaxation that included myogenic contraction and relaxation amplitude, stress relaxation phase and tension strain time (TST) were evaluated. Membrane current changes in each tension step were evaluated by glass microelectrodes and MultiClamp 700B Amplifier and pClamp 10 analyzer software. The membrane current changes after L0+1 and L0+10 were analyzed. Results 1) Stretch induced preload increasing induced a shortening phase of stress relaxation. High preload conditions induced detrusor relaxation phase were shorter than aorta. 2) Membrane current significantly increasing in high preload steps. 3) High calcium concentration (3% CaCl2) was relative to the enlargement of spontaneous contraction amplitude and frequency.Conclusions Increasing of preload in smooth muscle preparations induced a directly decreasing of preparation compliance with an enlargement of myogenic spontaneous contraction amplitude in BL-420s recording system. Membrane current were significantly enhanced in spontaneous contraction period, nevertheless L-type calcium channel blockade suppressed the enhancement of the amplitude and frequency.

Program Abstract #96

MiRNAs as regulators of metabolism in skeletal muscle fibers

Francesco Chemello[1], Stefano Cagnin[1,2,5], Francesca Grespi[1], Alessandra Zulian[3], Pasqua Cancellara[3], Etienne Hebert-Chatelain[1], Paolo Martini[1], Camilla Bean[1], Paolo Laveder[1], Enrico Alessio[1], Paolo Bernardi[3], Chiara Romualdi[1], Carlo Reggiani[3], Luca Scorrano[1,4], Gerolamo Lanfranchi[1,2,5]
[1]Department of Biology, University of Padova, Italy; [2]CRIBI Biotechnology Centre, University of Padova, Italy; [3]Department of Biomedical Sciences, University of Padova, Italy; [4]Venetian Institute of Molecular Medicine, Italy; [5]CIR-Myo Interdipartimental Research Center of Myology, University of Padova, Italy

Skeletal muscle is the most abundant tissue of the body and one of the major players in regulating systemic energy metabolism. It consists of different cell types, but the motor units are myofibers that possess different contractile and metabolic properties. Slow-twitch myofibers are rich in mitochondria and prefer fatty acids as substrate for ATP production, whereas fast-twitch myofibers have a low oxidative capacity and prefer glucose for energy production. Expression profiles, performed at the unprecedented level of individual isolated myofibers, has allowed the transcriptional classification of hindlimb mouse myofibers in 3 groups (slow, intermediate, and fast) and the identification of a complete myofiber-specific transcript catalogue. In addition, we proposed a limited set of transcriptional markers useful for a rapid

and unambiguous classification of myofibers in one of the 3 groups. Using this novel transcriptional classification, we investigated also the differential expression of miRNAs in single fibers. The integration of mRNA and miRNA expression data at single cell level enabled us to describe fiber-specific regulative transcriptional networks and to discover the role of specific miRNAs in the modulation of myofiber metabolism. In particular, we focused our attention on two miRNAs that could modulate myofiber metabolism. Results indicate that over-expression of either miRNAs induces mitochondrial fragmentation with a consequential deficiency in maximal mitochondrial respiration. However, they exhibit opposite effects on fatty acid metabolism: one promotes the utilization of fatty acids as energy substrate, while the other inhibits it, leading to an accumulation of lipid droplets in the cells. These findings demonstrate the importance of miRNAs to regulate metabolism at single fiber level in accordance with their metabolic properties and plastic changes of muscle physiology and could also have relevant impact on treatment of metabolic disorders.

Program Abstract #97

Regulation of muscle fiber-type differentiation by Pbx homeodomain proteins

Gist H. Farr III[1], Nathan Johnson[1], Lisa Maves[1,2]

[1]Seattle Children's Research Institute, USA; [2]University of Washington, USA

Vertebrate skeletal muscles are composed of slow- and fast-twitch fiber types. How the differentiation of distinct fiber types is activated during embryogenesis is not well characterized. Skeletal muscle differentiation is initiated by the myogenic bHLH transcription factors MYF5, MYOD1, MYF6 and MYOG. MYOD1 functions as a muscle master regulatory factor and directly activates both slow and fast muscle differentiation genes. We previously showed that PBX TALE-class homeodomain proteins bind with MYOD1 on the promoter of the zebrafish fast muscle gene *mylpfa* and are required for proper activation of *mylpfa* expression and the fast-twitch muscle-specific differentiation program in zebrafish embryos. However, the requirements for PBX proteins in mammalian skeletal muscle differentiation have not been previously addressed. We have examined the requirements for *Pbx* genes in embryonic mammalian skeletal muscle differentiation. Removing *Pbx1* function from skeletal muscle, in *Myf5$^{Cre/+}$;Pbx1$^{fl/fl}$* mouse embryos, has little effect on embryonic muscle development. However, *Myf5$^{Cre/+}$;Pbx1$^{fl/fl}$;Pbx2$^{-/-}$* mouse embryos show delayed activation and reduced expression of fast muscle differentiation genes, similar to our studies of Pbx function in zebrafish. The mouse *Pbx1/Pbx2*-dependent fast muscle genes closely match those that have been previously shown to be dependent on mouse *Six1* and *Six4*. We are using our zebrafish model to demonstrate synergy between Myod, Pbx, and Six factors in fast muscle differentiation. Additionally, our combined zebrafish and mouse work identifies new factors potentially involved in fast muscle differentiation and also reveals roles for Pbx proteins in repressing non-muscle differentiation fates. Taken together, our studies demonstrate conserved requirements for PBX factors in skeletal muscle differentiation. Our studies are revealing how PBX homeodomain proteins act with other transcriptional regulators to direct specific cellular differentiation pathways.

Program Abstract #98

Pervasive recruitment of myogenic factors by REST/NRSF transcriptional complexes repress non-myogenic lineage programs during myogenic differentiation

Vahab Soleimani[1], Hang Yin[3], Hong Ming[2], Zenghui Wu[1], Florian Bentzinger[2], Marie-Claude Sincennes[2], David Wilson[2], Michael Rudnicki[2]

[1]McGill University, Canada; [2]University of Ottawa, Canada; [3]University of Georgia, USA

Sequential transcriptional waves orchestrated by the temporal induction of the myogenic factors transition muscle progenitors through the myogenic differentiation program. The role of myogenic factors in activation of myogenic gene regulatory networks is extensively studied. However, their role in the repression of the alternative pathways to commit muscle cells to the myogenic program is largely unknown. Using gene expression analysis and genome wide occupancy of myogenic factors and by analysis of key histone marks, we have found that RE1 Silencing Transcription Factor (REST) recruits Myocyte Enhancer Factor 2A (Mef2a) and Myogenin to repress a vast repertoire of non-muscle genes during myogenic differentiation. Deployment of the myogenic factors in the REST complex leads to the recruitment of the SWI/SNF chromatin remodeling factor SMARCA2 (BRM) to the non-muscle genes, stabilizes REST complex on chromatin and leads to complete gene repression. Removal of myogenic factors by RNAi leads to re-acetylation of REST targets and causes leaky expression of non-muscle genes. Taken together, our data shows that in addition to activating the myogenic transcriptional network, myogenic factors play an unexpected role in suppression of non-muscle lineage genes during muscle cell differentiation.

Program Abstract #99

Skeletal muscle control of systemic metabolism: a role for Transcription Factor E-B (TFEB) signaling

Constanza Cortes[1,2]

[1]University of California San Diego, USA; [2]Sanford Consortium for Regenerative Medicine, USA

Skeletal muscle is the most abundant tissue in the human body and a major site for metabolic activity and energy homeostasis. Furthermore, skeletal muscle is a key organ in glucose metabolism and is an active endocrine tissue, serving

as both a target and a source of insulin signaling peptides and other 'myokines'. Transcription factor E-B (TFEB), a master regulator of cellular catabolism and energetics, is highly induced in skeletal muscle during starvation, but its function in muscle physiology remains largely unknown. To understand TFEB function in skeletal muscle, we generated transgenic mice carrying floxed-STOP Flag-TFEB, allowing for skeletal muscle-specific expression of TFEB after crossing with a Human-Skeletal-Actin (HSA)-Cre driver. We observed specific TFEB target gene induction and enhanced autophagy signaling in skeletal muscle from bigenic mice with no evidence of muscle atrophy or degeneration. Interestingly, fxTFEB-HSA-Cre mice weigh more than non-transgenic littermates, and display higher levels of insulin signaling pathway genes. Bigenic fxTFEB-HSA-Cre skeletal muscle has increased glycogen storage, and TFEB knock-out cells have impaired phosphorylation of key insulin signaling pathway components. Additionally, TFEB muscle also has higher mitochondrial content and activity, with no alterations in PGC-1α levels. This suggests a novel pathway for TFEB-modulation of mitochondrial function and glucose metabolism in muscle, both of which are currently under exploration. Importantly, TFEB overexpression in skeletal muscle also protected against proteostatic and metabolic age-related phenotypes. Our findings thus reveal a novel role for TFEB in regulating insulin response, glycogen storage, and mitochondrial function in skeletal muscle, suggesting that modulation of TFEB in skeletal muscle may have important effects in systemic metabolism and aging.

Program Abstract #100

Prmt7 deficiency causes reduced skeletal muscle oxidative metabolism and age-related obesity

Hyeon-Ju Jeong, Jong-Sun Kang
Sungkyunkwan University School of Medicine, Republic of Korea

Maintenance of skeletal muscle function is critical for metabolic health and the disruption of which exacerbates many chronic diseases such as obesity and diabetes. Skeletal muscle responds to exercise or metabolic demands by a fiber-type switch regulated by signaling-transcription networks that remains to be fully defined. Here, we report that protein arginine methyltransferase 7 (Prmt7) is a key regulator for skeletal muscle oxidative metabolism. Prmt7 is expressed with the highest levels in skeletal muscle and decreased in skeletal muscles with age or obesity. *Prmt7*$^{-/-}$ muscles exhibit decreased oxidative metabolism with decreased expression of genes involved in muscle oxidative metabolism, including PGC-1a. Consistently, *Prmt7*$^{-/-}$ mice exhibited significantly reduced endurance exercise capacities. Furthermore, *Prmt7*$^{-/-}$ mice exhibit decreased energy expenditure, which might contribute to the exacerbated age-related obesity of *Prmt7*$^{-/-}$ mice. Similarly to *Prmt7*$^{-/-}$ muscles, Prmt7 depletion in myoblasts also reduces PGC-1a expression and PGC-1a-promoter driven reporter activities. Prmt7 regulates PGC-1a expression through interaction with and activation of p38MAPK which in turn activates ATF2, an upstream transcriptional activator for PGC-1a. Taken together, Prmt7 is a novel regulator for muscle oxidative metabolism via activation of p38MAPK/ATF2/PGC-1a.

Program Abstract #101

Improvement of muscle strength and regenerative capacity in aged mice through application of a novel PGC-1a inducer

Hye-Been Kim, Hyeon-Ju Jeong, Jong-Sun Kang
Sungkyunkwan University School of Medicine, Republic of Korea

Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1a) plays essential roles in mitochondrial biogenesis and oxidative muscle metabolism. Reduced PGC-1a levels are linked to skeletal muscle weakness in aging or pathological conditions, while muscle-specific overexpression of PGC-1a results in a protective effect against aging-related muscle weakness. Thus we have performed a high-throughput screening for PGC-1a inducer and identified several compound. In this study, we report the beneficial effect of a newly identified PGC-1a inducer EX2 in muscle metabolism and function. EX2 treatment elevates the expression of PGC-1a isoforms including PGC-1 [illegible]4i, EX2 and enhances muscle mass, oxidative muscle capacity and muscle strength in young and aged mice. Consistently, the gene expression profile of EX2-treated aged muscle had a signature similar to that of control young muscles. Furthermore the muscle regenerative capacity improved greatly in EX2-treated muscles after repeated cardiotoxin injuries. In summary, these results indicate that EX2 mimics the effects of exercise which has a potential application to prevent muscle weakness related to aging or pathological conditions.

Program Abstract #102

Maintenance of a robust neuromuscular system as a novel strategy to protect skeletal muscle from sarcopenia

Alice Pannérec, Margherita Springer, Jérôme Feige
Nestlé Institute of Health Sciences, Switzerland

Declining muscle mass and function is one of the main drivers of loss of independence in the elderly. Sarcopenia, the age-related loss of skeletal muscle mass and function, is associated with numerous cellular and endocrine perturbations, and it remains challenging to identify those changes that play a causal role and could serve as targets for therapeutic intervention. Using a rat model of natural aging, we have found a differential susceptibility of certain muscles to age-related decline as aging rats specifically lose muscle mass and function in the hindlimbs, but not in the forelimbs. By

performing a comprehensive comparative analysis of these muscles, we demonstrate that regional susceptibility to sarcopenia is dependent on neuromuscular junction fragmentation, loss of motoneuron innervation, and reduced excitability. Remarkably, muscle loss in elderly humans also differs in vastus lateralis and tibialis anterior muscles in direct relation to neuromuscular dysfunction, suggesting that maintenance of the neuromuscular system is key for healthy muscle aging. We further found that circulating levels of one neurotrophic factor are decreasing with age in rats. *In vitro* analysis using a nerve/muscle co-culture assay revealed that this neurotrophic factor protects the neuromuscular system from damage, suggesting that maintaining proper circulating levels with advancing age would have beneficial effect on muscle. Indeed, when rats were treated with this specific factor at the onset of sarcopenia we observe a protective effect on muscle mass. Taken together, our results demonstrate that maintenance of the neuromuscular system is key to protect from sarcopenia and we provide evidence for the role of one neurotrophic factor in this process.

Program Abstract #103

Extracellular ATP as a mediator of musculoskeletal homeostasis at the masticatory system

Manuel Arias-Calderón[1], Camilo Morales[1], Julián Balanta[1], Carolina Beato[1], Fernán Gómez[1], Nathalie Vicencio[1], Carolina Verdejo[1], John López[1], Nadia Hernández[1], Paola Llanos[1], Mariana Casas[2], Sonja Buvinic[1]

[1]Institute for Research in Dental Sciences, Faculty of Dentistry, Universidad de Chile, Chile; [2]ICBM, Faculty of Medicine, Universidad de Chile, Chile

Molecular basis of muscle-bone crosstalk is an unsolved issue in the whole musculoskeletal system, but specially in head and neck tissues, that are embryological and biochemically different than the trunk and limbs ones. In hind leg muscles we have previously demonstrated that extracellular ATP is a relevant mediator between membrane depolarization and gene expression. ATP is also known as a bone remodeling regulator. The aim of the present work is to assess the expression of the purinergic signaling components at the mouse masticatory system, and the role of extracellular ATP as a signaling molecule in muscle-bone crosstalk. In masseter and digastric muscles, mandible or maxilla from BalbC mouse, mRNA for purinergic receptors (P2Y, P2X and adenosine receptor) was detected. The components of a multiprotein complex related to excitation-transcription coupling, that we previously described in flexor digitorum brevis muscle, were also expressed in masseter muscle (dihydropyridine receptor, pannexin 1 and $P2Y_2$ receptor). Tetanic electrical stimulation of masseter muscle evoked ATP release, with 2.5 fold maximal increase 1min after stimulus. Exogenous 100 µM ATP regulated the expression pattern of interleukin6, troponinI fast/slow, PGC1alpha and citrate synthase in mouse masseter muscle. Conditioned medium derived from masseter muscle (MST-CM) resembled the effect of 1 µM ATP in osteoclastogenesis of the pre-osteoclast RAW264.7 cell line. Differentiation to a giant multinucleated phenotype with increased expression of osteoclastogenic markers (tartrate resistant acid phosphatase, cathepsinK, metalloprotease9, carbonic anhydrase and lysosomal ATPase) was observed. We demonstrated that masticatory musculoskeletal system have a functional purinergic signaling pathway, placing ATP as a putative mediator for muscle plasticity and muscle-bone crosstalk.

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Program Abstract #104

Mechanical and molecular signals underlying tendon cell differentiation

Ludovic Gaut[1,2,3,4,5], Marie-Ange Bonnin[1,2,3], Nicolas Robert[1,2,3], Mathias Mericskay[3,4,5], Delphine Duprez[1,2,3]

[1]CNRS UMR 7622, IBPS-Developmental Biology Laboratory, F-75005, Paris, France; [2]Inserm U1156, F-75005 Paris, France; [3]Sorbonne Universités, UPMC Univ Paris 06, IBPS, F-75005 Paris, France; [4]CNRS UMR 8256, IBPS-Biological Adaptation and Ageing Laboratory, F-75005, Paris, France; [5]Inserm U1164, F-75005 Paris, France

Tendons are unique forms of connective tissue of the musculoskeletal system. They are composed of a dense extracellular matrix of type I collagen fibrils that are hierarchically organized to withstand tensile forces transmitted from muscle to bone. Tendon development, homeostasis and repair rely on specific combinations of mechanical parameters, transcription factors and growth factors that regulate the production and assembly of collagen fibers. Our objective is to decipher the mechanotransduction pathways underlying tendon cell differentiation. The zinc finger transcription factor EGR1 is a mechanosensitive gene and is involved in *Col1a1* and *Col1a2* transcription during tendon development and repair. We hypothesize that EGR1 senses mechanical signals to regulate tendon cell differentiation.

Tendon mechanobiology was studied *in vivo* during development. We showed that mechanical signals are required to maintain the expression of EGR1 and tendon markers during chick limb development. Mechanobiology was also studied *in vitro* using a 3-dimensional (3D) cell culture system (made of mouse mesenchymal stem cells) that mimics tendon formation. The expression of the mechanosensitive gene *Egr1*, components of TGFβ and FGF signaling pathways and tendon genes was increased in 3D cultures under tension compared to 2D cultures. Tension release in 3D-engineered tendons induced a drop of the expression of *Egr1* and tendon genes. Forced-expression of *Egr1* was able to prevent the downregulation of tendon gene expression in detensioned 3D-engineered tendons. All together, these results highlight the importance of EGR1 downstream of mechanical forces during tendon cell differentiation. However, the precise relationship between mechanical and molecular signals remains to be further explored.

Program Abstract #105

Measuring molecular tension at developing muscle attachment sites

Sandra B. Lemke, Katharina Austen, Carleen Kluger, Carsten Grashoff, Frank Schnorrer
Max Planck Institute of Biochemistry, Germany

During muscle development, muscle cells establish integrin-mediated attachments to tendon cells that allow the generation of mechanical tension across developing muscle fibers. This tension has been demonstrated to be important for myofibrillogenesis and the formation of regularly spaced sarcomeres within myofibrils. However, to date, tension at the molecular level cannot be directly measured in the living organism. Therefore, we adapted a fluorescence resonance energy transfer (FRET)-based tension sensor from cell culture and introduced it into the *Drosophila* genome by CRISPR/Cas9-mediated genome engineering. By inserting the tension sensor module into the endogenous locus of the integrin adaptor protein Talin, which localizes to muscle attachment sites, we ensure proper expression levels and timing in all tissues, including the indirect flight muscles. The flies generated this way are viable and able to fly, which shows that the Talin tension sensor fusion protein is fully functional, allowing the flight muscles to work properly. We established a protocol for fluorescence lifetime imaging (FLIM) and data analysis, which enables us to measure FRET in a reproducible manner in developing muscles of living pupae. We tested three different sensor modules to identify the best-suited sensor for the range of forces present in the muscle tendon system. We then applied this sensor to determine how molecular tension across Talin at muscle attachment sites changes during attachment formation and maturation, the latter coinciding with myofibrillogenesis, in the living organism. In the future, we will quantify the effects of genetic perturbations or drug treatments on tension levels and myofibrillogenesis during muscle development. This will allow us to study how force is transduced molecularly at muscle attachment sites, and thus provide insights into the molecular mechanism how tension build-up and myofibrillogenesis are functionally linked during muscle morphogenesis.

Program Abstract #106

TGFß signaling via Smad2/3 contributes to multiple aspects of tendon repair following Achilles rupture

Yue Lu, Sally Johnson
Virginia Tech, United States of America

Tendonitis, tears and ruptures are common maladies in man resulting in a substantial burden on the health care system due to the length of time required for structural repair. A barrier to effective tendon repair is the formation of fibrotic scar tissue of inferior strength and tensile properties. The objective of this study was to define the mechanisms underlying fibrotic tissue formation following tendon rupture. The Achilles tendon of adult (6-8 wks) *scleraxis*-GFP (SCX-GFP) reporter mice was transected and the dynamics of neotendon cell migration, proliferation and gene expression was measured during the immediate repair period (28 d). A leading edge of mitotic (EdU^+) cells was present at the base of the surgically resected Achilles at 7-d post-tenotomy (PT7). Mitotic cell numbers were virtually absent at PT21 coincident with a significant (P<0.05) increase in the number of SCX-GFP(+) cells. Quantitative PCR demonstrated greater expression (P=0.07) of *TGFβ1* at PT7 in neontendons compared to sham controls; *TGFβ2* and *TGFβ3* expression was unaffected. *Scx*, *collagen1a1* (*Col1a1*), *collagen3a1* (*Col3a1*) and *tenomodulin* (*TNMD*) expression was greater (P<0.05) in neotendon RNA isolates at PT14 than shams. Neotendon cells were isolated at PT7 and cultured for 4 d in TGFβ1 (10 ng/mL) supplemented media in the presence or absence of the TGFβ receptor (ALK4, ALK5, ALK7) inhibitor, SB431542, to suppress Smad2/3 signal transduction. Tenocyte differentiation was stimulated by TGFβ as noted by an increase (P<0.05) in *Scx* and *Col1a1* expression. Chemical inhibition of Smad2/3 activity blocked (P<0.5) growth factor mediated increases in *SCX* and *Col1a1* expression. TGFβ supplementation did not affect *TNMD* expression. However, treatment of neotendon cells with SB431542 resulted in a 8-fold increase (P<0.05) in *TNMD* expression. These results indicate that early formation of the neotendon is controlled by TGFβ mediated signals that affect multiple aspects of tenocyte differentiation.

Program Abstract #107

Acute p21 suppression in the skeletal muscle tissue causes proliferation of multiple cell types, fiber neoformation, and increases in strength and endurance

Maria Grazia Biferi[1], Carmine Nicoletti[2], Germana Falcone[1,3], Eleonora MR Puggioni[1], Nunzia Passaro[1], Alessia Mazzola[1], Deborah Pajalunga[1], Germana Zaccagnini[4], Emanuele Rizzuto[2], Alberto Auricchio[5,6], Lorena Zentilin[7], Gabriele De Luca[1], Mauro Giacca[7], Fabio Martelli[4], Antonio Musio[8,9], Antonio Musarò[2,10], Marco Crescenzi[1]
[1]Italian National Institute of Health, Italy; [2]Sapienza University of Rome, Italy; [3]Institute of Cell Biology and Neurobiology, National Research Council, Italy; [4]Policlinico San Donato, Italy; [5]Federico II University of Naples, Italy; [6]Telethon Institute of Genetics and Medicine, Italy; [7]International Centre for Genetic Engineering and Biotechnology, Italy; [8]Tumour Institute of Tuscany, Italy; [9]Institute of Biomedical and Genetic Research, National Research Council, Italy; [10]Italian Institute of Technology, Italy

Although in the last decades the molecular underpinnings of the cell cycle have been unraveled, the acquired knowledge has been rarely translated into practical applications. We have investigated the feasibility and safety of triggering proliferation *in vivo* by temporary suppression of the cyclin-dependent kinase inhibitor, p21. AAV-mediated, acute

knockdown (KD) of p21 in intact, adult skeletal muscles elicited proliferation of multiple, otherwise quiescent cell types, notably including satellite cells. Compared with controls, p21-KD muscles exhibited a striking two-threefold expansion in cellularity and increased fiber numbers by 10 days posttransduction, with no detectable inflammation. These changes partially persisted for at least 60 days, indicating that the muscles had undergone lasting modifications. Furthermore, morphological hyperplasia was accompanied by 20% increases in maximum strength and resistance to fatigue. To assess the safety of transiently suppressing p21, cells subjected to p21 KD *in vitro* were analyzed for γ-H2AX accumulation, DNA fragmentation, cytogenetic abnormalities, ploidy, and mutations. Moreover, the differentiation competence of p21-KD myoblasts was investigated. These assays confirmed that transient suppression of p21 causes no genetic damage and does not impair differentiation. Our results establish the basis for further exploring the manipulation of the cell cycle as a strategy in regenerative medicine. Furthermore, the behavior of the p21-KD muscle system makes it exquisitely suitable to investigate the homeostatic mechanisms that regulate cell number, organ size, and energy expenditure *in vivo*.

Program Abstract #108

The inhibitory core of the myostatin prodomain: implications for myogenesis

Yutaka Ohsawa[1], Yuta Fukai[1], Tatsufumi Murakami[1], Shin-ichiro Nishimatsu[2], Yoshihide Sunada[1]
[1]Neurology, Kawasaki Medical School, Japan; [2]Molecular and Developmental Biology, Kawasaki Medical School, Japan

Myostatin, the muscle-specific transforming growth factor-ß (TGF-ß) negatively regulates skeletal muscle mass. The N-terminal prodomain of myostatin non-covalently binds to and suppresses the C-terminal mature domain (ligand) as an inactive circulating complex. However, which region of the prodomain is required to inhibit the biological activity of myostatin has remained unknown. We identified a 29-amino acid region that suppresses myostatin-inducedtranscriptional activity by 79% compared with the full-length prodomain. This inhibitory core resides near the N-terminus of the prodomain and includes an α-helix that is evolutionarily conserved among other TGF-ß family members, but sinhibits activation of myostatin and growth and differentiation factor 11 (GDF11) that share identical membrane receptors. Interestingly, the inhibitory core co-localized and co-immunoprecipitated with not only the ligand, but also its type I and type II membrane receptors. Deletion of the inhibitory core in the full-length prodomain removed all capacity for suppression of myostatin. Asynthetic peptide corresponding to the inhibitory core ameliorates impaired myoblast differentiation induced by myostatin and GDF11, but not activin or TGF-ß1. Moreover, intramuscular injection of the peptide amelorated muscle atrophy and decreased the absolute force in caveolin 3-deficient limb-girdle muscular dystrophy 1C model mice. The injection suppressed activation of myostatin signaling and restored the decreased numbers of muscle precursor cells caused by caveolin 3 deficiency. These indings indicate a novel concept for the inhibitory core of the prodomain of myostatin: that it not only suppresses the ligand, but also prevents two distinct membrane receptors from binding to the ligand. This study provides a strong rationale for the use of the inhibitory core peptide in the alleviation of skeletal muscle atrophy in various clinical settings.

Program Abstract #109

PKA and mTOR mediate the inhibitory actions of calcitonin gene-related peptide (CGRP) on autophagy-lysosomal system in denervated skeletal muscles of rodents

Juliano Machado[1], Leandro H. Manfredi[3], Wilian A. Silveira[2], Dawit A.P. Gonçalves[1], Danilo Lustrino[4], Lilian C. Heck[2], Elza A. Filippin[1], Neusa M. Zanon[2], Maria A.R. Garófalo[1], Luiz C.C. Navegantes[2]
[1]Department of Biochemistry and Immunology, Ribeirão Preto Medical School - University of São Paulo, Ribeirão Preto, SP, Brazil; [2]Department of Physiology, Ribeirão Preto Medical School - University of São Paulo, Ribeirão Preto, SP, Brazil; [3]Federal University of Fronteira Sul, Chapecó, SC, Brazil; [4]Department of Physiology, Federal University of Sergipe, São Cristóvão, SE, Brazil

The role of calcitonin gene-related peptide (CGRP) on muscle excitation–contraction coupling is well known. However, its role in skeletal muscle protein metabolism remains unclear. We examined the *in vitro* and *in vivo* effects of CGRP on protein breakdown and signaling pathways in control skeletal muscle and following 7-days of skeletal muscle denervation (DEN) from rodents. In isolated rats skeletal muscles, CGRP (10^{-10} to 10^{-6}M), reduced basal and DEN-induced activation of overall proteolysis in a concentration-dependent manner. The *in vitro* anti-proteolytic effect of CGRP was completely abolished by CGRP8-37, a CGRP receptor antagonist. CGRP downregulated the lysosomal proteolysis, the mRNA levels of LC3b, Gabarapl1 and cathepsin L, LC3 lipidation and activated the PKA/CREB and Akt/Foxo signaling pathway in control and denervated muscles. In denervated rats skeletal muscles and starved C2C12 cells, H89, Rp-8-Br-cAMPs or PKI, three PKA inhibitors, completely abolished the direct inhibitory effect of CGRP on Foxo1, 3a and 4 and on autophagy-related genes, but had not the same effect on autophagic flux. In denervated rats and mice, a single injection of CGRP ($100\mu g.kg^{-1}$) increased the phosphorylation levels of PKA/CREB and Akt/Foxo, inhibited Foxo transcriptional activity, the LC3 lipidation as well as the mRNA levels of LC3b and cathepsin L, two bona fide targets of Foxo. Because CGRP increased the phosphorylation levels of S6, a mTORC1 target, and inhibited the autophagy in denervated mice and starved C2C12 cells, we analyzed the role of mTORC1 on autophagic flux using the pharmacological inhibitor of this kinase, rapamycin. We observed both *in vitro* (200nM) as *in vivo* ($1.5mg.kg^{-1}.d^{-1}$) rapamycin treatment completely abolished the direct inhibitory action of CGRP on autophagic flux. This study shows for the first time that CGRP direct

inhibits the autophagic-lysosomal proteolysis by a transcriptional mechanism Foxo-dependent and post-translational mTOR-dependent.

Program Abstract #110

Requirement of Myomaker-mediated satellite cell fusion for overload-induced skeletal muscle hypertrophy

Qingnian Goh, Douglas Millay

Cincinnati Children's Hospital Medical Center, USA

Myomaker, a muscle-specific membrane protein, is required for proper myoblast fusion during development and regeneration. However, the requirement of Myomaker, and to a larger extent myonuclear addition, for facilitating adult muscle fiber growth remains unclear. To investigate the involvement of Myomaker-mediated fusion in augmenting postnatal muscle hypertrophy, we rendered satellite cells fusion-incompetent through genetic disruption of Myomaker, and performed muscle overload via synergist ablation. Expression analysis of a Myomaker-LacZ allele revealed Myomaker to be upregulated in both satellite cells and myofibers throughout the 14 days of muscle overload. Targeted ablation of Myomaker in satellite cells resulted in a significant reduction in muscle mass and a complete attenuation of myofiber hypertrophy. Assessment of fusion using BrdU incorporation and genetic lineage tracing of satellite cells demonstrated that the majority of wild-type myofibers undergo fusion with satellite cells after muscle overload. In contrast, we observed a dramatic lack of nuclei incorporation into overloaded myofibers when Myomaker is deleted in satellite cells. This blunted hypertrophic response was associated with an acute reduction in Akt and p70s6k signaling. Furthermore, fusion-incompetent muscle exhibited increased formation of fibrotic tissue, indicating a role for normal satellite cell activity in maintaining muscle architecture. Taken together, our findings establish an essential role for Myomaker-mediated fusion during physiological postnatal muscle growth.

Program Abstract #111

The role of the Warburg effect-associated enzymes Phgdh and Pkm2 in myotube hypertrophy

Lian Stadhouders[1], Ilse Vogel[1], Gerard de Wit[1], Carla Offringa[1], Willem Hoogaars[1], Sebastian Gehlert[2], Henning Wackerhage[3], Richard Jaspers[1]

[1]Laboratory for Myology, MOVE Research Institute Amsterdam, Vrije Universiteit Amsterdam, The Netherlands; [2]Department of Molecular and Cellular Sport Medicine, German Sport University Cologne, Germany; [3]Faculty of Sport and Health Sciences, Technical University of Munich, Germany

A key component of the growth strategy of cancer cells is the Warburg effect. The Warburg effect refers to an increased glycolytic rate by which cancer cells provide glycolytic intermediates and other metabolites as substrates for biosynthetic reactions such as amino acid, nucleotide and lipid synthesis. Mechanisms do not evolve to promote cancer so we investigated whether the two Warburg effect-associated enzymes Phgdh and Pkm2 are expressed in skeletal muscle and whether their loss-of-function reduces growth factor-induced skeletal muscle hypertrophy. IGF-1 induced a 37% myotube hypertrophy and a 51% increase in medium lactate concentration. Blocking glycolysis by 2 deoxyglucose (2DG) reduced medium lactate concentration by 56%, caused a 30% myotube atrophy and ablated IGF-1-induced hypertrophy. This latter could not be explained by a reduced phospho-p70S6k, enhanced activation of Ampk or elevated Murf1 and Mafbx expression. Basal mRNA levels of the Warburg mediators Phgdh and Pkm2 as well as Phgdh protein were upregulated by IGF-1 in C2C12 myotubes. Knock down of Phgdh and Pkm2 expression using siRNA reduced C2C12 myotube diameter by 27% and 41%, respectively, and ablated IGF-1 induced hypertrophy. These effects were accompanied by increased mRNA levels of Mafbx and PGC-1α. Effects of downregulation of Phgdh on myotubes size was confirmed in primary mouse myotubes. Together these results indicate that Phgdh and Pkm2 are expressed in C2C12 myotubes and that this expression is regulated by IGF-1. These Warburg effect-associated enzymes not only contribute to the regulation of growth in cancer but also contribute to C2C12 myotube hypertrophy.

Program Abstract #112

Pitx2 is required for postnatal myofiber maintenance

Chrissa Kioussi

Oregon State University, USA

We have generated a conditional null allele of Pitx2 ($Pitx2^{FL}$) and used it in conjunction with a muscle creatine kinase (MCK)-Cre transgene ($Pitx2^{MCK}$) to disrupt the Pitx2 gene in the postmitotic nuclei of myofibers, where the MCK control sequences are thought to selectively drive CRE expression. While complete *Pitx2*-null mice can only rarely survive until embryonic day 14.5, the $Pitx2^{MCK}$ mutant were born alive without gross anatomical abnormalities. One week after birth, the myofibers of mutant muscles had only one third of the normal cross-sectional area. After one month, electron micrographs showed myofibrils and sarcomeres that were modestly reduced in thickness, and length, respectively. Longitudinal striations of the contractile apparatus were less electron dense, while Z-discs, I bands, M line, and H zones were clearly abnormal. The junctional sarcoplasmic reticulum (jSR) and mitochondria between myofibrils were vestigial or absent. Pitx2 protein normally occupies sites 8kb upstream of the FoxO3 gene and RNA for this autophagy regulator increased 20-fold in mutants, consistent with the loss of mitochondria. These results indicate that Pitx2 plays a significant,

cell-autonomous, non-redundant, functional role in postmitotic myofiber assembly. After nine months, mutants exhibited phenotypes similar to metabolic disease. They were much heavier, with prominent abdominal white adipose tissue. PPARg , a regulator for adipogenesis, gene expression was elevated in muscle. Blood glucose and insulin levels were also elevated. Metabolomic analyses demonstrated that a general increase in fatty acid metabolite levels was accompanied by a general decrease in amino acid metabolite levels in dissected muscles. The conditional mutant will therefore provide an additional model system to study the effect of muscle loss on energy physiology.

Program Abstract #113

Regulation of Hspb7 by MEF2 and AP-1 in muscle atrophy

J McDermott[1], S Tobin[1], A Blais[2], D Yang[2], J Girgis[2], A Farahzad[1]
[1]York University, Canada; [2]University of Ottawa, Canada

A genetic interaction between MEF2 and AP-1 in skeletal muscle cells has not previously been reported. Using MEF2A ChIP-exo, c-Jun and Fra-1 ChIP-seq data and predicted AP-1 consensus motifs, we identified common MEF2 and AP-1 target genes, several of which have a function in regulating the actin cytoskeleton. Since muscle atrophy results in remodelling or degradation of the actin cytoskeleton, we characterized the expression of putative MEF2/AP-1 target genes (Dstn, Flnc, Hspb7, Lmod3 and Plekhh2) under atrophic conditions using Dexamethasone (Dex) in skeletal myoblasts. Hspb7 was induced by Dex treatment and further biochemical analyses revealed that loss of MEF2A using siRNA prevented Dex-regulated induction of Hspb7. Ectopic Fra-2 or c-Jun expression prevented Dex-mediated upregulation of Hspb7, and AP-1 depletion resulted in upregulation of Hspb7. *In vivo*, Hspb7 expression was upregulated, along with other autophagy-related genes, in response to atrophic conditions in mice. Also, manipulation of Hspb7 levels in mouse TA muscle impacted gross muscle mass. Collectively, these data indicate that MEF2 and AP-1 confer rheostatic regulation of Hspb7 gene expression in skeletal muscle with implications for autophagy and muscle atrophy.

Program Abstract #114

HDAC4 modulates the response of skeletal muscle to long term denervation

Eva Pigna[1], Elena Simonazzi[1], Emanuela Greco[1], Eric N Olson[2], Sergio Adamo[1], Viviana Moresi[1]
[1]Sapienza University of Rome, Department of Anatomical, Histological, Forensic & Orthopedics Sciences, Histology & Medical Embryology Section, Italy; [2]UT Southwestern Medical Center, Department of Molecular Biology, TX, USA

Histone deacetylase 4 (HDAC4), a member of the class II HDACs, regulates skeletal muscle response following denervation, by promoting muscle atrophy and repressing reinnervation (1-2). Denervation induces accumulation of oxidative stress (OS) in skeletal muscle and HDAC4 shuttling from the nucleus to the cytoplasm is influenced by OS (3). However, the role of HDAC4 in mediating the response to OS is yet unknown. In this study we investigated the role of HDAC4 in skeletal muscle in response to long-term denervation, by using skeletal muscle-specific KO mice (HDAC4mKO). Skeletal muscles of HDAC4mKO mice do not respond to OS, are resistant to atrophy until two weeks, but degenerate four weeks following denervation, showing higher levels of reactive oxygen species (ROS) than control mice. Skeletal muscle degeneration is not caused by accumulation of OS, since treatment with an antioxidant does not ameliorate HDAC4mKO phenotype. Since compromised catabolic pathways may lead to muscle degeneration, we analyzed the ubiquitin-proteasome and the autophagic pathways in our system, finding that neither one is induced following denervation in HDAC4mKO mice. In light of these results, we triggered both the proteasome and the autophagic pathways in HDAC4mKO following denervation and, strikingly, both treatments ameliorated HDAC4mKO muscle degeneration and reduced ROS levels. These results indicate that HDAC4 mediates the OS response and activation of both proteasome and autophagic pathways in skeletal muscle following denervation and that long-term inhibition of HDAC4 leads to skeletal muscle degeneration in conditions of long-term denervation.

1. *Science*. 2009,326(5959):1549-54; **2.** *Cell*. 2010,143(1):35-45; **3.** *Am J Physiol Cell Physiol*. 2012,303(3):C334-47.

Program Abstract #115

RNA-seq and metabolomic analyses of Akt1-mediated muscle growth reveals regulation of regenerative pathways and changes in the muscle secretome

Chia-Ling Wu, Kenneth Walsh
Molecular Cardiology and Whitaker Cardiovascular Institute, Boston University School of Medicine, USA

Skeletal muscle is a major regulator of whole body metabolism. With aging, cachexia and myositis, there is a preferential loss of fast glycolytic muscle fibers. We previously reported a mouse model in which a constitutively-active Akt transgene is induced to express in a subset of muscle groups leading to the hypertrophy of type IIb myofibers. This muscle growth protects mice in various cardio-metabolic disease models. However, little is known about the underlying cellular and molecular mechanisms by which glycolytic muscle impacts disease processes and regulates distant tissues. In this study, mRNA-Seq and non-targeted metabolomics were performed to characterize the gastrocnemius muscle growth in Akt-transgenic mice. Combined metabolomic and transcriptomic analyses revealed that Akt1-induced muscle growth mediated a metabolic shift involving reduced oxidative phosphorylation, but enhanced branch chain amino acid (BCAA) accumulation and pentose phosphate pathway activation. Pathway analysis identified growth/cell cycle regulation and

inflammation as major enriched signaling pathways. Consistent with a regenerative transcriptional signature, the transgenic muscle was enriched for fibers with centralized nuclei that were positive for embryonic MHC. Signal peptide prediction analysis revealed 241 differentially expressed transcripts potentially encode for secreted proteins. A number of these secreted factors have signaling properties that are consistent with the cardio/ metabolic-protective properties that have previously been associated with glycolytic muscle growth. This study provides the first extensive transcriptome/ metabolome analyses for a model of type IIb muscle growth. These data revealed enhanced pentose phosphate and BCAA accumulation that are important for nucleic acids and protein production. We also identified numerous known and novel secreted proteins transcripts, indicating the importance of glycolytic muscle in inter-tissue communication.

Program Abstract #116

A physiological role of skeletal muscle-derived adiponectin in C2C12 differentiation

Katsumasa Goto[1], Rika Ito[1,2], Shingo Yokoyama[1], Takao Sugiura[2], Hirofumi Miyata[2], Yoshinobu Ohira[3], Toshitada Yoshioka[4]

[1]Graduate School of Health Sciences, Toyohashi SOZO University, Japan; [2]Yamaguchi University, Japan; [3]Doshisha University, Japan; [4]Hirosaki Gakuin University, Japan

Adiponectin, one of adipokines, is considered as a key mediator of obesity-associated insulin resistance as well as metabolic syndrome, and exhibits insulin-sensitizing effects to stimulate the utilization of glucose and lipids in skeletal muscle cells. Although it is generally considered that adiponectin is exclusively synthesized in adipocytes, is secreted into circulation, binds to adiponectin receptors (AdRs), especially AdR1, and thereafter acts on skeletal muscle cells. Recently, several reports showed that adiponectin expression in C2C12 cells as well as skeletal muscle tissues at mRNA and protein levels. However, the physiological role of adiponectin expressed in skeletal muscle cells remains unclear. Therefore, in the present study, we investigated a role of skeletal muscle-derived adiponectin in myogenic differentiation. Knockdown of adiponectin and/or AdR1 in C2C12 myoblasts was performed by the infection of small interfering RNA (siRNA). After the infection for 24 h, differentiation of C2C12 cells was initiated. Knockdown of adiponectin or AdR1 inhibits myogenic differentiation, in part. Simultaneous knockdown of adiponectin and AdR1 also partially depressed the up-regulation of creatine kinase (CK) expression during differentiation of C2C12. Muscle-derived adiponectin may stimulate myogenic differentiation via AdR1-associated signaling pathway in an autocrine and/or paracrine manner. This study was supported, in part, by Grant-in-Aid for Scientific Research (26560372, 26350818, 16K13002) from Japan Society for the Promotion of Science, the Uehara Memorial Foundation, the Naito Foundation, The Descente and Ishimoto Memorial foundation for the Promotion of Sports Science, All Japan Coffee Association, and Graduate School of Health Sciences, Toyohashi SOZO University.

Program Abstract #117

Are there intrinsic differences between satellite cells on fast or slow myofibers?

Laura Arnold

The University of Missouri, USA

Eph receptor tyrosine kinases and their membrane-associated ligands, ephrins, are expressed in almost every tissue and regulate multiple key processes during development, homeostasis, and regeneration, particularly in establishing tissue organization. Their diverse biological functions are achieved primarily by changing cell adhesion or promoting cell repulsion following cell-cell contact. Multiple groups have noted that myoblasts derived from fast vs. slow muscles, either in the embryo or in the adult, maintain a differential identity *in vitro* and *in vivo*, and preferentially form differentiated myotubes with a fiber type corresponding to the source of the cultured myoblasts, but to date no candidate mediators of this self-sorting have been identified. Data from our group showing that satellite cells exhibit classical repulsive behavior in response to ephrin guidance ligands, and that one such ligand (ephrin-A3) is specifically expressed by slow (Type I myosin heavy chain-expressing) myofibers in adult muscle, led us to hypothesize that satellite cells may also have an intrinsic fast vs. slow identity that can be identified by Eph/ephrin expression and activity. We have recently identified an Eph that is preferentially upregulated following activation by satellite cells resident on fast myofibers, is maintained cell-autonomously after activation, and can mediate cellular repulsion from ephrin-A3 *in vitro*. If this cell surface receptor is a marker of 'fast' satellite cells, it would suggest that subpopulations of satellite cells are segregated by fiber type during development and raise the possibility that repulsive interactions with myofibers, other satellite cells, or nonmuscle cell types may contribute to regeneration and repatterning following acute injury. Ideally, it will also provide a tool to prospectively separate them for further analysis.

Program Abstract #118

Hedgehog signaling regulates satellite cell function.

Caroline E Brun[1,2], Marie-Claude Sincennes[1,2], Fabien P Chevalier[1,2], Natasha Mercier[1,2], Michael A Rudnicki[1,2]

[1]Medicine Regenerative Program, Ottawa Hospital Research Institute, Canada; [2]Department of Cellular and Molecular Medicine, University of Ottawa, Canada

The regenerative capacity of skeletal muscle relies on a subpopulation of muscle stem cells, termed satellite stem cells.

Tracking the activation of Myf5 with Myf5-Cre:ROSA26-YFP mice revealed that YFP-negative satellite stem cells can perform symmetric cell divisions, which give rise to two identical daughter cells to self-renew and expand the satellite cell pool, or asymmetric divisions, which generate one self-renewing stem cell and one YFP-positive committed cell that express Myf5 and will progress through the myogenic lineage. However, the behavior and the myogenic fate of the YFP-negative satellite stem cell population are still unclear. By immunostaining on cultured myofibers, we discovered that satellite stem cells can progress through the myogenic lineage without expressing Myf5 but other myogenic markers of differentiation, such as MyoD and myogenin. Moreover, we showed that freshly sorted YFP-negative cells are able to differentiate *in vitro*. Cellular and molecular characterization of the differentiation potential will be presented. Our preliminary data support the hypothesis that multiple myogenic lineages coexist. Expression analysis by RNA-sequencing revealed that Sonic hedgehog (Shh) signaling genes are differentially expressed in YFP-negative stem cells and YFP-positive committed cells. Shh is a key regulator of myogenesis since the signaling pathway triggered by Shh leads to the activation of Gli transcription factors and expression of Myf5. We showed that activation of Shh signaling enhances asymmetric satellite cell divisions while its inhibition leads to satellite stem cell expansion. Therefore, we proposed that Shh signaling pathway could regulate the satellite stem cell fate decision to give rise to two distinct types of committed cells.

Program Abstract #119

GATA4 and GATA6 are required for skeletal muscle regeneration through regulating satellite cell fate by antagonizing MyoD

Tomohide Takaya, Yusaku Kodaka, Michael Baumrucker, Yoko Asakura, Atsushi Asakura
Stem Cell Institute, Paul and Sheila Wellstone Muscular Dystrophy Center, Department of Neurology, University of Minnesota Medical School, USA

The balance between myogenic differentiation and self-renewal of satellite cells, skeletal muscle stem cells, is tightly regulated during muscle regeneration. We notice that the transcription factors GATA4 and GATA6, which are expressed in activated satellite cells and downregulated during myogenic differentiation, promote satellite cell proliferation and inhibit differentiation into multinuclear myotubes. Microarray and ChIP-Seq analyses revealed that GATA4-induced cell cycle gene transcription is inhibited by MyoD occupying E-boxes. In contrast, MyoD-induced muscle gene transcription is attenuated by GATA4 binding to GATA sites, indicating that GATA4 prevents MyoD occupancy on their target loci in satellite cells to suppress myogenic differentiation. *Pax7*^{CreER}- or *MyoD*^{Cre}-mediated muscle-specific *Gata4/Gata6* double knockout mice displayed the delayed muscle regeneration with decreased satellite cell replenishment after injury. These results provide a novel mechanism wherein GATA4 and GATA6 regulate the gene expression program by antagonizing MyoD for proper equilibration of satellite cell differentiation and self-renewal during muscle regeneration.

Program Abstract #120

Unraveling STAT3 mechanisms of action in muscle stem cells

David Sala, Alessandra Sacco
Sanford Burnham Prebys Medical Discovery Institute, USA

MuSC (Muscle Stem Cell) functional exhaustion is intimately associated with defective muscle regeneration in chronic pathological conditions such as Duchenne Muscular Dystrophy or aging. Our laboratory has recently provided evidence that STAT3 activation in MuSCs stimulates a switch between self-renewal and myogenic lineage progression. Moreover, transient pharmacological inhibition of STAT3 improves muscle regeneration in dystrophic and aged mice, suggesting that STAT3 signaling pathway is a promising target for the treatment of different myopathies. However, the mechanisms of action of STAT3 in MuSCs are currently unknown. In order to identify the STAT3 target genes that regulate MuSC function, we performed RNAseq analysis to compare the expression pattern between activated control and STAT3 ablated MuSCs. Results showed the existence of 373 differentially expressed genes. Pathway analysis revealed that pathways involving glucose and lipid metabolism, and mitochondrial function were among the most enriched ones. Among the differentially expressed genes, we selected 30 candidates as potential target genes that mediate STAT3 effect in activated MuSCs. Consistent with pathway analysis and our previous work, most of the candidates regulate mitochondrial function, metabolism, cell proliferation and differentiation. We are currently performing candidate validation by qPCR and assessing their role in regulating MuSC function *in vitro*. Future experiments will focus on validating candidates' function *in vivo* and on assessing their potential as more specific targets to restore MuSC proper function in dystrophy and aging. Overall, this work will help us to define the role of STAT3 signaling pathway in MuSCs in both healthy and pathological conditions.

Program Abstract #121

Evaluation of novel extracellular matrix proteins to maintain the differentiation potential of mouse and human satellite cells during long-term cell culture expansion

Christopher Penton[1], Vasudeo Badarinarayana[1], Elaine Powers[1], Joy Prisco[1], Ronald Allen[1,2], Paul August[1]
[1]Sanofi, United States; [2]University of Arizona, United States

Large-scale expansion of myogenic progenitors is necessary to support the development of high throughput cellular assays *in vitro* and to develop cellular therapies for rare muscle diseases. A significant challenge exists to significantly expand myogenic progenitors since they progressively lose their ability to differentiate when cultured and passaged long term *in vitro*. To overcome this challenge, we evaluated the consequence of propagating mouse and human myogenic stem cell progenitors on various extracellular matrices to determine if they could enhance long-term myogenic potential. For the first time reported, we comprehensively examined the effect of physiologically relevant laminins, laminin 211 and laminin 521, compared to traditionally utilized ECMs (e.g. laminin 111, FN, and matrigel) to assess their capacity to preserve myogenic stem cell potential. Laminin 521 supported the increase of myogenic proliferation in early phases of expansion and was the only substrate facilitating high-level fusion following more than 8 passages in mouse cultures. In human cultures, laminin 521 also supported increased proliferation during expansion and superior differentiation with myotube hypertrophy and increased nuclear spacing. Significantly, these results demonstrate laminin 521 is a superior substrate for both short-term and long-term myogenesis.

Program Abstract #122
Characterizing the quiescent satellite cell translatome
Jeffrey Ehmsen[1], Ruifa Mi[1], Riki Kawaguchi[2], Giovanni Coppola[2], Ahmet Hoke[1]
[1]Johns Hopkins School of Medicine, USA; [2]University of California, Los Angeles, USA
Skeletal muscle satellite cells are considered resident stem cells of skeletal muscle, capable of emerging from a "quiescent" or dormant state to proliferate and differentiate into new muscle fibers in response to muscle injury or disease. These cells are found interspersed among muscle fibers, nestled between the sarcolemma and basal lamina, and are thought to comprise ~1-4% of total muscle nuclei depending on muscle type and age. Satellite cells are thought to reside within a distinct "niche," generated through autonomous and non-autonomous production of extracellular matrix components and adhesion molecules, which are critical for structural integrity and for localizing signaling molecules that regulate satellite cell behavior. Alterations to this niche, as may occur with disease, injury, or aging, are thought to influence capacity for satellite cell activation, migration, and differentiation, all of which are crucial for muscle regeneration and potentially important for muscle maintenance as well. To identify genes that may be important for maintaining the quiescent satellite cell niche, we characterized the translatome of satellite cells using RNA from acutely disrupted skeletal muscle from mice designed to express hemagglutinin (HA)-epitope-tagged ribosomes specifically in satellite cells. Here we provide a baseline characterization of this analysis, identifying putative satellite cell-enriched genes with potentially unique roles in satellite cell adhesion and signaling.

Program Abstract #123
Rapid satellite cell responses to the local environment are mediated by post-transcriptional mechanisms
Kate Gadek[1], Jason Doles[1,2], Bradley Olwin[1], Melissa Hausburg[1,3]
[1]University of Colorado at Boulder, USA; [2]Mayo Clinic, USA; [3]Ampio Pharmaceuticals, USA
Satellite cells (SCs) must transition from a non-dividing quiescent state to an activated state for maintaining adult muscle or to repair damaged skeletal muscle. Activated SCs can either enter the cell cycle and divide symmetrically, asymmetrically, or fuse directly into myofibers without an intervening cell cycle. How these decisions are made during SC activation is not yet understood but involves post-transcriptional regulation of critical mRNA targets via mechanisms that include microRNA-mediated decay, RNA sequestration, and mRNA decay. An mRNA binding protein, tristetraprolin (TTP) targets MyoD mRNA for degradation in quiescent satellite cells and is immediately inactivated by p38α/β MAPK signaling promoting a feed forward switch to accumulate MyoD protein and promote the transition of SCs to myoblasts. The AU-rich TTP binding site is abundant in mRNAs whose proteins regulate cell cycle and cell fate decisions. To query the landscape of TTP-regulated mRNAs in satellite cells we performed a TTP RIP-seq and identified transcripts that respond to environmental signals. Thus, post-transcriptional regulation of TTP target mRNAs during SC activation may rapidly alter SC fate dependent on the local environmental signals permitting SCs to immediately respond to the needs of the surrounding tissue.

Program Abstract #124
Satellite cell behavior following neonatal brachial plexus injury (NBPI)
Sia Nikolaou, Liangjun Hu, Roger Cornwall
Cincinnati Children's Hospital Medical Center, United States
Neonatal brachial plexus injury (NBPI) is a neurological injury at birth (1.5 per 1000 live births) that leads to permanent deficits in 20-30% of cases. These children develop muscle contractures that restrict limb range of motion and require corrective surgery. Surgery, which is not curative, could be avoided with a better understanding of how contractures occur. To aid in this, we developed a NBPI surgical mouse model and showed impaired longitudinal muscle growth, correlating with degree of contracture. Contractures developed by 2 weeks post-NBPI, which is within the myonuclear accretion period of mouse postnatal muscle growth. Satellite cells (SCs) are important in this process and are depleted in long-term neonatally denervated muscles. However, when we cultured SCs from NBPI-contracture muscle, they were not

only still present, but also capable of activation, proliferation, differentiation and fusion. Since normal function *in vitro* does not suggest normal function *in vivo*, we then characterized the *in vivo* behavior of SCs following NBPI. A larger than normal proportion muscle nuclei were quiescent SCs ($Pax7^+/MyoD^-$) 2 weeks post-NBPI. This may be due to (1) apoptosis of muscle nuclei decreasing the denominator, (2) failure of SC activation from the quiescent state, (3) increased self-renewal expanding the quiescent SC pool, or (4) failure of differentiation of activated SCs into the myonuclear denominator. Anti-activated Caspase 3/PARP and TUNEL assays failed to find increased apoptosis. Post-NBPI BrdU administration found normal proliferation rates indicating normal activation, with no disproportionate increase in SC self-renewal. However, we found an increased proportion of SCs that had never proliferated ($BrdU^-/Ki67^-$), suggesting a smaller than normal denominator of myonuclei 2 weeks post-NBPI. We are currently using the Pax7-ROSA26R-LacZ transgene to assess SC differentiation and fate in order to further investigate this failure of myonuclear accretion.

Program Abstract #125

Protection of the stem cell state of muscle stem cells *in vivo*

Federica Berti[1], Michal Kovác[1,2], Ricky-Lee Harris[1], Frank R. Schubert[1], Matt Guille[1], Susanne Dietrich[1]
[1]*University of Portsmouth, UK;* [2]*Slovak University of Agriculture in Nitra, Slovak Republik*

Muscle stem cells lose stem cell properties and the ability to efficiently produce muscle when cultured *in vitro*. *In vivo*, muscle stem cells, while muscle-competent are prevented from premature differentiation. We thus reasoned that their stem cell state is protected. To explore the underlying mechanism, we challenged muscle stem cells in the chicken embryo *in vivo* with molecular constructs suited to trigger myogenic differentiation. We show that in a certain time window, indeed, premature differentiation is suppressed. Interestingly, we found substantial differences between vertebrate species, suggesting that in anamniotes, cells are geared up towards muscle differentiation whereas in amniotes, they are not.

Program Abstract #126

iPSC modeling of human skeletal myogenesis and muscle disease

Meng-Jiao Shi[1], Jennifer Chen[1], Jing Yan[1], Emily Rozsahegyi[1], Carrie Spencer[1], Leslie Caron[2], Alex Kiselyov[3], Uli Schmidt[3], Haowin Zhou[5], Anne Bang[5], Kathryn Wagner[4], Charles Emerson[1]
[1]*Umass Medical School, Worcester, MA, USA;* [2]*enea Biocells, Sydney, Australia;* [3]*Genea Biocells, San Diego, CA, USA;* [4]*Kennedy Krieger Institute, Baltimore, MD, USA;* [5]*Sanford-Burnham Medical Resesarch Institute, San Diego, CA, USA*

Our lab collaborates with Genea Biocells to develop iPSC myogenesis technologies to investigate developmental and epigenetic mechanisms controlling human myogenesis and pathologies associated with muscular dystrophy. Utilizing Genea's step-wise, gene free myogenesis induction protocol, our findings show that human iPSCs derived from control subjects and FSHD and FKRP dystrophic patients undergo programmed activation of *PAX3* and *MYOD* and associated epigenetic changes in DNA methylation, leading to specification of myogenic "reserve" cell lineages. iPSC-derived myogenic reserve cells can be maintained as a proliferative population that can be induced to undergo myotube differentiation in response to environmental signals as well as can be xenografted into injured TA muscles of immunodeficient mice. Myotubes of FSHD and FKRP reserve cells express disease phenotypes, including misexpression of DUX4-fl target genes and deficiencies in laminin binding, respectively. These iPSC myogenesis technologies are being utilized to develop disease models for therapeutic development.

This work was supported by the NIH, FSH Society, and LGMD2i Fund.

Program Abstract #127

Receptor tyrosine kinase Tie2, a direct target of Notch signaling, maintains adult muscle stem cell quiescence

Qiang Gan[1,2], Antoine de Morrée[1,2], Thomas Rando[1,2]
[1]*VA Palo Alto Health Care System, 3801 Miranda Avenue, Palo Alto, CA 94304 USA, USA;* [2]*Department of Neurology and Neurological Sciences, School of Medicine, Stanford University, USA*

Notch signaling has been reported to maintain the quiescent state of muscle stem cells (QSCs). However, it is unclear how Notch signaling is required for QSC maintenance. To answer this question, we performed ChIP-seq experiments to identify Notch signaling target genes in QSCs. We identified the receptor tyrosine kinase Tie2 as a novel Notch signaling target, which we confirmed by expressing luciferase reporter constructs in C2C12 cells. Because Tie2 is best described as an endothelial cell receptor tyrosine kinase, we used three independent approaches to confirm that Tie2 is expressed in QSCs: 1) Using the SC-specific Pax7-creER and an YFP lineage tracer we isolated QSCs with flow cytometry and could detect *Tie2* transcript by RT-qPCR. *Tie2* transcript levels decrease dramatically after injury; 2) Using a Tie2 antibody we identified Tie2 high and Tie2 low subpopulations of QSCs, suggesting that a subset of QSCs expresses Tie2 protein; 3) Using single molecule RNA FISH, we could similarly identify Tie2 high and Tie2 low subpopulations in isolated QSCs. The QSCs that express high levels of Tie2 protein are also high in *Tie2* transcript. Therefore, we conclude that a subset of isolated QSCs expresses Tie2. To study its function, we inhibited Tie2 kinase activity with I.P. injections of a Tie2 kinase inhibitor, followed by EdU injections in uninjured mice. Treatment with the inhibitor doubled the number of QSCs that incorporated EdU, suggesting that receptor tyrosine kinase Tie2 plays a role in maintaining muscle stem cell quiescence.

Our results provide the first evidence that Notch signaling maintains quiescence in adult muscle stem cells partially through targeting receptor tyrosine kinase Tie2.

Program Abstract #128

The intracellular control of Thyroid hormone signaling in the biological activity of muscle stem cells and in Duchenne Muscular Dystrophy

Monica Dentice, Raffaele Ambrosio, Daniela Di Girolamo, Maria Angela De Stefano, Tommaso Porcelli, Giuseppina Mancino, Caterina Miro, Emery Di Cicco, Domenico Salvatore

University of Naples, Italy

Duchenne muscular dystrophy (DMD) is the most common form of muscular dystrophy, causing a severe, progressive muscle wasting due to satellite cells exhaustion and the inability of dystrophic muscles to compensate for the loss of muscle tissue. Precise control of the thyroid hormone (T3)-dependent transcriptional program is required by multiple cell systems, including muscle stem cells. We found that TH metabolism is dynamically modified during myogenesis and muscle regeneration by two TH-regulator enzymes, D2 and D3. This modulation is essential for proper muscle repair and satellite cell amplification and differentiation. Satellite cells-specific genetic ablation of D2 and D3 severely impairs skeletal muscle regeneration. By using genetically-manipulated mouse lines and primary culture of stem cells, we found that modulation of TH signal by deiodinases has a deep impact on satellite cells proliferation and their self-renewing ability. We generated D2-depleted mdx mice, which showed increased number of regenerating fibers and Pax-7 positive cells compared to control mdx mice. Furthermore, satellite cells-specific D2-depleted mice showed enhanced activated satellite cells, while reducing the percentage of quiescent satellite cells. Similar results were obtained in CTX-injured muscles, in which D2 depletion increased the number of activated Pax7 positive cells. Together, these results open a new scenario of TH involvement in the control of the niche microenvironment and stem cells renewal capacity in the dystrophic context. These studies suggest that the selective modulation of thyroid hormone concentration could be used to enhance rate-limiting steps in the muscle regeneration process, modulating stem cells expansion and/or differentiation, this might contribute to optimizing regenerative events in dystrophic context.

Program Abstract #129

A unique Twist-dependent progenitor cell contributes to adult skeletal muscle

Ning Liu, Glynnis Garry, Svetlana Bezprozvannaya, Stephen Li, Efrain Sanchez-Ortiz, Beibei Chen, Rhonda Bassel-Duby, Eric Olson

UT Southwestern Medical Center, United States

Skeletal muscle is composed of heterogeneous myofiber types that differ in contractile and metabolic properties. Skeletal muscle also possesses remarkable regenerative potential due to satellite cells, a stem cell population located beneath the muscle basal lamina. While satellite cells give rise to all fiber types, it remains unknown whether fiber-type specific progenitors might also exist. The Drosophila basic helix-loop-helix transcription factor Twist is expressed in muscle progenitors during embryogenesis and is essential for the formation of mesoderm and muscle. Within differentiated Drosophila muscles, Twist expression is restricted to adult muscle precursors (AMPs), a stem cell population that is normally quiescent but becomes activated in response to extracellular cues to regenerate the adult musculature during metamorphosis. Two mammalian Twist genes, Twist1 (Tw1) and Twist2 (Tw2), are expressed in various mesenchymal cell types, but not in differentiated myofibers. The potential contributions of Tw1 and Tw2 to muscle formation or regeneration in mammals have not been explored. We sought to determine whether mammalian Twist genes might participate in aspects of adult myogenesis. We traced the fate of Tw2-dependent cell lineages by analyzing the Tw2-CreERT2; R26-tdTO mice and discovered that Tw2 expression marks a previously unrecognized interstitial myogenic progenitor cell that forms type IIb/x myofibers in adult muscle. Tw2+ progenitors are molecularly and anatomically distinct from satellite cells *in vivo*, but transition through a Pax7+ state to form multinucleated myotubes *in vitro*. We show that Tw2 maintains progenitors in an undifferentiated state that is poised to initiate myogenesis in response to appropriate cues. Tw2-expressing progenitors represent a previously unrecognized, fiber-type specific progenitor cell involved in muscle growth and regeneration.

Program Abstract #130

ERK/CaMKII crosstalk: A novel mechanism controlling myogenesis.

Tamar Eigler, Eldad Tzahor

Weizmann Institute of Science, Israel

We previously reported that blockade of Extracellular Signal-Regulated Kinase (ERK) nuclear translocation induces myogenesis. We have since sought to identify novel ERK targets that mediate the downstream effect of ERK signaling in skeletal muscle derived satellite cells. We have identified a novel system of signaling crosstalk between the ERK and Ca^{2+}/Calmodulin-dependent protein kinase II (CaMKII) pathways that determines myoblast differentiation and fusion into multinucleated myotubes. Upon ERK inhibition with SCH772984, gastrocnemius muscle derived primary mouse myoblasts acquire a phenotype of fused multi-nucleated myotubes. The phenotypic progression from myoblasts to

differentiated myotubes is accompanied by the activation of $CaMKII^{T286}$, the inhibition of GSK3α/β, and the upregulation of markers associated with late myogenesis, MEF2C, MRF4, MYH1, and MYH2. Co-treatment with the CaMKII inhibitor KN-93 inhibits myocyte fusion and attenuates MEF2C, MRF4, MYH1, and MYH2 mRNA expression induced upon ERK inhibition. Moreover, myogenesis induced upon ERK inhibition and the activation of CaMKII is calcium dependent as treatment with BAPTA-AM, and the Ryanodine receptor antagonist Dantrolene, attenuates differentiation. Finally, we have identified Ca2+/CaMKII inhibitor-alpha (CAMK2N1) as a potential mediator of differentiation in primary myoblasts, as CAMK2N1 expression is significantly suppressed following ERK inhibition, as well in response to increasing doses of the GSK3α/β inhibitor CHIR999021. Taken together, we demonstrate that ERK activity suppresses Ca^{2+} dependent activation of CAMKII in primary myoblasts, whose activity is likely fine-tuned by expression level of CAMK2N1, and that activation of CaMKII in myoblast cultures drives myocyte fusion.

Program Abstract #131

Semaphorin 3A promotes activation of Pax7, Myf5, and MyoD through inhibition of emerin expression

Mulan Qahar[1], Wataru Mizunoya[1], Ryuichi Tatsumi[1], Seong-Kyu Choe[2], Mako Nakamura[1]

[1]Faculty of Agriculture, Kyushu University, Japan; [2]Department of Microbiology, Wonkwang University School of Medicine, Korea

Semaphorin-3A (Sema3A) has originally been identified as a repulsive axon guidance molecule and plays an essential role in neurogenesis, angiogenesis, and immune response. Recently, we have demonstrated that Sema3A expression was induced when quiescent satellite cells were stimulated by HGF and became active satellite cells (ASCs). However, how Sema3A can regulate genes in the early phase of ASCs remains unclear. We investigated whether Sema3A signaling can regulate the early phase of ASCs. Pax7 and Myf5 expression were decreased in myoblasts transfected with Sema3A siRNA. These cells failed to activate MyoD expression during differentiation. We also observed that Pax7 and Myf5 expression were increased in Myc-Sema3A overexpressing myoblasts. BrdU analysis indicated that Sema3A regulated ASCs proliferation. Myf5 and MyoD expression were totally inhibited in Pax7 siRNA transfected. These findings suggest that Sema3A signaling can modulate expression of the myogenic proliferation regulatory factors Myf5 and MyoD through Pax7. In addition, we found that emerin protein expression was regulated by Sema3A signaling. Emerin is an inner nuclear membrane protein that was identified by positional cloning as the gene responsible for the X-linked form of Emery-Dreifuss muscular dystrophy (X-EDMD). In conclusion, our results support a role for Sema3A in maintaining ASCs through regulation, via emerin, of Pax7, Myf5 and MyoD expression.

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